POPULATION GENETIC ANALYSIS OF NEOLITHIC TO BRONZE AGE HUMAN REMAINS FROM TRENTINO-ALTO ADIGE (NORTHERN ITALY)

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1 INTRODUCTION AND BACKGROUND

1.1 The Neolithisation of Europe

The spread of the Neolithic to central Europe, leading to a shift in human subsistence from hunting and gathering to an agricultural way of life, is undoubtedly one of the most important developmental processes in the history of humankind, and has therefore been the focus of research for more than a century, even before Gordon Childe created the term "Neolithic Revolution".

The endeavour to understand neolithisation processes focuses on several questions: These include the matter of cultural or demic diffusion, the Neolithic contribution to the modern European gene pool, the route of neolithisation and the relative contributions of male and female Near eastern pioneers from the Near East. Although the first two questions can, to a certain extent, be addressed using a multidisciplinary approach involving archaeological methods, genetic analyses can also provide objective data where typological analyses can only make assumptions.

The degree to which the transmission to agriculture was the result of cultural or demic diffusion (Cavalli-Sforza et al., 1993) is an example for the additional insights provided by genetic data. The former represents the transfer of knowledge, the adoption of innovative agricultural technologies and lifestyles by local, previously hunter-gatherer populations (acculturation), i.e. new agricultural technologies and lifestyles were adopted by the local, previously hunter-gatherer populations. The latter denotes the replacement of the existing hunter-gatherer gene pool by that of new settlers originating from the Near East, bringing with them new agricultural technologies.

The traditional archaeological approach to this problem is a typological one: Substantial continuity of artefact traditions such as distinctive stone tool

shapes or pottery designs with a certain degree of innovation is usually cited as an indication of acculturation. Abrupt changes toward artefact types known from established Neolithic cultures elsewhere, such as the transition from trapezoid, asymmetric arrowheads to symmetrical blades east of the Rhine and Neckar in the early Neolithic, or the sudden advent of *Linearbandkeramik* type pottery in Germany, are often interpreted as evidence of demic diffusion (Barfield, 1971). Based on classical genetic markers, which indicated a geographic cline, Cavalli-Sforza (1993) argued for the demic diffusion or "wave of advance" model, i. e. replacement of existing hunter-gatherer populations by the incoming farmers with little admixture between the groups, thereby inferring that modern European populations are descended almost solely from these Neolithic pioneers.

However, it is unlikely that demic and cultural diffusion can be strictly divided: In most cases the true question would probably not be which scenario is correct, but to what percentage the various models contributed to demographic development in the region in question. And indeed, ancient DNA studies (most based on mitochondrial DNA, but also an increasing number of palaeogenomic studies in more recent years) indicate very different patterns of relative hunter-gatherer and Neolithic contributions and general admixture and highlight the necessity of addressing this question on a regional basis. Indeed, a study by Bollongino et al. (2013) demonstrated a co-existence of hunter-gatherers and farmers for over two millennia, indicating that the entire question of cultural versus demic diffusion is by no means as simple as originally thought. Furthermore, genetic studies indicate that the Neolithic "revolution" was by no means a necessarily sudden and uniform event, but rather seems to have taken place in a series of waves and along different coastal and land routes (Pinhasi et al., 2012). Regarding the source of these migrations, two recent studies indicate a direct genetic link between the first farmers and throughout Europe with Greek/Anatolian Neolithic individuals (Hofmanová et al., 2016; Omrak et al., 2016).

In general, ancient DNA studies indicate widespread demic diffusion throughout Europe, revealing genetic discontinuity and the appearance of new mitochondrial haplogroups which were not present in the previous hunter-gatherer populations. For example, Bramanti et al. (2009) demonstrated that mitochondrial haplogroup U was predominant in hunter-gatherer populations (an observation that has been consistently confirmed through subsequent analyses, see Section 2.3.14.1.1), while the Neolithic LBK groups showed haplogroups which had been previously absent, such as H, T, J and J. On the other hand, mitochondrial haplogroup N1a was observed at high frequencies in LBK individuals, but is almost completely absent in modern European populations (Haak et al., 2005). Some studies, however, observe a certain degree of continuity between Neolithic and modern populations, especially in South-Western Europe (Sampietro et al., 2007). In addition, a later influx from the Steppe region (see Section 1.2) reintroduced certain hunter-gatherer haplogroups absent in LBK populations, so that the presence of these haplogroups need not necessarily indicate local continuity.

Percentages of relative contribution calculated in population genetic studies vary, depending on the type of data analysed (mtDNA or Y-chromosomal DNA, reflecting maternal or paternal lineages, or autosomal markers/genomic DNA) and the populations studied. A comparison of the currently available European Neolithic genomes with a large number of modern data sets (Lazaridis et al., 2014) shows that this research question is far more complicated: According to their findings, modern European populations are derived from three ancestral groups: Ancient North Eurasians (ANE), early European Farmers (EEF) and West European Hunter Gatherers (WHG) in varying ratios. The EEF group, which resulted from admixture between incoming Near Eastern farmers and European hunter-gatherer populations (although the specific times and places of this admixture is not determined) constitutes up to 90% of Mediterranean population ancestry (even >100% in Sicilians, indicating that this population

has more Near Eastern ancestry than can be explained by EEF admixture alone).

In addition, not just the presence of a Near Eastern genetic signature is decisive, but the time at which it was introduced into the population in question. For example, Achilli et al. (2007) analysed the present-day mitochondrial DNA from three regions in Tuscany (Casentino, Murlo and Volterra), one of which showed much higher Near Eastern haplogroup frequencies that the other two. As the region in question, Murlo, is of putative Etruscan origin, the authors inferred that the Near eastern signature was introduced by incoming Etruscans, who were interpreted by the authors as being of direct Near eastern origin. However, a later direct analysis of Etruscan ancient DNA by Ghirotto et al. (2013) indicates that the genetic affinity between the Etruscan population and the Near East is over 5,000 years old, indicating a local origin of the Etruscan culture. This demonstrates the necessity of taking later demographic changes and population histories into account when inferring Neolithic ancestry.

The routes upon which the "Neolithic package" was introduced to the various regions of Europe is also subject to debate. The archaeological record (pottery styles) indicates that the cultural innovations were spread along different routes: On the one hand via the Danubian/Balkan route, spreading into northern and central Europe as the *Linearbandkeramik* culture, and on the other hand via a seafaring route along the Mediterranean coast (Adriatic Impressed Ware), through Greece and the Mediterranean islands, around Italy and up the Adriatic coastline to the Po Plain and the later Cardial spreading into the Iberian peninsula and the South of France (Burger and Thomas, 2011; Olalde et al., 2015).

Paschou et al. (2014) performed a population network analysis using genome-wide data from modern populations throughout the Mediterranean and Anatolia. Their data indicate a higher rate of Neolithic gene flow via the southern route, by means of "island hopping" especially via Crete and Dodecanese.

1.2 Bronze Age Europe and the Pontic steppe migration

The Bronze Age is characterised by the advent of metallurgy – beginning with the Copper Age (or Eneolithic) and, after the addition of tin to copper to form the harder bronze, followed by the actual Bronze Age. Whether metallurgy was imported to Europe from other regions or whether it was developed independently in various places of origin is still a point of debate in archaeology. However, recent palaeogenomic studies (Allentoft et al., 2015; Haak et al., 2015) support the "Steppe hypothesis", i.e. a mass migration during the early Bronze Age from the Pontic steppe in various directions, including Europe. Specifically, the steppe herders of the Yamnaya Culture appear to have expanded their range, heavily influencing the Corded Ware and Bell Beaker cultures, possibly representing the origin of the Indo-European language (Haak et al., 2015). The second notable technological advancement, namely domestication of the horse and use of chariots, can also be attributed to the incoming steppe herders (Chechushkov et al., 2018).

The Bell Beaker culture spread throughout Europe from the Copper Age to the early Bronze Age from approximately 2900 to 2100 cal. BCE (Müller and van Willigen, 2001). Bell Beaker assemblages are not only characterised by their distinct, bell-shaped pottery style, but also specific metal objects such as "Beaker-type" copper daggers and axes as well as arrowheads (Barfield, 1971; Price et al., 1994). Furthermore, Price et al. note that Beaker burials are often those of robust males, and are frequently associated with horses: either through animal bones (Azzaroli, 1972, 1985) or through distinct osteological features in the region of the femur and acetabulum indicative of horseback riding, such as Quedlinburg individual 26 (Mathieson et al., 2015a). This has led to theories of the Bell Beaker folk representing a type of "warrior elite". The origin (as well as the general interpretation) of the Bell Beaker "phenomenon" has been a matter of long debate, with the Iberian Peninsula having been proposed as a possible origin, as the earliest sites with Bell Beaker-type assemblages were

found here. However, Barfield (1971) states that the Bell Beaker folk are more likely to have their roots in the Corded Ware cultures of central Europe, based on the typology of the pottery, arrowheads, daggers and mortuary houses. This is supported by recent genomic analyses, which revealed Bell Beaker samples, like those of the earlier Corded Ware groups, to have significant Yamnaya ancestry (Allentoft et al., 2015; Haak et al., 2015). However, as the proportion of Yamnaya ancestry was lower in Bell Beakers (approx. 50%, with around 35% early Neolithic ancestry and the rest Western hunter-gatherer) than in Corded Ware samples (approx. 75% Yamnaya, with less than 20% early Neolithic), this may indicate that the Bell Beakers were the result of admixture between Corded Ware groups with local farming communities (Haak et al., 2015). In particular, the Bell Beaker burials are associated with Y-chromosomal haplogroup R1b, which is exceedingly rare in pre-Bronze Age European contexts, but frequent in Yamnaya males (Allentoft et al., 2015; Haak et al., 2015). However, more recent studies give rise to the theory that the Bell Beaker culture may have been spread not only through demic, but also cultural diffusion: a genomic analysis by Olalde et al. (2018) involving 224 individuals from various areas with Beaker-type burial assemblages indicate that the spread of the Beaker culture is likely to have been based on both demic diffusion as well as cultural transmission, with some groups with clearly Beaker-associated material typologies lacking the Steppe genetic signature (e.g. in the Iberian peninsula), whereas others (notably Britain) showed high levels of Steppe ancestry. Focusing on Italy, Raveane et al. (2019) identified a north-south cline with regard to relative Anatolian Neolithic ancestry in modern Italy, ranging from 56% in the South and 72% in the North. The residual percentages were composed of WHG (Western Hunter-Gatherer), CHG (Caucasian Hunter-Gatherer) and Eastern Hunter-Gatherer) lineages, with the CHG/EHG that predominated in Northern Italy being interpreted as being a signature of the Steppe dispersal. Sardinia represents something of an exception: Neolithic ancestry was determined by the same authors to be 80%; an observation that is supported by a genomic analysis of Neolithic to

Bronze Age Sardinians by Marcus et al. (2019), which confirmed the Neolithic origins of the Bronze Age group with relatively little influx from Steppe-related groups.

The spread of peoples originating from the Pontic Steppe represents the second of two "mass dispersals" to significantly shape the genetic and cultural landscape of prehistoric Europe after the original migration of anatomically modern humans out of Africa, and one of the three most frequently postulated origins of modern-day European populations: Western Hunter-Gatherers, Northern Anatolian Neolithic and the groups originating from the Pontic Steppe (Raveane et al., 2019).

1.3 Relative male/female contribution during Neolithic/Bronze Age migrations

Apart from investigating the extent of demic diffusion during these two large migration processes, bioarchaeological studies also aim to establish the relative contribution of male and female migrants. Apart from genetic analysis based on non-recombinant uniparental markers (mtDNA and Y-chromosomal DNA), archaeological methods to address this purpose include osteological and strontium isotope analysis. Based on and admixture model using modern and ancient DNA datasets, Rasteiro & Chikhi (2013) established a similar dispersal history in which both males and females from the Near East admixed with local hunter-gatherer populations, but with a higher mitochondrial gene flow and diversity, indicating a higher effective female population size and mobility.

A possible explanation put forward by the authors is that the transition from a hunter-gatherer to an agriculture-based lifestyle may have brought promoted patrilocality as a result of the accumulation of wealth permitted by the new lifestyle: males inherited wealth and remained at their birthplace, whereas females were more mobile. Several ancient DNA studies by Lacan et al. (2011a; b) on Spanish and French Neolithic samples come to a similar result, namely a higher diversity for mtDNA than Y-chromosomal DNA.

However, in this case, the mitochondrial signatures are interpreted as a pre-Neolithic hunter-gatherer population, whereas the Y-chromosomal haplogroups are not associated with incoming Neolithic farmers, Neolithic, but also with a very low diversity, with the majority (5 of 6 in Spanish samples, 20 of 22 in French samples) of males belonging to haplogroup G2a, the others bearing haplogroups I2a and E1b1b1a1b. From this, it is inferred that a small effective incoming male population had a very high reproductive impact on the extant population.

The same high frequency of G2a has been observed in other Neolithic datasets, notably Starčevo (Szécsényi-Nagy et al., 2015), LBK (Haak et al., 2010, 2015; Mathieson et al., 2015b; Szécsényi-Nagy et al., 2015) and Near eastern Neolithic sample groups (Mathieson et al., 2015b; Hofmanová et al., 2016; Lazaridis et al., 2016). Analyses of strontium isotope signatures from over 300 Neolithic skeletons also indicated far lower geographic variance in males, which also supports the theory of patrilocality becoming established during this time (Bentley et al., 2012). These findings are contradicted, however, by a recent study by Goldberg et al. (2016) in which X-chromosomal data was compared to autosomal data for 20 early Neolithic and 16 late Neolithic/Bronze Age individuals. From this comparison, the authors deduce no difference in relative admixture for male and female during spread of the Neolithic, one explanation being the migration of entire family groups. However, the authors do not comment on how the pattern observed in many other Neolithic datasets (not only DNA, but also isotopes), namely that of low male diversity and higher female diversity and mobility, can be explained in the light of their proposed scenario. This discrepancy may be due to the limited geographical scope of the samples used by Goldberg et al.: of the 20 early Neolithic samples, all but one (the Iceman, who is described by the study in question as “early Neolithic” although the individual is in fact late Neolithic/Copper Age) stem either from the Hungarian Plain or from German LBK groups in Saxony-Anhalt. Four of the samples in this group are significantly younger than the others

(mid-fourth millennium BCE) – why these are termed early Neolithic is not clear.

In the same study, the authors infer a high male bias for the Bronze Age samples, with 5 to 14 migrating males per female, which is interpreted as a male-driven migration from the Pontic Steppe. However, the same geographical constraints apply here – all samples included in the ENLB group are Corded Ware or Únětice, all but three from the same sites from Saxony-Anhalt mentioned above. It is also notable that, although the mean X-chromosome ancestry is far lower in the ENLB group (0.366) than in the early Neolithic group (0.913), the individual values obtained for X-chromosomal ancestries are extremely variable in the ENLB collective, ranging from 0.01 (Únětice sample RISE577) to 1.0 (Corded Ware sample I0103). The observations put forward by Goldberg et al. were not replicated by Lazaridis et al. (Lazaridis and Reich, 2017) on the basis of the Bronze Age European population. One should therefore be cautious of applying theories of generalised migration patterns to specific regions.

Other analyses for Bronze Age samples yield evidence for widespread patrilocality and female exogamy (outward marriages) during this period: for example studies of craniofacial morphology in 171 early Bronze Age individuals from Austria (Pellegrini et al., 2008) as well as isotope analyses from South German Corded Ware collectives (Sjögren et al., 2016) and multidisciplinary analyses of Neolithic to Bronze Age remains from Bavaria (Knipper et al., 2017). The phenomenon of "*fremde Frauen*" (foreign women) is not uncommon in the Bronze Age (e.g. Frei et al., 2015), with women interred in Bronze Age cemeteries with garb and grave goods that are noticeably foreign to that region or culture.

Overall, the varying results of ancient DNA studies underline the necessity of investigating neolithisation processes and subsequent Bronze Age population dynamics processes on a regional level in order to aid construction of the overall picture.

1.4 Prehistory of South Tyrol and the Adige Valley

This section describes the current state of knowledge regarding the chronology of the South Tyrol and Adige Valley region from the Mesolithic onwards. Information is based largely on archaeological excavations, supplemented by bioarchaeological analyses (radiocarbon dating, palynological analyses, archaeobotany, archaeozoology and osteology).

1.4.1 Mesolithic

After Semi-Alpine environments became increasingly accessible during the Bølling-Allerød interstadial at the end of the last ice age, groups of Epigravettian hunter-gatherers probably originating from the valley bottom of the Adige migrated toward these mountainous regions (Bassetti et al., 2009). Characterisation of the Italian Mesolithic, in itself, is still a point of debate amongst some researchers: While some see the Mesolithic as having no separate cultural identity, preferring to term it the Epipalaeolithic, others regard it as a phase in which increasing shortages of available food sources, due to rising forest lines and associated retreat of ibex and chamois to far higher altitudes (Broglio and Lanzinger, 1990), caused the economy to shift toward more diverse subsistence strategies such as foraging and marine resources (Biagi, 2003).

The Mesolithic in northern Italy consists of the earlier Sauveterrian phase, characterised by very small, double-backed points (Mussi, 2006), dating from 7950-5800 BCE during the Preboreal and Boreal and the later Castelnovian from about 5800 BCE during the Atlantic (Biagi, 2003) characterized by trapezoid blades (Mussi, 2006). During the Sauveterrian, mountain sites were generally above 1800 m (Broglio and Lanzinger, 1990; Dalmeri and Pedrotti, 1992).

Migration may have been seasonal (Broglio and Lanzinger, 1990) with temporary habitation of rock shelters in the valley and higher altitude open air sites up to around 1500 m, less frequently higher. As few faunal remains have been preserved due to the high soil acidity of the mountain sites (Biagi,

2003), no generalised statements can be made on the type of fauna hunted. The Sauveterrian layers at Mondeval de Sora (Fontana et al., 2012; Thun Hohenstein et al., 2016), situated in the Belluno Dolomites, present an exception: The most common remains were from red deer and ibex, to a somewhat lesser extent roe deer and chamois. The assemblages also include occasional remains from wild boar as well as from carnivores such as bear, wolf and fox (Thun Hohenstein et al., 2016). This site, which also includes the burial of a 40-year-old male[1], also supports the perception of the Mesolithic as a culturally separate phase from the latest Palaeolithic, showing discontinuity with regard to flint assemblage, ritual and subsistence (Fontana et al., 2013).

Further climate warming in the Mesolithic led to a further northward migration, due to the rising treeline, and possibly the first cultural contacts with the region north of the Alps, as testified by raw materials originating from the south found at North Tyrolean (Schäfer, 1998) and Bavarian (Gehlen, 2010) sites.

Notable Mesolithic sites in the Trentino region include Romagnano, Pradestel, Colbricon and Vatte di Zambana, starting in the earliest Mesolithic, at around 8000 BCE (Alessio et al., 1984), and showing long Mesolithic traditions from the Preboreal to the Atlantic. However, the sites with Mesolithic and Neolithic layers that have undergone extensive radiocarbon dating, namely Riparo Gaban and Romagnano III, show a conspicuous hiatus from the end of the Castelnovian to the earliest Neolithic (Gaban) layers, spanning from approximately 5500-5000 BCE (Bagolini and Biagi, 1990; Perrin, 2009). Perrin (Perrin, 2009) argues that this chronological gap indicates that the early Neolithic was brought about by new settlers replacing the previous Mesolithic communities, rather than a gradual acculturation process amongst local groups. However, this view is challenged by the continuation of the lithic industry, with Castelnovian

[1] While some of the faunal remains found at the site (European Bison) have undergone palaeogenetic analysis, the human remains have not.

blades appearing in Gaban (early Neolithic) layers (Bagolini and Biagi, 1988; Pedrotti, 2001; Cristiani et al., 2009), whereby the technology is too similar to be put down to mere copying (Cristiani et al., 2009). However, comparable ^{14}C data is not (yet) available from several other sites which have Mesolithic and Neolithic layers. Furthermore, a similar phenomenon is observed in various regions throughout Europe and need not necessarily indicate population discontinuity, but may instead be caused by varied site usage or simply imprecise radiocarbon results due to disturbed stratigraphy. Some authors have also proposed climate change, indicating that the gaps are sedimentary in nature (Mlekuž et al., 2008a; b; Cristiani et al., 2009; Perrin, 2009). The modifications may also date to the Neolithic, i.e. Mesolithic layers were disturbed during the Neolithic (Mlekuž et al., 2008b). This may also be a result of the changed usage: throughout the Adriatic, caves showing anthropomorphic traces are more commonly used as animal shelters during the Neolithic (Mlekuž et al., 2008b).

1.4.2 Neolithic

Radiocarbon dates indicate that the Neolithic transition spread in a northward direction, starting from the late 8th millennium to the mid-6th millennium BCE in southern Italy (Malone 2003) and not detectable until around 4000 BCE in some of the remotest mountainous regions in northern Italy. The islands of Sardinia and Corsica also underwent first Neolithisation processes before these are observed in northern Italy (Tykot, 1999; Malone, 2003).

According to Biagi (Biagi, 1977), first Neolithisation processes are attributable to the Gaban group from approximately 5000 BCE, starting somewhat later than in the Po region (Pedrotti, 1998a). However, Annaluisa Pedrotti notes that a barley seed from Isera dates to 5300 BCE, possibly indicating an earlier coexistence of Mesolithic and early Neolithic groups. Pottery is of a typically impressed and incised ware with handles of Fiorano and Vhò types, which also document a technology spread from the Po Plain, i.e. the spread of the Neolithic via the Mediterranean route

(Adriatic Impressed Ware). However, some stylistic elements are reminiscent of LBK patterns, indicating that this area may also have been influenced from the Balkan region via the Pusteria Valley (Bagolini & Biagi, 1985). At the same time, Mesolithic traditions persist in the earliest Neolithic: not only do the lithic assemblies reflect a Castelnovian tradition, but the subsistence strategies indicate a largely hunter-gatherer lifestyle. Domesticates (primarily sheep, goats and cattle bones and coprolites) are observed in the later layers of the early Neolithic. With regard to the more recent layers, both at Gaban (Pedrotti, 1998a) and La Vela (Bazzanella, 1998): the faunal remains from the latter still include wild animals, but domesticates become more common in the later phases of the early Neolithic. According to A. Pedrotti (Pedrotti, 1998a), the chronological developments at the respective sites indicating advanced agricultural processes in existing settlements (such as the construction of a ditch at La Vela, or terraced agricultural area at Aica de Fié) with simultaneous persistence of Mesolithic traditions (e.g. microliths) would seem to indicate incoming cultures bringing farming traditions to small communities already present in the area, and a process of gradual acculturation rather than replacement.

In the course of the full Neolithic, the artefacts typical of the Gaban group are gradually replaced by a new typology: Square-mouthed pottery, vasi a bocca quadrata (VBQ) rather than the impressed ware characteristic of the early Neolithic; the typically Mesolithic microliths are replaced by flat-retouched arrowheads and scrapers (Pedrotti, 1998a). The proportion of domesticated animals increased further, with an emphasis on cattle, goats, sheep and (to a lesser extent) pigs (Bazzanella, 1998). Hunting focused mainly on red deer. Much information on the full Neolithic in the Trentino region was obtained during several excavations of the La Vela site. While transhumance (seasonal use of high-altitude pastures) was thought to have started during the Neolithic, recent palynological studies (Festi et al., 2014) indicate that, at least in the Ötztal mountains, widespread grazing activity at high altitudes was not practiced until the middle Bronze Age. Whether or

not this was also the case for the plateaus to both sides of the Adige Valley is not clear. Plant remains include both cultivated wheat and barley, as well as gathered nuts and berries (Pedrotti, 1998a). Pottery is of the VBQ type with flat bottoms. An obsidian blade originating in Lipari, as well as small ollas in the typical Serra D'Alto style, document cultural contacts with southern Italy (Pedrotti, 1998a). Similar trade connections are documented by various other objects not derived from the vicinity (e.g. jadeite axe and "*Schuhleistenkeil*" type implement). Trade contacts with southern Germany are indicated by pottery of South German origin at Rocca di Rivoli (Barfield et al., 1976), Münchshofen-type pottery at Isera and North Italian flint and VBQ pottery North of the Alps (Pedrotti, 1998a). Influences from the central Balkan region are documented by pintaderas (stamp seals), figurines and pottery decoration styles (Bagolini & Biagi, 1985), indicating that Neolithisation of Northern Italy tool place not only via coastal routes, but possibly also from LBK/Starčevo sources from the Balkan mainland.

One notable aspect is that rock shelters are apparently almost all (with the exception of Romagnano (Perini, 1971)) abandoned during this time. Although this is attributed to the demand for increased space for animal husbandry and crops, use of rock shelters is resumed later on, at least for burial purposes. The last elements of the VBQ pottery style have been dated to between 4500 and 4300 cal. BCE. Later on in the course of the Neolithic, a deterioration in craftsmanship is observed both north and south of the Alps, resulting in a simple vessel shape at the start of the Copper Age. Pedrotti (1998a) regards this as an indication of the arrival of new people, whose origin cannot be deduced from the archaeological typology.

1.4.3 Copper Age (Chalcolithic)

From the mid-4th millennium BCE onward, the Neolithic was gradually replaced by the Copper Age, which ranged from 3400-2200 BCE (Bagolini and Biagi, 1990; Pedrotti, 2004), and the copper artefacts giving the epoch its name began to appear. The oldest copper artefact in a Neolithic level in

northern Italy to date was found in Alba (Piedmont) and dates to 3430 ± 40 BCE (Zoppi et al., 2001).

One particular find has become synonymous with the North Italian Chalcolithic: the Tyrolean Iceman "Ötzi", discovered by climbers on 19. September 1991 on the Tisenjoch on the Italian side of the Ötztal Alps. Since its find in 1991, this excellently preserved ice mummy has yielded a wealth of information, not only on the personal life circumstances of the individual itself, but also on the Copper Age in general. As this individual is, in a broader sense, a subject of this study, he is described in more detail in Section 2.1.2.13.

Another site that provided much information on the North Italian Copper Age is Remedello di Sotto in Brescia, approximately 120 km south-west of Trento and around 140 km south of the Iceman's find spot. This site, first discovered in 1883, yielded 123 individual burials. Males were generally buried in a crouching position, facing north-west direction, and were usually accompanied by flint daggers, stone arrowheads and polished stone axes. Some individuals had copper axes and daggers. Women were more commonly buried in a supine position in east-west direction; their grave goods were largely restricted to pottery (Barfield, 1971; De Marinis, 1997, 2013). The proportion of copper axes and daggers to those made of stone, however, is still low, indicating the beginnings of a possible social stratification or "elite warrior class" (De Marinis, 1997; Pedrotti, 20042004). Chemical analyses of the copper objects found in Remedello di Sotto revealed different compositions: While the axes tended to consist of pure copper, the daggers contained a higher proportion of arsenic (De Marinis, 1992). This differentiation seems to have been intentional (as heated arsenic is easily detectable by smell) and has been proposed as a further indicator of a social hierarchy (Pearce, 1998).

The copper axes found in grave 102 of the Remedello necropolis was almost identical in type to that found with the Tyrolean Iceman. This is not the only typological link between the Iceman and the Remedello necropolis – both

the Iceman's dagger as well as the arrowhead later found lodged in his shoulder both had parallels in the Remedello di Sotto cemetery. On the basis of the Remedello finds, a chronology was developed for the Copper Age in northern Italy, discerning phases Remedello I and II for the early and late Copper Age, respectively (De Marinis, 1997). On the whole, the metal objects found at Copper Age sites from northern Italy show a variety of external influences. For example, the dagger shapes are reminiscent of those found at late 3rd millennium Aegean and Anatolian sites, silver pins apparently copy Corded Ware-type pins and the pottery of the Remedello I phase has parallels in the South of France (Barfield, 1971). While Copper Age sites are more numerous in the Po Valley, Barfield (1971) suggests that the metals (both silver and copper) were obtained from the known deposits in the Alpine foothills and in the vicinity of the Adige Valley.

Cattoi et al. (2000) analysed Copper Age slag residues from sites in the Trentino region, including Gaban, Acquaviva and Romagnano. The metal composition was unlike that of the surrounding geological deposits, leading the authors to conclude that the ore cannot have been mined in the direct vicinity, have been obtained some distance away: most probably from the phyllite basement of Val Sugana, of which the most important deposit was located at Calceranica, a walking distance of approximately 20 km east of Acquaviva and Romagnano. Other potential provenance sites are Vetriolo (35 km walking distance east from Acquaviva and Romagnano) and Val Sella (45 km walking distance east from Acquaviva and Romagnano) (Cattoi et al., 2000).

How and from where the metalworking technology was introduced to northern Italy is still a matter of debate, as so many different external influences are seen in the Copper Age of northern Italy, while still retaining several aspects of the local VBQ culture (Barfield, 1971). As described above, the typology of the early copper objects shows different external influences. The earliest evidence for copper metalworking in central Europe, however, was found at a late Neolithic site belonging to the Münchshofen

culture in Brixlegg in the Inn Valley of the Austrian Alps. Excavations at this site revealed copper slag, as well as copper beads and a copper band, in a late Neolithic layer. Radiocarbon dating on charcoal fragments contained in a lump of clay which also held green copper minerals revealed an age of 3960-3650 cal. BCE (Höppner et al., 2005), predating the earliest radiocarbon dated Copper Age layers in the Trentino region. A copper strip found at Isera 2 may be the earliest copper object found in the Trentino region – however, no ^{14}C dates are available for this layer. As Isera 1 has been dated to 4500-4300 cal. BCE and Isera 3 to 3800-3600 cal. BCE, the Isera 2 copper strip may be older than the Brixlegg objects (Pedrotti, 1996, 2001). However, lacking any evidence for actual metallurgy in Alto Adige-Trentino at this time, the copper strip is likely to have been imported, whereas the Brixlegg site documents actual smelting processes. Although the slags themselves seem to have been derived from local ores, the copper strip could not have been produced from local ores, but was apparently an import originating from the Carpathian Basin, the metal composition most closely matching that of the ores found in Majdanpek in Serbia (Höppner et al., 2005). Furthermore, the typology of the metal strip has close parallels in the late Neolithic Jordanów culture of Moravia (Pernicka et al., 1997; Höppner et al., 2005). These contacts are underlined by several other typological parallels (mainly pottery) between the Münchshofen culture and the Carpathian Basin. This led the authors to conclude that the technological knowledge of metalworking was introduced to the Alpine region through contacts with the Carpathian Basin, which encouraged the local population to experiment with the ores found in the region (Höppner et al., 2005). As both Brixlegg and the Trentino region are situated directly upon the main route traversing the Alps via the Brenner Pass, which is known to have been used since Mesolithic times, it is therefore highly likely that there were contacts between the Brixlegg and the Trentino groups. The knowledge regarding metalworking may well have been obtained from an eastern direction via this north-south contact and then established in the Trentino region due to the wealth of ores in the vicinity. As with the spread of the

Neolithic, the advent of metalworking gives rise to the question whether the technology was simply adopted by local communities, or whether new incoming groups who already possessed metallurgic knowledge settled in the Adige valley due to the accessibility of ore metals, either mixing with or replacing the previous populations.

In northern Italy, the Bell Beaker sites (see Section 1.2) are generally situated throughout the Po Plain (Barfield, 1971). Although Bell Beaker burials were found in the direct vicinity of the Remedello di Sotto necropolis, they were culturally distinct from the latter. However, Marzatico and Tecchiati (2002) point out that some artefacts that are described as having been influenced by the Polada Culture may just as well be ascribed to the "campaniforme" (bell-shaped) tradition. Whether or not this may have been due to admixture between Bell Beaker groups and local communities is not clear. The earliest Bell Beaker ware found in the Adige Valley is a handled pitcher of type 34/35, one of the most significant Bell Beaker forms, found at the Pigloner Kopf near Pfatten, approximately 50 km north of Trento, dating to approx. 2500 BCE (Piguet and Besse, 2009) and represents the earliest occurrence of this type in Italy. This specific type first appears in pre-Bell Beaker groups in Poland, Slovakia, Hungary and Moravia from about 2700 BCE, spreading through Central Europe to Northern Italy in 2500 BCE, then to Switzerland southern France by 2300 BCE, and also to Sardinia, although no radiocarbon dates are available for the latter, and the admixture with the preceding Neolithic population in Sardinia appears to have been minor (Raveane et al., 2019; Marcus et al., 2019).

1.4.4 Bronze Age

The start of the Bronze Age, beginning with the first stage of the Polada Culture, is generally said to be approximately 2200 BCE, although some sites (e.g. Romagnano) show slightly earlier dates. Based on radiocarbon dates, the early Bronze Age lasts until approximately 1600 BCE (De Marinis, 1999).

Early Bronze Age sites are characterized by an increased amount of evidence for large-scale metallurgic activity, such as copious amounts of slag deposits and smelting ovens at the sites of Montesei di Serso and Vela Valbusa di Trento (Marzatico and Tecchiati, 2002). Elements from the Copper Age are observed, with an increasing eastern influence on metal objects such as awls (Marzatico and Tecchiati, 2002). In particular, the groups in the Adige valley showed an advanced metalworking technology, far beyond the rest of Italy, leading to an accumulation of wealth (Barfield, 1971).

A certain influence from the Bell Beakers is seen in Polada burials, especially regarding archery equipment: triangular barbed or hollow-based arrowheads as well as stone armguards show parallels to the Bell Beakers. A Polada site south of Lake Garda, Barche di Solferino, not only yielded the oldest Italian evidence for horse domestication (as wild horses appear to have died out at the end of the Pleistocene, the animal in question can only have been a domesticate) (Azzaroli, 1972, 1985; Riedel, 1976), but also what may be the oldest European spoked wheel (Barfield, 1971), both very probably a result of the North-eastern influence via Bell Beaker/Corded Ware groups. The metal objects associated with Polada sites are also firmly linked with cultures north of the Alps, particularly Únětice and Straubing. Transalpine contacts are further documented through finds from South German lakeside dwellings, such as Bodman-Schachen and Hegau: e.g. through the use of similar building technologies as those observed in Fiavè, similarly decorated "*Brotlaibidole*" (Ital. *tavolette enigmatiche*, fired clay plaques with line and dot patterns, which may have been used as a counting device) to those found at Polada sites in northern Italy as well as tub-shaped crucibles and globe-headed needles most commonly found in the Trentino region (Köninger and Schlichtherle, 1999). According to Barfield (Barfield, 1971) similar "*Brotlaibidole*" were also found in Slovakia and Hungary – although the exact origin and purpose of these tablets is still a mystery, it documents trade networks between these regions. On the other hand, the Polada pottery, with its "elbow" handles, is reminiscent of types used in

southern Italy during the Copper Age. However, local subsistence and settlement patterns dating back to the Neolithic still persist, as does the predominant use of flint over metal (Barfield, 1971; Riedel and Tecchiati, 2000).

Bronze artefacts seem to be rare in early Bronze Age sites. An early Bronze Age grave excavated in Piedmont revealed a silver ring and a bronze blade. The radiocarbon dating revealed an age of 2210-2030 cal. BCE, confirming placement in the early Bronze Age. A bronze pin with a tin proportion of 7.32% was found in a Bronze Age layer at Ledro; however, allocation to the early Bronze Age was excluded (de Marinis and Valzolgher, 2013). This relative scarcity of the use of bronze may well be due to the limited availability of tin. In contrast to copper ore, which is available from numerous sites throughout Europe (including the Valsugana site near Trento), only a few tin deposits exist. The nearest to the Trentino region is Monte Valerio in Tuscany, over 400 km from the Adige Valley (Venerandi-Pirri and Zuffardi, 1981; Benvenuti et al., 2003). Other known deposits exist in the Erzgebirge (Ore Mountains) along today's German-Czech Republic border, which would have been over 700 km from Trentino, as well as deposits in Serbia and Romania, which would have been even further away. On the other hand, copper alloy with a high arsenic content, such as that found in certain artefacts from Remedello di Sotto, is often termed "arsenical bronze" (Charles, 1967; Lechtman and Klein, 1999), is harder than pure copper and may have been regarded as equivalent for the purpose. No actual evidence for bronze (as opposed to copper) smelting exists for Bronze Age Trentino – the few artefacts that were found during later phases are likely to have been imported.

The middle Bronze Age in northern Italy sees continued use of existing early Bronze Age settlements, with innovations being adopted gradually. One new development, for example, is the use of "*Brandopferplätze*" – sacrificial bonfire sites, often located at high-altitude sites and used until well into the Iron Age or even Roman times (Haupt, 2010). Another new

practice is that of cremation, which is observed for the first time during the middle Bronze Age, as is the funerary style of sword burial (Barfield, 1971). Copper mining and metalworking continued to develop throughout the middle and final Bronze Age, as documented by veritable hoards of copper objects (Marzatico and Tecchiati, 2002). During the late Bronze Age from about 1300 BCE, the Laugen-Melaun (Luco-Meluno) Culture became established, bringing a variety of new pottery styles and cremation burials in urns, analogous to the Urnfield culture of central Europe. Despite the new impulses, continuity was observed with regard to settlement sites, subsistence and the popularity of copper metallurgy (Riedel and Tecchiati, 2000). The Laugen-Melaun Culture lasted well into the Iron Age, despite the declining popularity of bronze since the acquisition of iron, until it was replaced in the 6th century by the Rhaetian Fritzens-Sanzeno Culture, a group with a strong Celtic influence (Megaw and Megaw, 2001; Marzatico, 2014). The existence of individual prehistoric cultures in the Alto Adige-Trentino region came to an abrupt end in 15 BCE, when it was occupied by the Roman Empire in the course of the Emperor Augustus's Alpine campaign, during which the local populations were either romanised or effectively destroyed (Barfield, 1971). The extent to which the pre-Roman populations in this region survived, perhaps by withdrawing to the more remote regions of Alto Adige-Trentino that are still populated to this day, and contributed to the modern gene pool of these regions is not known and is one of the research questions to be addressed in the course of this study.

1.5 Population genetics of South Tyrol and the Adige Valley

Ancient DNA data from northern Italy remains sparse. A study by Di Benedetto et al. (2000) attempted ancient DNA analysis of two Pre-Neolithic (Villabruna, Vatte di Zambana[2]) and three Neolithic

[2] Vatte di Zambana is described as Neolithic in the cited publication. However, the radiocarbin dates provided in the same publication for Vatte di Zambana clearly indicates Mesolithic origin, and is described as such by Alessio et al., 1984

(Mezzocorona, Borgonuovo, Fiavè) and obtained reproducible HVR1 sequences for the first two sites. However, the results for Villabruna are ambiguous: Using a standard PCR approach, Di Benedetto (Di Benedetto et al., 2000) reported this individual as having mutations at positions 16261T and 16274A. However, data from the more recent genomic analysis by (Fu et al., 2016) show 16270T (14x coverage) and no mutations at either site reported by Di Benedetto. Figure 3 in the earlier paper shows the obtained sequences per PCR/clone. The second fragment clearly shows that one of the two PCRs did indeed amplify 16270T, whereas the other yielded 16261T and 16274A. Why the latter was selected to be the correct one without further analyses is not specified in the paper – however, in the light of the more recent findings by Fu et al., the 16270T variant seems to be far more likely, especially as this indicates U5, a haplotype far more common in Palaeolithic individuals. The results obtained by Benedetto et al. for Mezzocorona, however, are unambiguous, with all three observed mutations reproduced in at least three PCRs.

The well-preserved mummy of the Tyrolean Iceman, discovered on the Tisenjoch at the Italian-Austrian border in 1991, has yielded further genetic data (albeit late Neolithic/Copper Age): The HVR1 sequence was sequenced in 1994 (Handt et al., 1994), the entire mitochondrial genome a few years later (Ermini et al., 2008) and recently the whole nuclear genome (Keller et al., 2012). Further information on the results of these analyses is provided in section 2.1.2.13.

Recently, genomic data was generated for three individuals from the Remedello di Sotto necropolis (Brescia, 25 km south of Lake Garda) described earlier (Allentoft et al., 2015). As all of the aforementioned samples are used for reference purposes, they are described in more detail in Section 2.3.14.10.

Extensive genetic analyses have also been performed for extant populations living in Alto Adige-Trentino. Initial studies of the HVR1 of Ladin groups (Stenico et al., 1996, 1998) reported a high mitochondrial variability within

Ladin groups, argued against any significant bottlenecks in the history of present isolates and indicated that genetic difference does not necessarily correlate with language difference. However, this study seems to have been affected by widespread sequencing errors (Vernesi et al., 2002). A broader genetic study, including German-speaking isolates and also observing Y-chromosomal DNA and microsatellites (Pichler et al., 2006), reported that the mtDNA diversity in the Ladin Val Badia group was slightly lower, and Y-chromosomal variation much lower than in the neighbouring German-peaking populations, indicating a patrilocal population structure. Overall, the authors identify reduced genetic diversity as well as significant differentiation between groups, which also correlates with language, i.e. a younger German-speaking and older Ladin contribution, but with significant differences between localities. Thomas et al. (2008) confirmed the lower diversity of these isolates as compared to other regions of Italy, high differentiation (also between the two Ladin groups sampled for that study) and a low effective population size leading to genetic drift. For Val Badia, the authors infer a higher Palaeolithic contribution than for the neighbouring groups.

While the above-named studies focused mainly on the Ladin and German-speaking groups from the upper Adige valley and Dolomites, Coia et al. (2012, 2013) analysed isolates from further south, namely in the Trentino region, observing a high level of mitochondrial differentiation between groups and variable levels of intra-group diversity, the highest found in the Adige Valley and lower levels (reflecting a smaller effective population size) in the more geographically isolated localities to either side of the Adige valley. A higher diversity for mtDNA than for Y-chromosomal DNA is observed, indicating different levels of gene flow for males and females, possibly as a result of local traditions in which agricultural holdings can be inherited one son only, encouraging the others to relocate elsewhere (Coia et al., 2013).

An osteological analysis, more specifically analysis of non-metric dental traits by Cucina et al. (1999) from sites including La Vela, Martignano, Solteri, Madonna Bianca, Moletta Patone, Paludei di Volano and Nogarole indicated high similarity between groups from the Neolithic to the Bronze Age.

1.6 Aim and objective

The aim of this study is to provide insights into the genetic structure of the Neolithic to Bronze Age population of the Alto Adige area of northern Italy, and area for which little ancient DNA data exists to date. Alongside analysis of maternally inherited mtDNA, the study will focus on the Y-chromosomal haplogroup and certain phenotypic autosomal markers such as single nucleotide polymorphisms determining lactase persistence and eye colour.

On the one hand, the resulting data should provide a more comprehensive image of prehistoric Europe as a whole, as data from northern Italy is generally sparse. Comparison of ancient data with modern populations from the same region could add information on the population history of these areas from prehistoric times until today. Not lastly, the obtained data should resolve the relationship between the Copper and Bronze Age Trentino populations with the Tyrolean Iceman, a find of substantial archaeological and public interest.

Specific questions this project aims to address are as follows:

Ancestry of ancient Trentino populations:

- Do the genetic data lend more weight to acculturation or replacement models, i.e. are the haplogroups of Neolithic, Copper or Bronze Age individuals more indicative of hunter-gatherer ancestry, lending weight to the hypothesis of gradual acculturation of local Mesolithic groups, or are they typically “Neolithic” in origin (see Section 2.2.1)?

- In case of a likely Neolithic origin: Are these more closely linked with the Mediterranean coastal wave of advance (Adriatic Impressed Ware/later Cardial) or with the Neolithic advance via Transdanubia into Central Europe (mainly LBK)?
- Is a genetic difference discernible after the migration movements originating from the Pontic steppe, and/or the Bell Beaker settlements in northern Italy?
- Which variants are observed for lactase persistence and blue eye colour in the ancient samples, can any inferences be drawn from these observations (especially regarding the higher frequencies of lactase persistence in modern north-eastern Italy)?
- What can be observed regarding the intra-group diversity and differentiation between local groups and what are the implications regarding population history and expansion?
- What similarities can be observed between the ancient Trentino samples and the genetic data from the Tyrolean Iceman?

Continuity

- Does the chronological gap between the late Mesolithic and the early Neolithic observed in the archaeological stratigraphy reflect actual the population situation at the time?
- Is any genetic continuity discernible from ancient Trentino to later/modern populations from the greater area?

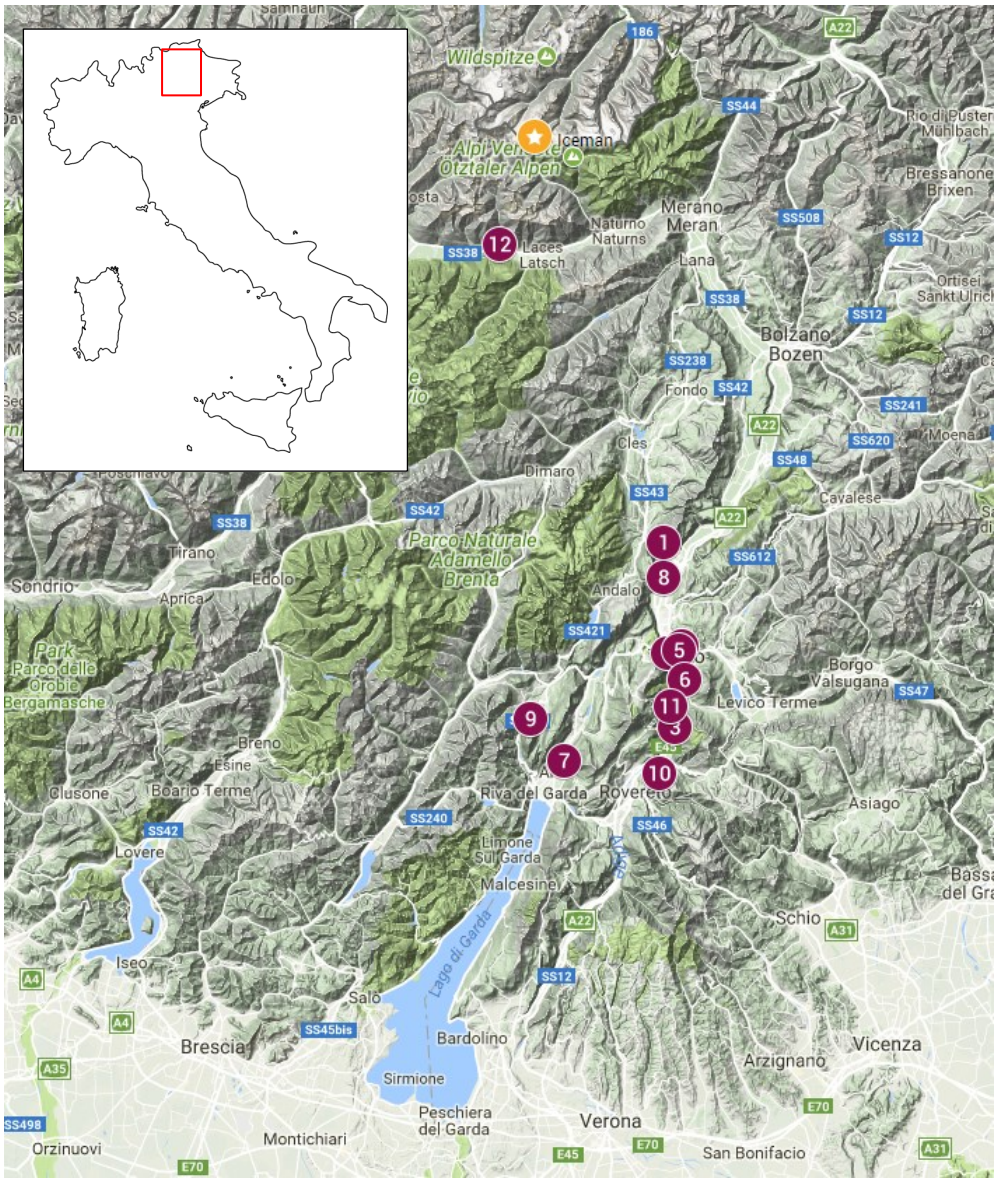
2 MATERIAL AND METHODS

2.1 Sample material

The samples used for this study were all derived from prehistoric human skeletal material dating from the Neolithic to Bronze Age. Samples were initially taken from a total of 66 individuals[3]. Where possible, at least one sample per site was subjected to radiocarbon dating if no reliable absolute or chronological data was available. Radiocarbon dates for the samples are given in Section 2.1.3. Some individuals which had previously been described as prehistoric proved to date to the Middle Ages or later and were thus not used for further analysis. The bulk of samples analysed in this study were kindly provided by the Museo Tridentino di Scienze Naturali in Trento and included skeletal human remains found in the Adige valley in the vicinity of Trento, mainly in rock shelters. Additionally, sample material from a Middle Bronze Age individual recently found near the village of Schlanders (South Tyrol) was provided by the Archaeological Museum in Bolzano. Finally, this general study also focuses upon genetic data of the Tyrolean Iceman, whose genome was published by a research group co-headed by the author of this thesis (Keller et al., 2012). The modern and ancient sample data used as references are listed in Section 2.3.14.

[3] This reflects the status quo during the sampling process: Later, some bones/teeth allegedly belonging to the same individual were from different individuals. After analysis and reconstruction of sampling/archiving circumstances, the actual number of individuals sampled was 76.

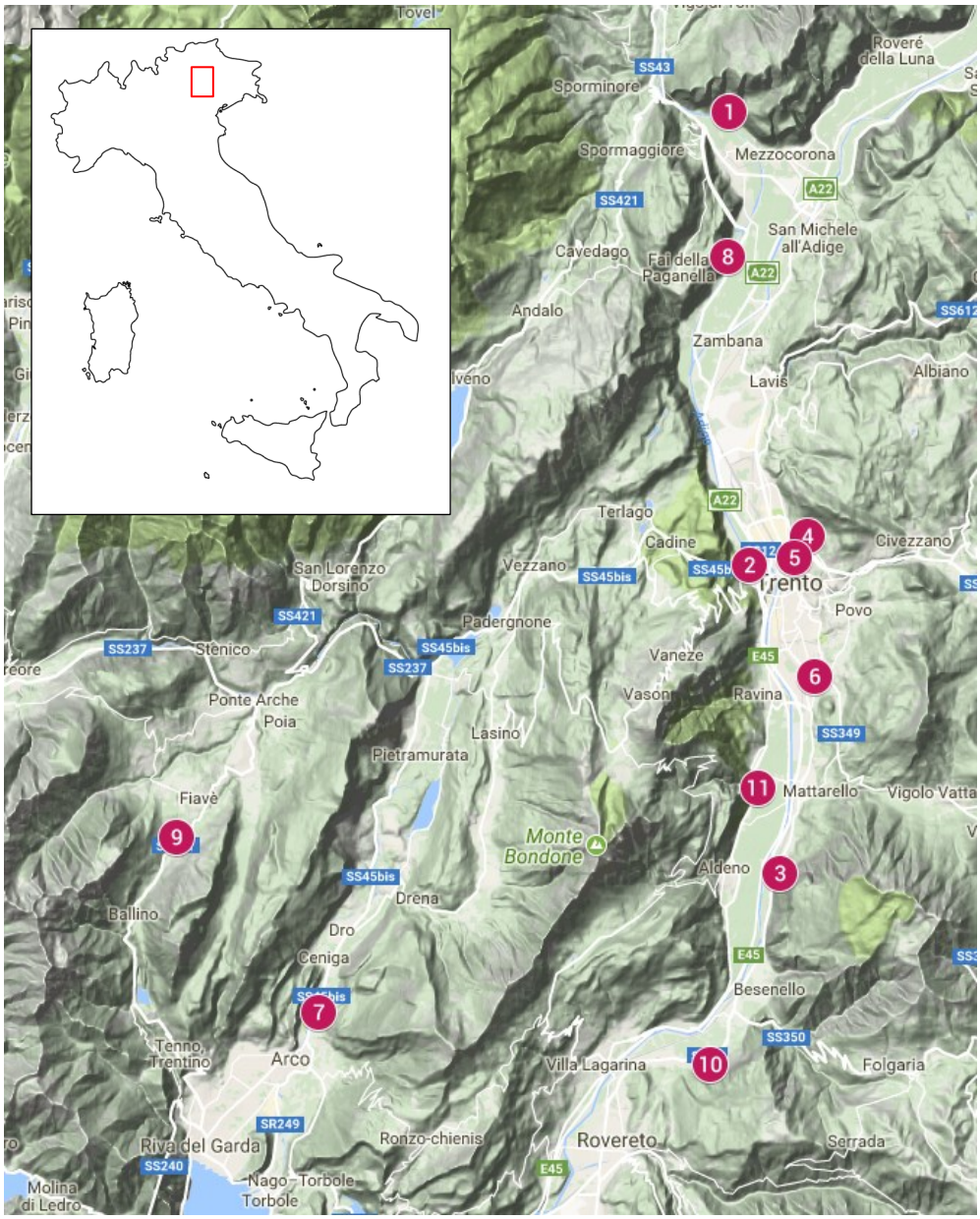
Figure 1: Map of Northern Italy showing location of sampled sites



1. Mezzocorona (NEO), 2. La Vela (NEO), 3. Acquaviva di Besenello (CA), 4. Martignano (CA), 5. Solteri (CA), 6. Madonna Bianca (CA), 7. Moletta Patone (CA), 8. Nogarole (CA & BA), 9. Fiavè (BA), 10. Paludei di Volano (BA), 11. Romagnano (BA), 12. Schlanders (LBA), ☼ Iceman find site

An enlarged map of the sites in the vicinity of Trento (1-11) is shown below. An interactive map of the sites in question can be accessed via the URL trento.bioarchaeology.de.

Figure 2: Map of the Adige Valley showing location of sampled sites



1. Mezzocorona (NEO), 2. La Vela (NEO), 3. Acquaviva di Besenello (CA),
4. Martignano (CA), 5. Solteri (CA), 6. Madonna Bianca (CA), 7. Moletta Patone (CA),
8. Nogarole (CA & BA), 9. Fiavè (BA), 10. Paludei di Volano (BA),
11. Romagnano (BA).

2.1.1 Characteristics of sampled sites

Table 1: Overview of sites and their archaeological characteristics

Site	Site type*	Ind. sampled here	Excavation year	Latitude Longitude
Acquaviva di Besenello	Rock shelter	1	1980	45.978993, 11.116682
Fiavè	Open lakeside	1	1947	45.990884, 10.831488
La Vela	Open, cist burials	6	1960-1976	46.080416, 11.102362
Madonna Bianca	Rock shelter	1	1986	46.043888, 11.133396
Martignano	Open field	1	1948	46.090131 11.129999
Mezzocorona	Rock shelter	2	N/A	46.229584, 11.092947
Moletta Patone	Rock shelter	11	1985	45.933248, 10.898639
Nogarole	Rock shelter	6	1985	46.182144, 11.092261
Paludei di Volano	Rock shelter	2	1978	45.916111, 11.084047
Romagnano	Rock shelter	30	1970- 1974	46.007295, 11.106394
Schlanders	Open-air site	1	2011	46.632845 10.772834
Solteri	Rock shelter	14	1962	46.082856, 11.123866
Tyrolean Iceman[4]	Alpine glacier	1	1991	46.779034, 10.840009

[4] The Tyrolean Iceman was not actually sampled in the course of this study (the history of this individual's genetic characterization is described in detail in section 2.1.2.13. However, as one of the aims of this study is to investigate the possible relationship between the newly studied samples and the Tyrolean Iceman (who was not only a contemporary of some of the samples, but also lived in the same greater region), site type and location are listed here to place the find into context.

2.1.2 Prehistoric samples

With the exception of Schlanders and the Tyrolean Iceman, all sampled individuals originated from sites along the Adige Valley in the vicinity of Trento.

2.1.2.1 Acquaviva di Besenello

Acquaviva di Besenello lies 10 km south of Trento along the Adige River. During construction works for a mineral water plant in 1977, anthropogenic layers were found within a rocky plateau. In the spring of 1980, the Tridentine Museum of Natural Sciences carried out excavation works of this area, which had already been partially disrupted by the construction works. The excavation brought to light several stratigraphic layers containing anthropogenic residues (Angelini et al., 1980). Trapezoid microliths found in the oldest layer document human use since the Early Mesolithic. This layer also contained red and roe deer, chamois and wild boar residues, as well as fish remains. Late Mesolithic layers contained traces of hearths, a Castelnovian lithic industry with bones from red and roe deer, wild boar and fish. Another late Mesolithic layer was difficult to differentiate from the above-lying layer: This combined part of the stratigraphy contained lithic implements of Castelnovian type, but also arrowheads of a shape similar to those observed in the VBQ. The faunal remains contain domesticates: sheep or goat as well as pig, but also red and roe deer. Although this does appear to indicate that Mesolithic traditions were upheld until well into the Neolithic, this cannot be determined with certainty due to the indistinct separation between the layers (Angelini et al., 1980; Riedel, 1982). Furthermore, it cannot be ascertained whether there is a "chronology gap" between the late Mesolithic and early Neolithic, as is the case for Romagnano III and Gaban, as no ^{14}C data have been published for these layers. Above Middle and Younger Neolithic layers (for which little description is provided), the Copper Age level contained a disturbed, incomplete burial of one individual, which was sampled for this study and dated to the Early Copper Age. The general osteometry and gracility of the

human skeletal remains (comprising several vertebrae, sternum, left humerus, radius, ulna and hand bones, left patella and several foot bones) indicated an adult individual of female sex (Corrain, 1982; Paladin, 2013). The Copper Age layer included the base of a copper smelting furnace. Grave goods included a flint knife, an almond-shaped flint tool and three arrowheads, one with a convex base and one with a shaft (Angelini et al., 1980). Analysis of the faunal remains showed that, although some domestic animal residues (caprines, pig), were found in the Chalcolithic strata, wild animals (primarily red deer) still formed the greater part of the Acquaviva subsistence strategy at that time, supporting the notion of a very long continuation of certain Mesolithic traditions (Riedel, 1982).

2.1.2.2 Fiavè-Carera

The prehistoric site of Fiavè, first described by Battaglia in 1947, was a lake-dwelling (stilt house) settlement near Trento. The settlement shows two phases of occupation: During the late Neolithic (3800- 3600 BCE, and again from the later early Bronze Age to the advanced middle Bronze Age (Perini, 1976) settlement was dated to the Bronze Age on the basis of the settlement characteristics and accompanying finds.

The sample used for this study could not be dated due to the small amount of sample material available (the other half of the molar analysed by Benedetto et al (Di Benedetto et al., 2000). This publication describes the sample as Neolithic, stating that this was defined on archaeological grounds; however, no specific publication was cited. Given that all found archaeological publications describing human remains from Fiavè refer to Bronze Age material (Battaglia, 1947; Corrain and Capitanio, 1967; Perini, 1987; Pedrotti, 1998a; Marzatico and Tecchiati, 2002), so it seems likely that this applies to the sample in question. This layer, Fiavè 2, coincides with the Polada A phase.

According to the preliminary investigation, the Bronze Age human remains found in the course of the excavations were those of 18 adults, 12 males and

6 females, mostly subadults of 14-18 years of age. Skulls were particularly abundant in contrast to other skeletal remains, and not always found in the vicinity of the postcranial elements, leading Battaglia to hypothesise a possible skull cult (Battaglia, 1947). A detailed osteological reinvestigation by Corrain and Capitanio (1967) largely confirmed the initial descriptions. The proximity to the lakeside led to the preservation of certain organic materials such as wooden cups or whisks (Dalmeri and Nicolodi, 2004). The site also revealed abundant faunal (mostly sheep, goats and cattle) and botanical remains, (including emmer and barley) indicating a well-established agricultural economy (Jarman, 1975; Jarman and Gamble, 1975).

DNA analysis for the sample used in this study was already attempted by Di Benedetto et al. (2000), but no DNA could be amplified from the sample.

2.1.2.3 La Vela

In 1960, during building work for a house in the town of La Vela near Trento, traces of a prehistoric settlement were brought to light. A first excavation was carried out in 1960, second and third campaigns were performed in 1975-1976 and 1987-1988. The earliest layers are attributed to the Early Mesolithic, containing typical lithic implements as well as roe deer remains, and to a lesser extent chamois, ibex, wild boar and badger. The Late Mesolithic layers are present but poorly documented, containing a few remains from red deer, chamois and ibex (Bazzanella, 1998; Bazzanella et al., 2000). Domesticates (caprines, pigs) first appear in the early Neolithic layers, but wild animals are still abundant, indicating that subsistence was based on both livestock and hunting (Bagolini, 1987; Pedrotti and Demetz, 1997; Bazzanella, 1998). while domesticates became more frequent in the Middle and late Neolithic. A clear shift in subsistence becomes apparent at the start of the VBQ, with oxen and other domesticates largely replacing deer (Bazzanella, 1998). In contrast to the Middle Neolithic layers, which have undergone extensive radiocarbon dating, no absolute dates have been

published for the Late Mesolithic. No statement can therefore be made regarding habitation during the phase in which a "gap" was observed for Romagnano and Gaban. The Middle Neolithic (VBQ) layers revealed a number of burials and were extensively excavated. A sandy layer (Layer E, 0.30-1.0 m) contained three burial cists of red limestone, placed in east-west orientation. One cist measured 1.10 m x 0.60 m, the other two could not be measured due to disruption by the excavation machinery. The former cist contained the skeleton of an adult, buried in a crouched position on its left side. Initial analysis described this individual as an adult male, whilst the two other cists contained the (disturbed) remains of two adults (ind. 1960/1). Furthermore, the remains of a child (ind. 1960/4) were found outside the cists. Osteological analyses of these human remains by Corrain and Capitanio (1967) confirm the sex of the undisturbed individual as male. The remains of the child are described as being approximately 5 years of age. The remains in the two disturbed cists are of (at least) two adults. The skull morphology of the adult individuals indicates male sex. Further excavations, carried out from 1975-76, revealed 3-more undisturbed skeletons, apparently all adult females, two aged 17-19, one aged 30-40 years (Capitanio, 1978). A third excavation campaign from 1987-1988 brought to light another 7 tombs containing remains of 4 adults and 4 subadults (Corrain and Capitanio, 1994). Some burials indicate a NW-SE orientation (Malone, 2003). The cist burials also yielded various artefacts: a polished, jadeite axe head, a dark stone chisel or axe, a large blade of grey flint, seven triangular arrowheads, a leaf-shaped arrowhead and a small triangular blade (Barfield, 1970). The layer above the anthropogenic layer contains alluvial material, which Barfield interprets as signs of erosion possibly due to deforestation for agricultural purposes (Barfield, 1970).

2.1.2.4 Madonna Bianca

In September 1985, a collection of lithic implements was discovered by chance in the hills near the town of Madonna Bianca-Malpensada, on the left bank of the Adige River. This material contained artefacts from the

Mesolithic to the Copper Age, initiating extensive survey work on the area. The following year, a small exploratory excavation was carried beneath a rocky outcrop and revealed a stratigraphic sequence, partially disturbed by building work. The site stratigraphy documents anthropogenic activity in the late Mesolithic, early Neolithic, Copper Age and Middle Bronze Age (Dalmeri and Nicolodi, 2004), although, lacking absolute data, it cannot be ascertained whether use was continuous from the Mesolithic to the Neolithic. Within a niche in the Copper Age layer, a burial consisting of several teeth, long bones and fragments of skull was discovered. The same area yielded a large arrowhead of the shafted Remedello type and a thin, rod-shaped bone pendant of a type traditionally interpreted as belonging to Bell Beaker culture and pottery sherds of Corded Ware type (Copper Age horizon Isera V). However, due to the disturbed stratigraphy, these artefacts cannot with certainty be associated with the burial (Angelini et al., 2002; Dalmeri and Nicolodi, 2004). As for other Copper and Bronze Age groups, Cucina (2002) documents a higher frequency of enamel hypoplasia for Madonna Bianca, inferring a possible focus on early agriculture (and thus a less varied diet).

2.1.2.5 Martignano

In March 1948, ploughing work in the north-west of Trento revealed a prehistoric grave. The owner of the farmland recovered the skeleton and handed it over to the archaeological cultural heritage department, who carried out a survey of the site. The poor preservation state of the skeleton is noted in the original report, characteristic for human remains buried in sand or exposed to the air. No mention is made in the original study of how the find was dated, but radiocarbon dating performed in the course of this study indicates placement within the Early Copper Age. The stratigraphic layer in which the remains had been found contained limestone slabs forming a rectangular cist of 85 x 60 cm, covered by an irregular slab. The skeleton itself lay in a crouched position on its right side directly on a sand layer. Part of the skull, mandible, the main long bones (incomplete), fragments of

scapula and pelvis were retrieved. Osteological characteristics indicated an adult male individual (Battaglia and Leonardi, 1950). No information is available regarding accompanying finds or faunal/plant remains.

2.1.2.6 Mezzocorona

Some confusion appears to have arisen with regard to the site name. Two Neolithic sites are known within the locality of Mezzocorona (slightly north of Nogarole): the Borgonuovo site with a documented chronology from the Sauveterrian and Castelnovian Mesolithic to the mid Neolithic/VBQ1 (Pedrotti, 1998b) and the Dos del la Forca site with known absolute dates from the Castelnovian Mesolithic to the early Neolithic (Gaban). As the first sample analysed in the course of this study (tooth) is the same individual studied by Benedetto et al. (Di Benedetto et al., 2000), it dates to the early Neolithic. Whether this sample was obtained from the Borgonuovo or the Dos de la Forca site could not be ascertained from the sample documentation and is not specified in the above-named publication. Although Di Benedetto et al. also analysed a different sample named "Borgonuovo", indicating that the first was more likely to be from the Dos de la Forca site, this remains ambiguous.

To add to the confusion, a second sample obtained for this study was labelled "Mezzocorona Tierknochen" (Tierknochen is German for animal bones), despite it being a human petrous bone[5]. Possibly due to some language misunderstanding, it seems to have been assumed at some point that "Mezzocorona Tierknochen" was the name of the site and was entered as such on the initial osteological report. According to Dalmeri & Nicolodi (2004), the exact location for "Mezzocorona Tierknochen" is indeterminate. The latter was ^{14}C dated to 4607-4536 cal. BCE in the course of this study, thereby dating to the mid Neolithic VBQ 2, thus somewhat younger than the sample studied by Benedetto et al (the other half of which was also analysed here), which was dated in the original study to the early Neolithic. Due to

[5] Identified as the petrous bone of an approximately 6-8-year-old female by A. Paladin, Master's Thesis

the ambiguity regarding the exact site, both samples are simply referred to as “Mezzocorona”. As the general locality and the date range are known, the precise allocation to one or the other site is of little consequence for the main purpose of this study. The molar studied by Benedetto et al. was sawn in half for that analysis; the second half was used for this study, thereby ensuring that any resulting sequences derive from the same individual. Di Benedetto et al. were able to identify mutations at positions 16126, 16292 and 16294 in the HVR1.

2.1.2.7 Moletta Patone

The site of Moletta Patone is a rock shelter near the town of Moletta which was excavated by B. Bagolini in 1981 (Angelini et al., 1982; Bagolini et al., 1984). The site yielded layers from the Late Mesolithic, the early Neolithic (Gaban), Middle Neolithic (VBQ) and the Copper, Bronze, Iron and Roman ages. Lacking data from the layers earlier than the Chalcolithic, no deductions may be made regarding continuity from the Mesolithic to the Neolithic, although there does seem to be a gap during the late Neolithic.

The Mesolithic layer is not described in detail, the only information provided is that the lithic implements date to the Castelnovian. The next layers date to the early Neolithic, containing pottery fragments characteristic of the Gaban group, and the Middle Neolithic, with VBQ potsherds and a spondylus pendant (Dalmeri and Nicolodi, 2004). The following Copper Age layer contained numerous disturbed burials, which were accompanied by stone implements (mainly knives and arrowheads) of Copper Age “Remedello” typology as well as other objects, such as a perforated jadeite plate, a bone awl as well as several copper spiral rings and beads wrapped in copper foil (Bagolini et al., 1984; Dalmeri and Nicolodi, 2004). The burials included the incomplete remains of 5 adults (3 females and 2 males, according to Corrain (1984)) and several subadults and infants. These appear to have been secondary burials. Dental analyses performed by Cucina et al. (1999; 2002) on samples from Neolithic to Bronze Age Trentino revealed an increasing rate of enamel defects such as hypoplasia in

the later samples, which is interpreted possibly resulting from a less varied, more carbohydrate-based diet (i.e. subsistence based on agriculture with little meat). However, the faunal analysis indicates no lack of meat, but rather an established animal husbandry. Faunal remains mainly consist of domesticated animals (63%), mainly cattle (11%), caprines (30%) and pigs (22%). The residual wild animals mainly comprise foxes, hares and cats which in all probability frequented the site when uninhabited by humans (Riedel, 1984). Several remains from this layer were sampled for this study. The remains were stored in individual boxes marked Scatolino 1-9. This was initially assumed to denote separate burials and sampled accordingly; however, at a later point, it became apparent on the basis of the osteological assemblies that there appears to have been some mixing due to the disturbed nature of the burials.

2.1.2.8 Nogarole

In March 1985, two archaeologists discovered a rock shelter with anthropogenic sediments near the locality of Nogarole di Mezzolombardo. The area is located on the right bank of the Noce River, just north of the Adige River. The spot itself is located at the base of the cliffs, in the region of the gravel quarries. The gradual depletion of one of these quarry pits revealed a series of rock shelters, approximately 30-35 metres above the current level of the quarry floor. Sondage trenches revealed three distinct areas with anthropogenic traces, which were named Nogarole I-III from south to north, the results of which are described in one publication to date (Bagolini et al., 1985). Directly south of these areas, traces of an ancient waterfall are visible in the rock. The first area, Nogarole I, consisted of a single level containing early Neolithic traces (the nature of which is not described in the initial report). The second sector, Nogarole II, contained several layers with anthropogenic traces. In the second level from the top, a tomb was discovered, covered by a mound of stones and placed against the rock face. The burials in this level of Nogarole II are placed in a flexed position on their left side, facing west and covered by rocks and can be

dated to the Copper Age. The grave goods included various necklace pendants (shells, beads and bones), an antler awl and a pedunculate flint. On the top layer, other items were found which may also be associated with this level, including two leaf-shaped blades, a boar's tusk with four drilled holes, an antler awl and a sherd of pottery with a horizontal corded pattern. The levels below the burial have not been excavated and might contain further inhumations. The burial can be attributed to the Eneolithic (Copper Age). The northernmost site, Nogarole III, has several anthropogenic layers. In a recess within the shelter, in the third layer from the top, a small and much disarrayed burial area with several burials was found against the rocky wall of the recess. The only accompanying finds were small clay objects. If front of the tomb, charcoal residues were found. The burials were attributed to the Bronze Age, although a more exact chronological or cultural placement was not possible due to lack of grave goods. The underlying anthropogenic level covers the entire floor surface of the shelter ($30m^2$) and contains the burials of babies and children in conical urns, reminiscent of those seen in the Polada A layer at Romagnano. These were in turn contained within a type of cist covered with a small stone pile. Similar urns with horizontal cording were found at the cliff edge, sometimes covered by stone piles, sometimes simply placed on the floor. These were surrounded by traces of charcoal, possibly indicating ritual fires as proposed for Romagnano (Marzatico and Tecchiati, 2002). While a Nogarole II sample was subjected to radiocarbon dating in the course of this study, Nogarole III was allocated to the Polada A phase of the Bronze Age based on the burial circumstances described above.

2.1.2.9 Paludei di Volano

Building work in the town of Paludei in the 1970s revealed prehistoric human traces near the rock wall on the eastern bank of the Adige valley. In 1978, excavations were carried out by the Tridentine Museum of Natural Sciences. The older levels revealed trapezoid blades and faunal remains (including fish) attributed to the Late Mesolithic, while younger layers contained the inhumations of three children, whose grave goods (two small

cups with handles) indicated a placement within the late Neolithic or Copper Age (Bagolini et al., 1980). Later layers revealed no anthropogenic materials (Dalmeri and Nicolodi, 2004). No faunal remains are reported. Radiocarbon dating of individual T3 revealed an age of 2281-2148 cal. BCE (Curt-Engelhorn-Zentrum Archäometrie gGmbH, 2012, this study), indicating that the individuals dated to the Early Bronze Age rather than the Copper Age. The burials contained three individuals, each in a good state of preservation. Tomb 1 (T1) contained the remains of either a small neonate or late foetus, with an incomplete and damaged skull, and fragments of vertebrae, pelvis, scapulae and long bones. Tomb 2 (T2) consisted of the remains of an approximately 1.5-year-old individual, the shape of whose schiatic notch indicated female sex. Tomb 3 contained the almost complete skeleton of a 12-year-old individual (age estimation based on osteometry of long bones), also described as female, based on general gracility, angle of frontal bone, size of mastoid and shape of the schiadic notch (Corrain and Erspamer, 1978).

2.1.2.10 Romagnano Loc

The prehistoric site of Romagnano Loc, adjacent to the Rio Bondone, was discovered at the foot of the cliffs in 1969 and excavated the following four years by the Tridentine Museum and the University of Ferrara. The excavation work revealed several areas with chronologies ranging from the Mesolithic to the Iron Age (Dalmeri and Nicolodi, 2004). Sector I (excavated in 1969) contained chronological layers from the early Neolithic, Bronze and Iron Ages. Sectors II and IV (excavated in 1970) brought to light various Bronze and Iron Age artefacts. Sector III yielded the most extensive chronology, with Mesolithic, Neolithic, Bronze and Iron Age layers (Bagolini, 1971; Perini, 1971, 1975).

Romagnano III, in particular, represents one of the most important Mesolithic sites in southern Europe. The radiocarbon dates for the earliest layers are the oldest Mesolithic dates yet determined, the oldest layer dating from the earliest Sauveterrian, 7880±90 BCE, with an uninterrupted

Mesolithic continuity until the mid-Castelnovian, 5550±160 B.C (Alessio et al., 1984). A layer dating to 4530±50 contains both Mesolithic-style microlith blades of local tradition and early Neolithic pottery bearing similarities to Impressed Ware and Fiorano type ceramics (Bagolini, 1971; Alessio et al., 1978; Broglio and Kozlowski, 1984), in combination with asymmetric arrowheads and rhomboid blades. This assemblage indicates both the persistence of Mesolithic traditions as well as contacts to early Neolithic groups from the Po Plain. This chronology reflects the general observation for the Mesolithic to Neolithic transition in the Trentino region: Although there is a "gap" in the chronology at the end of the Castelnovian from about 5500-5000 BCE, the lithic traditions seem to continue, reappearing at the start of the Neolithic.

The stratigraphy spans the Early and Mid-Neolithic, yielding pottery of the VBQ tradition, sparse final Neolithic/early Copper Age pottery and then an extensive early Bronze Age necropolis in sectors III and IV, dating to approx. 2300-2100 BCE 13 separate tombs containing 34 individuals, most (but not all) placed in flexed positions were determined, as well as two vessels: one larger jar containing two skulls, and several vases containing the bones of infants (Harding and Fokkens, 2013). A few scattered bones, not in any anatomical connection (*ossa erratiche*), were also found (Capitanio, 1970, 1973; Perini, 1975). The grave goods and the date clearly indicate affinity to the Polada A culture. Some aspects of the inhumations, with the body in a crouched position under a pile of stones, infant burials in vases, skull burials and secondary inhumations are reminiscent of those found in Borgonuovo di Mezzocorona, Nogarole di Mezzolombardo and Colombo di Mori (Marzatico and Tecchiati, 2002).

2.1.2.11 Schlanders

This individual was discovered in 2011 in a semi-alpine spot above Schlanders (Val Venosta, Vinschgau). The description of the find is as yet unpublished (personal information Andreas Putzer, Archaeological Museum Bolzano), but radiocarbon dates have attributed the skeleton an age of

1690-1530 cal. BCE, placing it within the latest stages of the early Bronze Age or the earlier phase of the Middle Bronze Age.

2.1.2.12 Solteri

The prehistoric cave-shelter site of Solteri, located north of Trento near the Martignano hill, was excavated by R. Perini from the Tridentine Museum in 1962 (Corrain and Capitanio, 1967). The excavation revealed anthropogenic traces in the Middle Neolithic (VBQ pottery) Bronze and Iron Ages (Mottes 1996). One undisturbed burial, that of an adult male, was found in the Copper Age layer. Three other graves contained numerous bones no longer in anatomical connection (Dalmeri and Nicolodi, 2004). A recent osteological analysis of these remains, which were dated in the course of this study to the Copper Age (Paladin, 2013) revealed a number of 13 individuals (11 adults and 2 children). Several individuals, including the undisturbed burial, were sampled for this study. No information is available on the exact typology of the accompanying finds in these layers or on any faunal remains.

2.1.2.13 Tyrolean Iceman

On September 19, 1991, two hikers discovered the body of a man, whose head, arms and torso were visible above the ice, at the Tisen Pass in the Ötztal Alps at an altitude of 3208 m. At first, it was assumed that the body was a more recent one (perhaps that of a climber who met with an accident) and the body was excavated with some haste from the shallow trench in which it lay, causing damage to the body and its accompanying finds (for example, the body's hip was damaged with a pickaxe when the body was freed from the ice, and both humeri were broken during recovery). The body was then transported to the Legal Medicine Department in Innsbruck in Austria, where archaeologist Konrad Spindler recognised the true age on the basis of the axe typology and thus the huge significance of the find.

The excellent preservation status of this find permitted insights into the Copper Age far beyond anything known to that point. The clothes were

intact, including a woven grass cape, leggings, a loincloth, shoes stuffed with grass and a bearskin cap. He had with him a hafted copper axe very similar in shape to those known from Remedello, a flint knife, birch containers, birch and tinder fungi and a small antler tool. The assembly also included an unfinished yew bow as well as a quiver containing 14 arrows, all but two yet unfletched.

Several morphological and radiological analyses were performed in Innsbruck. (general morphology and radiology) As exact measurements at the find site revealed that the mummy was in fact on the Italian side of the border, in 1998 the mummy was transferred to the Archaeological Museum of Bolzano, Italy, where specially equipped conservation facilities had been established.

Analysis of contents of what was initially thought to be the stomach (but later turned out to be the small intestine) revealed red deer, ibex and cereals (Rollo et al., 2002). More recent radiological investigations identified the stomach, which was not empty, as previously thought, but was in fact well-filled and had become displaced due to taphonomic processes (Gostner et al., 2011). As postgastric digestion of the stomach contents had not yet begun, the meal must have been eaten very shortly before death. An in-depth analysis combining microscopy and genomic/proteomic methods determined the stomach contents to consist of approx. 46% fat (muscle/adipose tissue of wild ruminants), ibex/red deer meat as well as einkorn grain (Maixner et al., 2018).

It was not until ten years after the find that Bolzano-based radiologist Paul Gostner discovered an arrowhead lodged in the mummy's shoulder (Gostner and Egarter Vigl, 2002). A multislice CT examination revealed extensive damage to the subclavian artery. Similar wounds are known from forensic case reports to lead to death from haemorrhagic shock within a short time if left untreated (Pernter et al., 2007), so this can certainly be regarded as the cause of death. Although the arrowhead has not been extracted, shape reconstructions from the CT images show that the arrowhead is also

reminiscent of Remedello typology. A head injury which had already been discovered in Innsbruck and was later studied in detail via proteomic analysis (Maixner et al., 2013). Several proteins related to blood, coagulation and stress response were identified. However, it is difficult to say whether this may indicate a certain time lapse between the blow and the time of death, or whether the blow may have occurred perimortally (not necessarily by the assailant, but possibly resulting from a fall after sustaining the arrow wound).

The additional discovery of a wound on the Iceman's right hand, between the thumb and the index finger (reminiscent of a wound sustained in a parry movement to ward off a knife), which showed first traces of a healing process, started to give an indication of a dramatic sequence of events during the Iceman's last days (Nerlich et al., 2003), and the pollen profile contained in the intestines documented that the Iceman had travelled from the valley bottom (around 1200 m) past the timber line (approx. 2500 m) to the high alpine glacier levels (above 3000 m) within the space of 32-33 hours before death (Oeggl et al., 2007).

This indicated a scenario in which the Iceman became involved in a conflict situation, in the course of which he sustained a wound to his hand fending off an attacker's knife. He then ascended the mountain, probably in flight from his pursuer. The fact that he partook of a large meal shortly before his death makes it likely that he thought himself safe. However, he was struck by an arrow – the entry angle indicated that he may have been crouched over his meal at the time, or that the assailant was standing further down (Zink et al., 2011).

Other analyses revealed insights into the Iceman's general health. The Iceman suffered from Helicobacter pylori (Maixner et al., 2016), gallstones (Gostner et al., 2011), degenerative arthritis and knee enthesopathies (Murphy et al., 2003; Gostner et al., 2011), dental pathologies (Seiler et al., 2013) which were possibly caused in part by the opportunistic parasite Treponema denticola (Maixner et al., 2014), arteriosclerosis (Murphy et al.,

2003), Lyme disease (Keller et al., 2012) and well as whipworm infestation (Dickson et al., 2000).

Since the find in 1991, the Iceman underwent several genetic analyses. The Iceman's mitochondrial HVR1 mutations were first sequenced by in 1994 (Handt et al., 1994) and identified as belonging to Haplogroup K1 in 2006 (Rollo et al., 2006). The complete mitochondrial genome was sequenced in 2008 (Ermini et al., 2008), permitting fine characterisation of the mitochondrial haplotype (and the insight, that the Iceman's mitochondrial haplotype – initially named K1*, later K1Ö (for "Ötzi") and ultimately K1f (Coia et al., 2016) – belongs to a novel branch not observed in other modern or ancient humans to date). The entire genome was sequenced four years later (Keller et al., 2012). Apart from revealing that the Iceman had brown eyes, belonged to blood group O, was lactose intolerant and had a predisposition for cardiovascular disease (which possibly facilitated the development of the arteriosclerosis detected radiologically), analysis of the Y chromosome showed that the Iceman belonged to Y-chromosomal haplogroup G2a-L91. Generally rare in modern populations, this haplogroup G2a is observed at higher frequencies in isolated regions such as Sardinia and Corsica (Keller et al., 2012; Rootsi et al., 2012; Francalacci et al., 2013) as well as in the Alpine region (Berger et al., 2013). This haplogroup, which has been detected in numerous prehistoric individuals, is described in more detail in Section 2.2.3. On a whole-genome level, the Iceman clustered most closely with extant Sardinian populations, an observation which was confirmed by Sikora et al. with other reference datasets (Sikora et al., 2014). This affinity indicates that the Iceman shares a more recent common ancestor with the Sardinian population than with other modern populations, from which it can be inferred that these variants were widespread during the late Neolithic, but disappeared in most areas as a result of demographic developments, only surviving at significant levels in isolated areas. Sardinia's largely Neolithic genetic heritage has been confirmed in studies involving both modern and ancient (Marcus et al., 2019) individuals.

To determine whether the Iceman's mitochondrial lineage (haplogroup K1f) still exists in populations from the same region, Coia et al. (2016) sequenced the mitogenomes of 42 individuals from the east Italian Alps and compared these and the Iceman's mitogenome with a worldwide database. Neither the Iceman's specific signature, nor a closely related line, was detected either in Alpine populations or in worldwide databases, leading the authors to conclude that the Iceman's branch no longer exists today.

2.1.3 Chronological overview

2.1.3.1 Radiocarbon dates determined for this study

As samples were selected and placed in a chronological context on the basis of the radiocarbon dates determined at the start of the study, these results are provided in this section.

With the exception of the Schlanders sample, the samples radiocarbon dated in the course of this study were analysed at the Curt-Engelhorn-Zentrum Archäometrie in Tübingen. The ^{14}C content was determined using the MICADAS AMS system. Collagen was extracted from the bone and the >30 kD phase was removed, freeze-dried and burned. The $C0_2$ was catalytically reduced to graphite. Calibration was performed using INTCAL09 and SwissCal 1.0 software.

The sample from Fiavè was not dated in the course of this study as insufficient material was available, date allocation was performed on the basis of the archaeological documentation.

Table 2: Radiocarbon dates determined in the course of this study

Site	Ind.	Sample material	^{14}C age (BP uncal)	±	^{13}C	Cal 1 sigma cal. BCE*	Cal 2 sigma cal. BCE*	Chrono-logical placement (according to Bagolini)
Acquaviva	A	Humerus diaphysis	4551	30	-19.8	3363-3125	3368-3104	Copper Age I
La Vela	60/1	Pars petrosa	5475	32	-16.7	4353-4270	4433-4257	mid Neolithic VBQ 2
	75/22	Tibia diaphysis	Insufficient collagen					
Martignano	73	Pars petrosa	4570	19	-23.2	3366-3200	3483-3129	Copper Age I
Mezzocorona Tierknochen	T1	Pars petrosa	5731	21	-22.7	4607-4536	4679-4501	early Neolithic Gaban
Moletta Patone	Sc. 1	Molar	4119	20	-16.2	2851-2626	2861-2581	Copper Age II
	Sc. 4	Molar	4046	19	-18.6	2617-2496	2826-2490	Copper Age II
Nogarole II	69 A	Pars petrosa	4207	19	-19.2	2886-2764	2891-2702	Copper Age
Paludei di Volano	5	Molar	3788	24	-19.2	2281-2148	2289-2141	Bronze Age
Romagnano III	89/T2	Femur diaphysis	3770	20	-23.9	2269-2142	2284-2136	early Bronze Age Polada A
Romagnano IV	58	Pars petrosa	3744	20	-17.9	2199-2066	2266-2043	early Bronze Age Polada A
Solteri	67	Femur diaphysis	4478	26	-27.4	3327-3097	3338-3032	Copper Age I

*cal. BCE unless stated otherwise

2.1.3.2 Radiocarbon dates obtained from available literature

Table 3: Available radiocarbon dates for relevant sites

Site	Ind.	calBCE	Chrono-logical placement	Laboratory	Reference
Madonna Bianca	B	2878-2836	Copper Age	Utrecht Van De Graaff Laboratorium UTC 10557.	Pedrotti 2001
Mezzocorona	2	6326–6444 BP (5478-5292 calBC)	Early Neolithic	University of Utrecht, 1998	Di Benedetto 2000
Romagnano	Rom. III 4.7, P2.3	3680-3580	Early Bronze Age	University of Rome, 1978 R-770a	Alessio 1978
Schlanders	1	1690-1530	Bronze Age	CEDAD – Centro di Datazione e Diagnostica, Dipartimento di Ingegneria dell'Innovazione, Università di Salento	Personal communication A. Putzer, Archaeological Museum Bolzano
Tyrolean Iceman		3370-3320 BC (34.3% probability), 3230-3100 BC (61.1% probability)[6] 3300-3100 supported by radiocarbon measurement of accompanying botanical material	Copper Age	Institute for Isotope Research and Nuclear Physics University of Vienna	Kutschera, 1994; Rom 1999

[6] The varying dates are due to so-called "wiggles" in the calibration curve, making it difficult to pinpoint a single age range in certain time periods (e.g. a curve plateau in the area of the Neolithic or Iron-Age "Hallstatt Plateau").

2.2 Genetic markers

The following section describes the loci analysed for the samples in question. Primer sequences are provided in Section 2.3.4.

2.2.1 Mitochondrial DNA

2.2.1.1 Hypervariable region 1 (HVR1)

The hypervariable regions of the mitochondrial DNA (mtDNA) include a high number of known polymorphic sites. As mitochondrial DNA is inherited maternally without recombination, these polymorphisms are used to reconstruct maternal relationships over distance and time. Due to the far higher copy number of mitochondrial DNA in each cell, amplification of the latter from ancient or forensic material is far more likely to be successful than nuclear DNA. For these reasons, until the more widespread application of whole-genome methods to ancient DNA research, a large part of the ancient DNA data obtained is limited to mtDNA, predominantly HVR1. For this study, the HVR1 stretch spanning from positions 15997-16409 was amplified, using a set of four standard primer pairs targeting fragments of 150-180 bp to accommodate the fragmented nature of ancient DNA[7]. As HVR1 testing permits general screening for all mitochondrial haplogroups, no "likely" haplogroups needed to be selected a priori (as is the case for Y-chromosomal haplogroups, as the defining mutations are spread across a far wider area). For this reason, information (such as frequency in other ancient and modern populations, possible origins) on the specific haplotypes detected in the Trentino samples are provided in the results section and reviewed in detail in the discussion section.

2.2.1.2 Haplogroup U (12308)

Haplogroup U is largely regarded as having developed in Europe and is therefore accepted as an indicator of Palaeolithic ancestry (Richards et al., 1998, 2000; Bramanti et al., 2009; Malyarchuk et al., 2010; Bollongino et

[7] Primer design methods and primer sequences are described in Section 2.3.4.

al., 2013), with almost all Eurasian hunter-gatherers tested to date having a sub-branch of U; mainly U5, but also U8 and U2 (Fu et al., 2016; Posth et al., 2016). However, as the main defining mutation for haplogroup U, position 12308A/G, lies outside the hypervariable region (Torroni et al., 1996), this was therefore tested in samples whose HVR1 mutations indicated a likely affinity to this haplogroup[8].

2.2.2 Functional and phenotypic markers

2.2.2.1 HERC2 (eye colour)

Since studies by Kayser et al. (2008), Eiberg et al. (2008) and Sturm et al. (2009) first demonstrated via genome-wide linkage studies that certain SNPs are highly predictive of eye colour, this technique has been developed further and used for forensic applications. One SNP that can reliably predict brown/non-brown eye colour even when regarded on its own is rs12913832 (A/G), a polymorphism located in the intron of the HERC2 gene on chromosome 15, with G/G almost always being linked with blue, green or grey eyes, and hetero- or homozygous A generally inferring a brown eye phenotype (Sturm et al., 2008). On the basis of frequency and distribution in modern populations, the derived allele is postulated by Eiberg et al. to have originated in the Black Sea region approximately 6-10 KYA and been spread to Europe in the course of the Neolithic. However, the detection of the G/G variant (denoting blue eye colour) in the Mesolithic Spanish La Braña individual (Lazaridis et al., 2014; Olalde et al., 2014) argues against a (sole) Neolithic origin for this trait. While the origin remains uncertain, it seems clear that the trait underwent strong positive selection throughout the next millennia in some regions. By comparing data from ancient Yamnaya and modern samples, Wilde et al. (2014) demonstrated that blue eye colour has been under strong selection for the last 5000 years. Mathieson et al. (2015a) observe differing patterns of selection, depending on region, with slightly negative selection in regions of southern Europe, indicating that the

[8] This test was introduced at a later stage of the study, at which point sample material was no longer available for all individuals and was therefore not routinely tested for all individuals.

trait may be more advantageous in northern than in southern regions. The derived blue eye colour variant was detected in farmer groups from Hungary (Gamba et al., 2014) as well as nomadic Bronze and Iron Age groups from Siberia and the Altai region (Keyser et al., 2009). A genomic analysis of Neolithic, Copper and Bronze Age individuals by Olalde et al. (2018) revealed a significant increase in frequency of this polymorphism between the Neolithic and the Bronze Age in Britain, occurring at the same time at which an almost complete genetic turnover occurred, associated with an influx of Beaker-related groups, indicating that the spread of this culture may well have contributed to increasing frequencies of the derived allele/blue eye colour.

In the course of the whole-genome analysis of the Tyrolean Iceman (Keller et al., 2012), specific analysis of pigmentation-related SNPs by this author revealed that the Iceman was homozygous for A/A at the rs12913832 site, indicating that this individual very probably had brown eyes. This stood in contrast with previous assumptions: Up to that point, the mummy's eyes had been described in popular literature as being blue, and the reconstruction of the individual displayed at the Archaeological Museum in Bolzano showed blue eyes accordingly. After the whole-genome results had been obtained and verified in separate PCRs, this author tried to determine how or when the blue-eyes hypothesis had originated. However, no specific source could be revealed. It may have arisen based on the fact that the mummy's irises now have a greyish colour as a result of pigment decomposition, which may have initially been interpreted as blue-grey colour (personal communication E. Egarter Vigl).

2.2.2.2 Lactase persistence (-13.910*T)

Lactase persistence, i.e. the ability to digest lactase (milk sugar) beyond early childhood, is closely associated with the derived T variant at the polymorphic site 13.910 bp upstream of the lactase gene (-13.910*T/C). Although rare in the early Neolithic, the derived allele underwent unparalleled positive selection until it became almost ubiquitous in many

regions of Europe (Burger et al., 2007; Itan et al., 2009). The spread of the derived allele is closely linked with the evolution of dairying, providing a selective advantage on those able to digest milk without the need of time-consuming processes to break down lactase, such as fermentation (Burger and Thomas, 2011). Computational models indicate that the trait was first selected approximately 7,500 years ago approximately between the Balkans and central Europe, and whose spread may have been linked to the *Linearbandkeramik* culture (Itan et al., 2009). However, more recent, extensive genomic studies of Bronze Age individuals show that the lactase persistence allele was (in contrast to light skin pigmentation) not yet particularly widespread in the Bronze Age, possibly indicating a more recent selection that previously thought (Haak et al., 2015; Olalde et al., 2018). In the latter study, Olalde et al. established an almost complete genetic turnover occurring in Britain between the Neolithic and the Bronze Age associated with the influx of the Beaker culture. However, at the same time, the lactase persistence allele remained rare, indicating that the increase of frequencies is not necessarily associated with the Beaker culture.

In modern European populations, the distribution follows a geographical cline, ranging from frequencies of as low as 10% in south-eastern Europe up to 95% in north-western Europe (Ingram et al., 2009).

Although lactase persistence is generally rare throughout most regions of the Italian peninsula, with frequencies of the derived allele in the region of 0.07-0.08 in Sardinia and southern Italy and 0.11-0.13 in central and northern-central Italy (Anagnostou et al., 2009; Santonocito et al., 2014), the region of North-eastern Italy (Bolzano, Bergamo, Udine, Castelmassa) shows much higher frequencies of 0.237 (Anagnostou et al., 2009). Whether this is due to differing selective pressure in this wider area or is a result of the ancestral population already possessing the derived allele is not clear. For this reason, the status of the C/T-13910 lactase allele in Neolithic/Bronze Age populations may help to clarify the origin of the allele in this region.

2.2.2.3 Amelogenin (sex determination)

The amelogenin locus, which is associated with tooth enamel production, is a useful tool for sex determination in forensic studies. Due to the length polymorphism (Y-chromosomal amelogenin is 112 bp in length, the x-chromosomal variant 106 bp), sex can be determined by means of gel electrophoresis alone without the need of sequencing the sample, provided that the nuclear DNA is sufficiently well-preserved (Mannucci et al., 1994). To be able to clearly distinguish the two bands from one another, either SDS-PAGE (polyacrylamide gel electrophoresis) or a higher concentration of standard agarose gel (3-4%) in combination with a low voltage current is required (see Section 2.3.7).

2.2.3 Y-chromosomal DNA

Due to the method used in this study (classical singleplex PCR rather than STRs, multiplex assay or genomic approach), samples could not be comprehensively screened for all possible haplogroups, as the defining mutations are spread across a large part of the Y-chromosome. Instead, Y-chromosomal analysis focused on the most likely haplogroups, which therefore had to be selected a priori, based on considerations described in the following.

Samples showing sufficiently promising nuclear DNA preservation and whose amelogenin samples and/or previous osteological analysis (Paladin, 2013) indicated male sex were first screened for M201, the defining marker for haplogroup G (a haplogroup observed in numerous prehistoric remains, including the Iceman). Samples showing the derived allele at this site were to be further subtyped for the markers described below; samples with the ancestral allele for M201 were to be screened for haplogroups E, I, R1a and R1b, which have also been observed in several ancient samples. As primers for the latter were only to be designed and set up when actually needed (i.e. haplogroup G subclade markers if any samples tested positive for G; other haplogroups if any samples tested negative for haplogroup G), only the primers actually used in this study are listed in Section 2.3.4.

2.2.3.1 Haplogroup G

Haplogroup G, defined by a G->T mutation at polymorphic site M201 (rs2032636) (Underhill et al., 2001), is strongly associated with the spread of the Neolithic to Europe (Battaglia et al., 2009; Rootsi et al., 2012), and has been found in numerous ancient samples to date. These include several Near Eastern Neolithic samples: one from Iran dating to the 8th millennium BCE (Broushaki et al., 2016), several from Neolithic Anatolia dating to the mid-7th millennium BCE (Mathieson et al., 2015a; Hofmanová et al., 2016; Lazaridis et al., 2016) and another from Iran from the early 6th millennium BCE. In Europe, this haplogroup was observed in high frequencies in individuals from the Starčevo culture, mid-6th millennium (Szécsényi-Nagy et al., 2015) as well as LBK and other individuals from Hungary and Germany (Haak et al., 2010, 2015; Brandt et al., 2013; Mathieson et al., 2015a; Szécsényi-Nagy et al., 2015; Lazaridis et al., 2016). Two Neolithic populations from France and Spain revealed this haplogroup in almost all individuals yielding autosomal DNA (Lacan et al., 2011a; 2011b). A late 4th/early 3rd millennium individual from Greece also showed this haplogroup (Hofmanová et al., 2016).

In the Copper and Bronze Ages, frequencies seem to be much lower: an overview of individuals from Bronze Age Eurasian cultures only found haplogroup G amongst Bronze Age Hungarians. The Iceman is one notable Copper Age individual with haplogroup G (Keller et al., 2012), which was also found in two individuals from the Copper Age El Mirador cave in Spain (Mathieson et al., 2015a). Haplogroup G seems to have been ousted in many regions by haplogroups R1a and R1b, which were spread as a result of the Steppe expansions, and survived, in particular, in isolated regions.

This specific haplogroup is most commonly observed today in the Caucasus (where it reaches a frequency of up to 70%), with lower frequencies (15-20%) in various regions of the Near East and southern Europe (Rootsi et al., 2012), but is rare in other parts in Europe, with the exception of geographic refugia such as Sardinia (Contu et al., 2008; Keller et al., 2012),

Corsica (Rootsi et al., 2012) and Tyrol (Berger et al., 2013), with particularly high frequencies (over 40%) in the more isolated Paznaun and Ötz Valleys in Austria. A similar pattern can be observed in the modern-day Trentino region, with frequencies reaching 49% in Val Primiero (Coia et al., 2013). On the basis of a calculated age for haplogroup G2 of 15,020 YBP, and its generally high frequency in Italy, Boattini (Boattini et al., 2013) suggests that G2 might actually indicate Mesolithic continuity rather than Neolithic introgression. However, this theory fails to explain the high levels of G2a in most Neolithic groups throughout Europe and in early Near Eastern Neolithic farmers, as well as its apparent absence in hunter-gatherers.

For individuals testing positive for G-M201, the following subclades are to be tested:

- Subclade G2, characterised by a G->T mutation at the P287 (rs4116820) SNP (Karafet et al., 2008) [9].
- Subclade G2a-L91, defined by a derived allele (C->T) at Y-chromosomal marker L91 (rs35474563). The haplogroup defined by this marker has been renamed several times (G2a4 in ISOGG Y-DNA haplogroup tree 2011, G2a1b2 in ISOGG Y-DNA haplogroup tree 2012, G2a21b in ISOGG Y-DNA haplogroup tree 2013). To avoid confusion, therefore, this haplogroup is simply termed G2a-L91 here. L91 was detected in the Tyrolean Iceman in the course of whole-genome sequencing (Keller et al., 2012), but is rare in modern populations, constituting only a small proportion of haplogroup G samples (Rootsi et al., 2012; Berger et al., 2013), although it was detected in several unrelated men in Tyrol (Austria) by Berger et al. (Berger et al., 2013). The frequency of this subtype in Alto Adige-Trentino has not yet been established.

[9] Nomenclature differs across various publications. In some cases, samples were described as G2a on the basis of the derived T allele at P287

2.2.3.2 Haplogroup E

Samples yielding nuclear (male) DNA (e.g. through amelogenin testing) are to be tested for haplogroup E, defined by marker M96 (rs930684), (Underhill et al., 2001). All haplogroup E individuals known from ancient sample sets belong to a sub-group of E1b1b. Haplogroup E has been identified in Mesolithic samples from Israel dating to approximately 11840-9760 BCE as well as early Neolithic samples (8300-7900 BCE) from Jordan (Lazaridis et al., 2016). Haplogroup E samples from the European Neolithic were found in Spain (Lacan et al., 2011b) and Hungary (Szécsényi-Nagy, 2015). This is consistent with the theory that haplogroup E was introduced to Europe in the course of the Neolithic (Semino et al., 1996; Hammer et al., 1998). In present populations, haplogroup E reaches relatively high frequencies in the Balkans and in Southern Italy. As is the case for haplogroup G, haplogroup E also seems to be rare in the Copper and Bronze Ages, only having been found in two middle Bronze Age samples from Armenia (Allentoft et al., 2015).

Samples testing positive for M96 are also to be tested for M215, which defines subclade E1b1b, the most common E subclade outside Africa.

2.2.3.3 Haplogroup I

Haplogroup I, characterised by marker M170, is occurs at high frequencies in modern European populations, especially in Scandinavia and the Balkans (Dupuy et al., 2006; Battaglia et al., 2009). Although overall levels in Italy are low (Di Giacomo et al., 2003), a few regions show higher rates. Frequency is particularly high in Sardinia, especially one particular subclade (M26) which is rare in other parts of Europe, consistent with a founder effect in Sardinia (Contu et al., 2008). According to simulations, the most recent common ancestor of M26 predates the start of the Neolithic in Sardinia by several thousand years. This gives rise to the assumption that – while the modern population of Sardinia shows a predominantly Neolithic origin (Marcus et al. 2019; Raveane et al., 2019) - Sardinia's Palaeolithic

and Mesolithic population must have been large and the lineage has since then undergone little drift.

Haplogroup I has been detected in several Palaeolithic samples from Germany, Switzerland, Italy and France (Fu et al., 2016), in the Mesolithic samples from Loschbour (Luxembourg) and Motala (Sweden) (Lazaridis et al., 2014). During the Neolithic, this haplogroup seems to become rarer, constituting only a small percent of the Y-haplogroups determined for Neolithic individuals to date. Several Copper Age individuals from the El Mirador cave in Spain had haplogroup I (Gomez-Sanchez et al., 2014), as did all three individuals from the North Italian Remedello necropolis who recently underwent genomic analysis, as well as Bronze Age individuals from Hungary and the Yamnaya group (Allentoft et al., 2015).

Due to the observations of haplogroup I in Palaeolithic remains and their distribution argue for a European Palaeolithic origin for this haplogroup (Semino et al., 2000). The varying clines of frequency and microsatellite diversity for the major subclades reveal that they probably originated in France and the Balkan region (Rootsi et al., 2004).

2.2.3.4 Haplogroup R1a and R1b

Together, haplogroup R1a (M420) and R1b (M343) represent the majority of haplogroups amongst modern Europeans, with R1b dominating in Western Europe and R1a in the East. These haplogroups were found in individuals from Russian steppe but appear to have been rare elsewhere until the Copper and Bronze Ages, where they were introduced into Europe in the course of the Yamnaya migrations from the Pontic Steppe during this time (Haak et al., 2015). Haplogroup G, which had been the predominant type during the European Neolithic, was largely replaced by R1a and R1a, indicating a significant population turnover (Allentoft et al., 2015; Haak et al., 2015; Underhill et al., 2015).

The earliest individual found to date with R1b was the Palaeolithic Villabruna sample (14,180-13,780 cal BP), indicating that R1 haplogroups

were not completely absent in Europe prior to the Bronze Age migrations. It was not until the Copper and Bronze Ages, however, that these haplogroups spread throughout Europe. The majority of Yamnaya samples belonged to R1b (Allentoft et al., 2015); the Corded Ware, on the other hand, showed higher frequencies of R1a (despite the high level of overall genetic similarity between the two) (Allentoft et al., 2015; Haak et al., 2015). A very high frequency R1b was observed amongst the Bell Beakers (Lee et al., 2012; Allentoft et al., 2015; Lazaridis et al., 2016; Olalde et al. 2018) which, bearing in mind this culture's expansion throughout Europe, is likely to represent the basis for this haplogroup's ubiquity in modern-day western Europe.

2.3 Ancient DNA analyses

2.3.1 General ancient DNA anticontamination and authentication measures

The analysis of ancient DNA required strict protocols to be observed in order to minimize the risk of contamination from exogenous DNA. This is particularly true when using PCR: high cycle numbers are required to achieve sufficient amplification of the fragmented and degraded genetic material which is typical for ancient samples, but this also co-amplifies any trace contaminants that can be targeted by the primers in question. General measures to minimise contamination included:

- Care was taken to avoid any possible introduction of contaminant DNA into the ancient DNA lab. This involved only entering the facilities when absolutely necessary; with only trained personnel allowed to enter the lab. All consumables, reagents, equipment etc. to enter the laboratory was externally cleaned with a soap solution and either bleach or AppliChem DNA-ExitusPlus.
- Protective clothing was worn at all times inside the ancient DNA lab area. These included a Tyvek overall with hood, a clean headscarf, paper shoes, a face mask, a visor, plastic sleeves and two pairs of

latex gloves (so that the superficial pair could be exchanged after critical steps without exposing the skin).

- A “one-way” laboratory routine: Steps were carried out in order of susceptibility to contamination, e.g. PCR setup would be performed before entering any other room in the ancient DNA lab, and neither the PCR nor the extraction area would be entered after handling samples in the milling/sawing or irradiation area. Furthermore, the ancient DNA area was never entered after work in the post-PCR area; the ancient DNA area was only entered after showering/changing clothes.

Measures to assess authenticity as described by Pääbo et al. (2004) included:

- Co-preparation and amplification of PCR and extraction controls (blanks without target DNA, but prepared from the same master mix and under the same condition)
- Wherever possible, repeated extraction from the same or from other extracts
- Reproduction in a second laboratory
- Comparison with modern samples: HVR1 sequences of all persons to enter the laboratory area were recorded in a database. These were routinely compared with sample results as well as with any occurring contaminants to identify possible contamination sources.

Further, step-specific anticontamination measures are described in detail in the next sections.

2.3.2 Selection and preparation of bone and tooth samples

Bones were visually inspected at their place of storage at the Museum in Trento. Even though surface contamination from excavators and museum personnel were to be expected, initial anti-contaminant measures in the form of face masks, hair nets and gloves were already implemented at this point to prevent further contamination. If available, teeth (preferably molars) or dense, solid bone such as from the femoral shaft or the pars petrosa of the temporal bone were selected for sampling, as removal of surface contaminants is easier and solid bone structure is more likely to yield intact genetic material. Bone material obviously discoloured by soil residues, as humic acids are known to inhibit PCR (Burger et al., 1999; Sutlovic et al., 2008), making successful and reproducible amplification less likely. Sampling was carried out in three campaigns: October 2011, November 2011 and April 2012. Selected bones and teeth were labelled according to the original documentation, individually packed and transported to the ancient DNA laboratory of the EURAC Institute for Mummies and the Iceman in Bolzano. Upon introduction to the ancient DNA laboratory, samples were first irradiated under UV light for 4 hours from each side.

Samples from which not all cancellous bone could be removed, or which appeared otherwise porous or damaged (e.g. cracked teeth) were additionally treated with bleach (immersion in 3% sodium hypochlorite for 30 seconds) to remove exogenous DNA that cannot easily be removed mechanically. The samples then underwent surface removal (and removal of cancellous bone, provided that enough compact bone material remained), either with the edge of a Dremel blade (e.g. for large, flat samples such as from the femur diaphysis) or with a sand blasting machine (for uneven surfaces such as teeth). The Dremel blades were exchanged prior to sawing in order to avoid transferring any residual surface contaminants to the interior. After surface removal, the bone/tooth samples were sawn into small fragments approx. 2-3 mm in size and irradiated once more from both sides. The small bone fragments were then pulverized in a Retsch mixer mill (oscillation speed and duration according to bone hardness) until a fine powder was obtained. This powder was divided into aliquots of 0.5 g each, filled into previously irradiated 15 ml Falcon tubes and frozen until further use. All surfaces and instruments coming into contact with the sample material (fume hood, saw blades, tweezers and spatulas, grinding jars and steel balls etc.) were treated with bleach for at least 15 minutes and all mobile parts were irradiated. Delicate machinery that could not be bleached was cleaned with detergent and treated with AppliChem DNA-ExitusPlus.

2.3.3 DNA extraction

All samples were extracted using a standard phenol-chloroform extraction technique. For initial lysis, 2500 µL/g pH8 0.5 M EDTA, 250 µL 5% sodium lauryl sarcosinate and 30 µL Proteinase K were added from a master mix to each 0.5 g powder aliquot intended for extraction plus to one empty Falcon tube as an extraction control. The caps were secured with parafilm against leakage and the contents mixed well by shaking. The Falcon tubes were then placed on a rotating tray in an incubator and left to incubate at 37°C overnight. After lysis, 3 mL phenol-chloroform-isoamyl alcohol (25:24:1) was added to each Falcon tube, shaken well for one minute and

subsequently centrifuged for 10 minutes at 4000 rpm. The resulting upper, aqueous phase of each sample was then transferred to a new 15 mL Falcon tube. 2.5 mL of phenol chloroform-isoamyl alcohol (25:24:1) was then added, the contents shaken, and the centrifugation step repeated. The upper aqueous phase was again transferred to a new Falcon tube and 2.5 mL of chloroform was added to remove phenolic residues. The shaking and centrifuging step was repeated once again. The aqueous phase was then transferred to previously irradiated 50 mL Amicons (50 kDa Amicon centrifugal filter unit, Amicon® Millipore), filled to 5 mL with irradiated HPLC water and centrifuged for approximately 3 minutes at 4000 rpm (somewhat longer in case of very discoloured samples) until approximately 150 mL liquid remained on the filter. Another 5 mL of water was added and the process repeated once, or several times in case of strongly coloured residues, indicating a high content of humic or fulvic acids which would otherwise inhibit PCR. Once the colour of the liquid was relatively clear, the residue was aliquoted into three 0.5 mL Eppendorf tubes and frozen until further use. The extraction control was subjected to the same procedure.

2.3.4 PCR primer design

Established primer pairs described in the literature or routinely used at the Mainz laboratory. Further primer pairs were designed in the course of this study using the DNAStar LaserGene PrimerSelect software. Primers used here are listed in the table below. For mitochondrial DNA, fragments of 160-200 bp are initially targeted. If these fail to amplify, smaller fragments (100-120 bp) are targeted. As nuclear DNA is generally present in far lower copy number, smaller fragments are usually targeted. The optimum annealing temperature for newly designed primer pairs was determined by means of a gradient PCR, starting with the temperature suggested by the primer design software.

As noted in Section 2.2.3, determination of the Y-chromosomal haplogroup followed a step-by-step approach, first focusing on haplogroup G and subsequently designing only those primers that were actually required (i.e.

primers targeting G subclades if G was confirmed, or primers targeting other haplogroups if samples tested negative for G). In other words, primer design was in part dependent on the first results (and not planned in full in advance). The results on which the primer design steps are based are provided in Section 3.4.3.

Table 4: Mitochondrial DNA primer pairs used in this study

Primer name	5' → 3' sequence	Annealing temp. [10]	Product length (bp)	SNP/locus	Reference
L15996	CTCCACCATTAGCACCCAAAGC	58°C	145	mtDNA HVR1 Standard	Endicott 2003
H16142	ATGTACTACAGGTGGTCAAG				Stone & Stoneking 1998
L16117	TACATTACTGCCAGCCACCAT	58°C	115	mtDNA HVR1 Standard	Haak 2005
H16233	GCTTTGGAGTTGCAGTTGATGTGT				Haak 2005
L16209	CCCCATGCTTACAAGCAAGT	58°C	138	mtDNA HVR1 Standard	Handt 1996
H16348	ATGGGGACGAAGGGATTTG				Haak 2005
L16287	CACTAGGATACCAACAAACC	58°C	122	mtDNA HVR1 Standard	Handt 1996
H16410	GCGGGATATTGATTTCACGG				Handt 1996
L16280	ACCCCTCACCCACTAGGATACC	55°C	52	mtDNA HVR1 Short	This study
H16333	AAGGGATTTGACTGTAATGTGCTA				This study
L16055	GAAGCAGATTTGGGTACCAC	58°C	87	mtDNA HVR1 Short	Handt 1996
H16142	ATGTACTACAGGTGGTCAAG				Stone & Stoneking 1998
L16209	CCCCATGCTTACAAGCAAGT	53°C	94	mtDNA HVR1 Short	Handt 1996
L16303	TGGCTTTATGTACTATGTAC				Handt 1996
L16117	TACATTACTGCCAGCCACCAT	58°C	100	mtDNA HVR1 Short	Haak 2005
H16218	TGTGTGATAGTTGAGGGTTG				Handt 1996
12303U	GATAACAGCTATCCATTGGTCTTAGGC	52°C	103	mtDNA 12308 A → G	Unterländer 2015
12352L	GGAAGTCAGGGTTAGGGTGGTTATAG				Unterländer 2015

[10] As specified in the original publication or as determined by gradient PCR. The annealing temperature specified by the primer design software sometimes differed significantly from the temperature that yielded the best results in a gradient PCR.

Table 5: Nuclear DNA primer pairs used in this study

Primer name	5' → 3' sequence	Annealing temp.	Product length (bp)	SNP/locus	Reference
Amel-A	CCCTGGGCTCTGTAAAGAATAGTG	55°C	106/112	Amelogenin X/Y	Mannucci 1994
Amel-B	ATCAGAGCTTAAACTGGGAAGCTG				Mannucci 1994
HERC2-A	GATCCAAGAGGCGAGGCCAGTT	55°C	33	Eye colour rs12913832 A → G	Graefen 2009
HERC2-B	GCCTCGGCCCCTGATGATGATA				Graefen 2009
Lac5u	GCGCTGGCAATACAGATAAGATA	53°C	111	Lactase rs4988235 C → T	Burger 2007
Lac5l	AATGCAGGGCTCAAAGAACAA				Burger 2007
Lact-A	CTGCGCTGGCAATACAGATA	55°C	118	Lactase rs4988235 C → T	Graefen 2009[11]
Lact-B	GTACTACTCCCCTTTTACCTCGTT				Graefen 2009

Table 6: Y-chromosomal primer pairs used in this study

Primer name	5' → 3' sequence	Annealing temp.	Product length (bp)	SNP/locus	Reference
L91-A	TTCTGGAGAGCACTAAGCCACTTCC	53°C	81	rs35474563 C → T	Graefen[12]
L91-B	CCAAAGCTGATCACATGAAAAAGATG				Graefen
M201-A	CTCAGATCTAATAATCCAGTATCAACTGA	59°C	72	rs2032636 G → T	Haak 2010
M201-B	CCTATCAGCTTCATCCAACACTAA				Haak 2010
P287-A	TTGCAACCCAGGGCACAAGAGAT	55°C	81	rs4116820 G → T	This study
P287-B	CTAAAGCCACTGGCACTGAATGCTC				This study

2.3.5 Polymerase chain reaction (PCR)

PCR setup, including preparation and aliquoting of reagents, was performed in designated ancient laboratories (at the EURAC Institute of Mummies and the Iceman in Bolzano, Italy, and later at the Palaeogenetics Group ancient DNA laboratory at the University of Mainz, Germany for replication purposes), both of which are spatially separate from post-PCR facilities. PCR set-up, as well as preparation/aliquoting of any necessary primers or reagents, was performed in a UV cabinet, which is closed on all sides with only two closable holes for the analyst's arms, which is cleaned with

[11] For singleplex amplification of the lactase locus, the Lac5u/Lac5l primer pair was used. The second primer pair for the lactase locus (Lact-A/Lact-B) had been originally designed for a multiplex PCR in order to align annealing temperatures of the primer pairs run together (Lact, HERC2 and Amel). The second lactase primer pair was therefore only used if a multiplex or duplex was run.

[12] In the course of the Iceman Genome study (Keller et al. 2012)

detergent. The cabinet's clear walls and the pipettes were cleaned with detergent and subsequently treated with AppliChem DNA-ExitusPlus, whereas less sensitive surfaces such as benchtop, pipette tip boxes, glove boxes and general surface areas were treated with 3% sodium hypochlorite solution after cleaning. The cabinet, as well as the pipettes and pipette tip boxes were irradiated for 4 hours prior to use. No consumables (tubes, pipette tips) or reagents were opened outside the ancient DNA laboratory area. Tube preparation protocol differed slightly between the two laboratories: In Mainz, tubes and pipette tips are irradiated prior to use. In Bolzano, tube and pipette tip boxes are wiped down with sodium hypochlorite solution and only opened inside the PCR box, based on the assumption that the irradiation procedure with open boxes may in itself pose a contamination risk. When working inside the PCR cabinet, the analyst wears two pairs of gloves and irradiated plastic sleeves. PCR was set up as a master mix, with target DNA added after the mixture is divided amongst the requisite number of tubes. At least one blank control containing HPLC-H_20 instead of target DNA was set up for each PCR. Additional tubes were reserved for positive controls. Modern DNA was added for the latter in a separate post-PCR area. As a general rule, thermocycling was performed at a low cycle number for ancient mtDNA to prevent co-amplification of contaminants. This cycle number was increased for nuclear DNA and experimentally for mitochondrial samples whose amplification was present but too faint to be successfully sequenced, although these were discarded if the blank controls were co-amplified.

2.3.6 Multiplex PCR

In some cases, multiplex PCR was applied (several primer pairs to amplify more than one target in one PCR). Multiplex PCR poses several advantages: several loci can be analysed in one step and it saves sample extract (which is often limited for ancient material), as a multiplex PCR requires the same amount on extract as a singleplex would. However, primer pairs used together must have similar annealing temperatures, and should not interact

to form undesired products. A nuclear multiplex had been established in an earlier study (Graefen, 2009) in which the HERC2, Amel and Lact primers (as described in Section 2.3.4) were used. Although a functioning primer pair for the lactase locus had already been available (Burger et al., 2007), a new pair was designed to obtain a higher annealing temperature in line with the other primers. In some cases, HERC2 and Lact were run together as a duplex (excluding Amel), as this did not require higher-resolution gel electrophoresis (see next Section).

2.3.7 Gel electrophoresis

PCR amplicons were visualised by means of standard agarose gel electrophoresis, using 2% agarose gel to accommodate the small fragment size (< 200 bp) of the amplicons studied here. A higher gel concentration (4%) was used for amelogenin products at lower voltage and higher runtime in order to be able to differentiate the 106/112 bp bands. Ethidium bromide (EtBr) was used as a dye and 1x tris-borate was used as electrophoresis buffer. To facilitate gel loading, 1 µl of loading dye was mixed to the 10 µl of PCR product added to each lane. 1.5 µl of a 50 bp ladder (GeneRuler) was added to one or both outermost lanes for size comparison purposes. The gels were then illuminated ultraviolet light (which causes the amplicons to fluoresce due to the intercalated EtBr) and photographed. The gels as well as all instruments, solutions and cleaning utensils coming into contact with EtBr were handled with nitrile gloves and disposed of accordingly.

2.3.8 DNA purification (PCR product)

PCR products were purified prior to cycle sequencing in order to remove substances that would have an adverse effect on sequencing (e.g. dNTPs or primers). The silica-based MSB®Spin PCRapace kit (Invitek) was used for this purpose according to the manufacturer's instructions: 250 µl was added to the PCR product, vortexed and transferred to the silica membrane, and then centrifuged at 12,000 rpm to remove superfluous substances (sufficiently sized DNA adhering to the membrane via salt bridging). After

addition of a low-salt elution buffer (to break up the aforementioned salt bridging) and a 10-minute incubation period, the DNA is centrifuged down into a clean tube by centrifugation at 10,000 rpm for one minute. The eluate containing the purified DNA is then frozen at -20°C until required for cycle sequencing.

2.3.9 Cycle sequencing

Cycle sequencing was performed according to the standard method described by Sanger (1977), involving the random incorporation of fluorescence-marked ddNTPs which can be detected via capillary electrophoresis. Cycle sequencing setup involved 1.5 µl of sequencing buffer (5x), 1.0 µl of BigDye® Terminator v1.1 Cycle Sequencing reagent, 1.0 µl of the respective primer (products were generally sequenced with each primer in turn), 1.0-2.0 µl of PCR product (1.0 µl for normal products, 2.0 µl for weaker products) and filled to 10 µl with HPLC-H_2O. The thermocycler program involved 25 cycles, each cycle involving a denaturation step at 92°C for 30 seconds, an annealing step at the primer's optimum temperature (53-59°C) for 15 seconds and an elongation step at 60°C for 2.5 minutes.

2.3.10 Purification II (cycle sequencing product)

Cycle sequencing products were purified prior to capillary electrophoresis to remove substances that would adversely affect the electrophoresis process, such as non-incorporated dNTPs and ddNTPs. Purification was conducted using a sephadex-based gel. To prepare the purification gel for sequencing on the ABI PRISM® 3100 genetic analyzer, Sephadex™ G-50 fine (GE Healthcare Bio-Sciences) powder was distributed into each well of a gel column plate by means of a distribution plate, with 300 µl HPLC-H_2O added to each and left to well up for 2.5 hours at 4°C. After centrifuging off surplus water for 3 minutes at 2750 rpm, the gel was cleaned by adding another 150 µl to each well and centrifuging off once more. For purification of the cycle sequencing products, 7 µl of formamide was added to each well

of a 96-well plate in order to prevent DNA renaturation and to ensure the minimum volume required for capillary electrophoresis. The gel column plate is placed on top of the 96-well plate and the cycle sequencing products pipetted into the centre of each gel column. The block was then centrifuged at 2750 rpm for 5 minutes, causing larger DNA strands to pass through the gel into the formamide-filled wells, while retaining smaller fragments under 10 bp (dNTPs and ddNTPs) in the gel matrix. After centrifugation, the gel column plate was discarded, and the 96-well plate labelled and sealed with a rubber lid and refrigerated until capillary electrophoresis. For sequencing using the ABI PRISM® 310 genetic analyzer, samples were purified using the same reagents, only using single tubes rather than a 96-well tray.

2.3.11 Capillary electrophoresis

Capillary electrophoresis was carried out using the ABI PRISM® 310 genetic analyzer (Applied Biosystems) at the Bolzano laboratory and the ABI PRISM® 3100 genetic analyzer (Applied Biosystems) at the Mainz laboratory. Injection time and runtime were set individually for each fragment/sample, based on the fragment length and sample concentration. Performance enhanced polymer (POP-6) was used as a separation matrix. The light signals emitted by the ddNTPs through stimulation by the instrument's argon laser are detected by a CCD camera, which is translated into an electropherogram by the ABI PRISM® DNA Sequencing Analysis Software.

2.3.12 Sequence analysis

Initial sequence analysis (general sequence quality, review and correction of basecalls) was performed using ABI PRISM® DNA Sequencing Analysis Software. Sequences were then aligned against the respective reference sequence using the DNASTAR Lasergene SeqMan application.

2.3.13 Scoring system

A variety of different criteria for establishing authenticity of ancient DNA results have been proposed and applied by different authors (Cooper and Poinar, 2000; Hofreiter et al., 2001; Pääbo et al., 2004; Willerslev and Cooper, 2005). These include assessment of fragment size, screening for typical degradation products (e.g. uracil resulting from the deamination of cytosine), blank controls, stringent cleanroom protocol and reproducibility. For the latter, confirmation of results in at least three PCRs from two different extracts (and, where possible, in two different laboratories) is desired. However, for the reasons given in Section 3.2.1, repeating the procedure with a second extract was not possible in each case.

Therefore, a scoring system was developed to determine overall sequence quality, taking the following factors into account:

A) Number of extracts from which results were obtained
B) Replication in two separate laboratories (2 points for samples replicated in a second laboratory, otherwise no points)
C) Overall sequence quality (score from 0=very poor to 5= very good)
D) Each amplicon reproduced in at least 2 PCRs

The overall score is calculated as follows:

2A + B + C = Score

In addition, each amplicon must be reproduced in at least 2 PCRs (D). If this is not the case, then the sequence does not meet the criteria, regardless of the score.

Samples gaining at least 8 points (of a maximum number of 11) are regarded as meeting authenticity criteria and were taken into regard for statistical analyses.

For example: If a sequence is obtained from only one extract (A=1), it can only meet the overall requirements if the result is reproduced in a second

laboratory, sequences are of good (=4) quality and the amplicons in question are covered in at least 2 PCRs. Samples obtained from only one extract and not replicated in another laboratory cannot achieve the required score, even with very good sequence quality.

Additional requirements apply with regard to HVR1 sequences, which are generated through four overlapping amplicons. Each of these amplicons must be reproduced in at least two PCRs in order to be considered in full. Sequences which could only be partially reproduced (i.e. only three of the four amplicons could be reproduced at least twice) were taken into consideration as shortened sequences (loci not reproduced twice were denoted as unknown), provided that the overall score was sufficient. Sequences lacking more than one of the four amplicons were not used for statistical analysis.

All samples which yielded results but were not used for statistical analyses for reasons described above are discussed individually, depending on the likelihood of authenticity (which will then be addressed on a case-by-case basis).

2.3.14 Reference data sets

The reference data sets listed below are used for population genetic analysis of mitochondrial sequences. Wherever a sufficient number of individual sequences were available, published sequences were only included in the respective reference data sets if they covered all positions in which polymorphisms were observed in the Trentino group (16037-16362; for details, see Section 3.4.1). Inclusion of too many samples with missing data leads to certain loci of interest being disregarded by the Arlequin software, potentially distorting the result.

Exceptions were made for sample sets which would otherwise include less than 20 individuals. To ensure that all significant loci were used by the Arlequin software, the allowed missing level per site was changed from the standard setting of 0.05 to 0.08.

2.3.14.1 Ancient DNA

Reference sample groups/populations were selected according to relevance to the population analysed in the course of this study, analysis/replication according to generally accepted standards and minimum sequence length of ~250 bp. Populations of direct relevance regarding ancestry of the Trentino groups are described individually in the following sections.

Table 7: Ancient reference sample groups

Group	Abbr.	n	References[13]
Western Hunter-Gatherers	WHG	30	Bramanti 2009, Hervella 2012, Fu 2013, Lazaridis 2014, Bollongino 2013, Haak 2015, Posth 2016, Fu 2016
Pre-Pottery Neolithic	PPN	18	Fernandez 2014, Kılınç 2016, Broushaki 2016
Anatolian and Aegean Neolithic	AAN	30	Hofmanová 2016, Lazaridis 2016, Mathieson 2015
Starčevo	STA	31	Szécsényi-Nagy 2015, Hervella 2015
Linearbandkeramik	LBK	72	Haak 2005, 2010, 2015, Brotherton 2013, Brandt 2013, Mathieson 2015
Yamnaya	YAM	39	Wilde 2014, Haak 2015, Mathieson 2015, Allentoft 2015
Corded Ware	CW	29	Adler 2012, Allentoft 2015, Mathieson 2015, Brandt 2013, Brotherton 2013, Haak 2008, 2015, Lazaridis 2016
Bell Beaker	BB	36	Allentoft 2015, Lee 2012, Brandt 2013, Mathieson 2015, Lazaridis 2016, Haak 2015
Únětice	UNE	28	Adler 2012, Haak 2015, Mathieson 2015, Allentoft 2015, Brandt 2013, Brotherton 2013, Lazaridis 2016
Neolithic France	NEF	29	Lacan 2011
Neolithic Spain	NES	58	Hervella 2012, Lacan 2011, Fregal 2017
Nuraghi (BA Sardinia)	BAS	32	Caramelli 2007, Der Sarkissian 2011
Etruscans	ETR	42	Vernesi 2004, Ghirotto 2013
Remedello	REM	3	Allentoft 2015

[13] Samples and references are described in detail in supplementary information S2

2.3.14.1.1 Western Hunter-Gatherers

Hunter-gatherer populations who inhabited Europe during the Palaeolithic and Mesolithic, prior to the advent of the Neolithic pioneers, generally show a very different genetic signature to the latter (documented by very high F_{ST} values between the two). Almost all hunter-gatherer individuals analysed to date belong to mitochondrial haplogroup U, predominantly U2, U5 and U8 (Bramanti et al., 2009; Bollongino et al., 2013; Fu et al., 2016; Posth et al., 2016), haplogroups that are rare in subsequent farming groups. In general, haplogroup U5 is regarded as an indicator for hunter-gatherer ancestry and is therefore suitable for identifying such genetic contribution in Neolithic and Bronze Age groups. Although Y-chromosomal data is naturally more sparse than mitochondrial data (due to the fact that cells of male individuals contain only one copy of the Y-chromosome DNA, but thousands of copies of the mitochondrial genome, making the latter far more likely to survive degradation processes in copy numbers sufficient for analysis), haplogroups I, C and F have been identified in several Palaeolithic and Mesolithic individuals (Fu et al., 2016; Posth et al., 2016) as well as R1b in the case of the Palaeolithic individual from Villabruna, Italy (Fu et al., 2016).

Archaeological data shows that, in some cases, hunter-gatherer subsistence strategies persisted long after the appearance of the first farmers in Europe. For example, at the Blätterhöhle site in Germany, one group of burials could be identified as Mesolithic hunter-gatherers solely on the basis of the ^{14}C data, while the other age group fell into the Neolithic period. However, stable isotope signatures indicated that this latter could be clearly divided into two subsistence groups: one comprising farmers and one deriving their food mainly from hunting and fishing, the other from domestic herbivores (Bollongino et al., 2013).

Western hunter-gatherer groups (WHG) show a somewhat different genetic composition to Caucasian hunter-gatherers (CHG), the latter constituting a significant part of the ancestry of the Yamnaya herders who, in turn, had a significant impact on western European populations during the Bronze Age

(Haak et al., 2015; Jones et al., 2015). In order to identify any possible local Palaeolithic and Mesolithic ancestry, and to differentiate this from potential Yamnaya lineages in the samples analysed in the course of this study, only Western hunter-gatherers are used in this reference sample set.

2.3.14.1.2 Pre-pottery Neolithic

The first Neolithic cultures emerged in the fertile crescent in the 10th millennium BCE, spreading to Anatolia by the 8th millennium (Ammerman and Cavalli-Sforza, 1984; Kılınç et al., 2016) and from there rapidly spreading to Europe via two main routes, namely the Danubian/Balkan route on the one hand and the Mediterranean route on the other (Price 2000; Burger and Thomas, 2011). Recent analyses have shown that genetic diversity was low in the early pre-pottery Neolithic (PPN) groups (Kılınç et al., 2016), and that modern haplogroup frequencies from the fertile crescent do not necessarily reflect the genetic composition of the original farmers from this region (Fernandez et al., 2014; Broushaki et al., 2016). Therefore, pre-pottery Neolithic individuals from Syria, Anatolia and Iran are observed as a separate reference group. Several samples from the data set of Fernandez et al. were disregarded due to the sequence length of <244 bp.

2.3.14.1.3 Anatolian and Aegean Neolithic

Recent studies (Kılınç et al., 2016) have shown that pottery Neolithic groups existing in Anatolia after the initial westward spread of the Neolithic had a higher level of genetic diversity than the pre-pottery groups, most likely due to the more isolated social structuring of the latter (comparable to hunter-gatherer groups). These analyses revealed that the early central Anatolian groups shared the same gene pool as modern Europeans, as did early Neolithic groups in the Aegean (Hofmanová et al., 2016). These groups can therefore be regarded as the predecessors of the early European Neolithic farmers, and are regarded as a separate reference group.

2.3.14.1.4 Starčevo

The Starčevo was an early Neolithic culture ranging throughout the Balkans and the Carpathian Basin during the 6th century BCE, and represents the earliest advance of the Neolithic from the fertile crescent into Europe. The theory that the Starčevo culture was the precursor of the LBK, which spread northward from Transdanubia into much of Central Europe, was confirmed in a genetic comparison of Starčevo and LBK samples, which revealed close affinity between the two (Hervella et al., 2015; Szécsényi-Nagy et al., 2015). The majority of the 31 Starčevo samples used for reference purposes in this study are from Hungary, with five from Croatia and four from Romania.

2.3.14.1.5 Linearbandkeramik

As elucidated in Section 1.1, the *Linearbandkeramik* (LBK, Linear Pottery) culture represent the spread of the Neolithic to Central Europe via the Danube/Balkan route, reaching Germany by about 5500 BCE Genetic studies on LBK individuals revealed that these earliest farmers in Central Europe are very different from the contemporary hunter-gatherers, reflected by a very high F_{ST} value. Instead of the mitochondrial U haplogroups observed in hunter-gatherers, LBK samples showed a variety of mtDNA haplogroups, including a variant of N1a which is exceedingly rare in modern populations (Haak et al., 2005; Bramanti et al., 2009). The same applies to Y-chromosomal haplogroup G2a, which seems to have been widespread in LBK populations, but is rare in most parts of modern Europe, with the exception of some geographically isolated regions (see Section 2.2.5.1). This “dilution” of LBK genetics can most probably be explained, at least to some extent, by the subsequent Bronze Age migrations from the Pontic steppe, the impact of which was demonstrated in recent studies (Allentoft et al., 2015; Haak et al., 2015), (see also Section 1.2). The LBK reference data set includes samples from archaeologically confirmed LBK sites mainly from Germany, with one individual from Austria and four from Hungary.

2.3.14.1.6 Yamnaya

The Yamnaya Culture originated in the Pontic Steppe during the late Copper and early Bronze Age (approx. 3,300–2,700 BCE), and are associated with "Steppe Hypothesis", a massive population migration from the Steppe region into central Europe during this time, which is regarded as the source of the Indo-European language (Anthony, 2010). According to recent genomic analyses (Haak et al., 2015), the Yamnaya population combined local hunter-gatherer with Near Eastern ancestry – and, in turn, contributed significantly to the Corded Ware Culture, which had an approximately 75% Yamnaya component.

Wagons and carts had been common in Yamnaya burial contexts in the Pontic Steppe, and horse domestication had already become established in this region. The westward Yamnaya migration introduced these new technologies to Western Europe and may also account for the re-emergence of hunter-gatherer haplogroups during the Bronze Age, after they had initially become scarce during the Neolithic (Brandt et al., 2013). Furthermore, the derived allele for lactase persistence was observed in some Yamnaya and other steppe samples (Wilde et al., 2014; Allentoft et al., 2015). Although the derived allele is still in the minority, these groups have the highest derived allele frequencies of any ancient groups, leading to speculation regarding a possible Steppe origin for this trait (Allentoft et al., 2015).

2.3.14.1.7 Corded Ware

The Corded Ware culture (German *Schnurkeramik*), named after the characteristic pottery decoration style by impressing a twisted cord into the clay, spread across a huge part of Central and Northern Europe during the late Neolithic to Early Bronze Age (approx. 2800-2200 BCE). Recent genomic analyses revealed that Corded Ware populations had a very high proportion of Yamnaya ancestry (approx. 75%), driven by migrations from the Pontic Steppe, although this may also have been in part from a pre-Yamnaya steppe population (Haak et al., 2015). Mitochondrial

haplogroup distribution is highly similar to the Yamnaya, whereas Y-chromosomal haplogroup frequencies differ with regard to R1a and R1b – the latter being the most frequent in Yamnaya groups, and the former more frequent in Corded Ware populations. Stable isotope analyses have demonstrated that Corded Ware groups were highly mobile, especially women, which is interpreted as a system of female exogamy (Sjögren et al., 2016). Corded Ware-type pottery found at some of the Trentino sites sampled for this study (see section 2.1.3) indicate that direct cultural contact probably existed.

2.3.14.1.8 Bell Beaker culture

As described in detail in Section 1.4.3, the Copper/Bronze Age Bell Beaker culture is of particular relevance to the Bronze Age samples analysed in the course of this study, not least because direct cultural contacts are documented from 2500 BCE onwards. The Bell Beaker samples used as a reference group for this study are derived from various sites from East and South Germany and the Czech Republic. As exemplified by the diagram below, the haplogroup distribution differs significantly from the first farmers in Central Europe: haplogroup U, which was predominant in hunter-gatherers but exceedingly rare during the Neolithic, becomes far more frequent, constituting over one quarter of all samples in the data set. This return of haplogroup U frequency was initially interpreted as a re-emergence of remaining local hunter-gatherer groups and their admixture with established farming populations. However, high haplogroup U frequencies in Yamnaya samples indicates that haplogroup U is likely to have been reintroduced to Central and Western Europe at significant levels during the migrations from the Pontic steppe of the Bronze Age, most likely through the Bell Beaker culture (Allentoft et al., 2015; Haak et al., 2015).

From a Y-chromosomal point of view, haplogroup R1b has become firmly established as a marker of the spread of the Bell Beaker culture, as documented, for example, by the recent study by Olalde et al. (2018): 84 out

of 90 of the male, non-Iberian, Beaker-associated individuals analysed belonged to this haplogroup.

2.3.14.1.9 Únětice Culture

This culture, named after the cemetery of Únětice in Prague (referred to as "*Aunjetitzer Kultur*" in German), was widespread throughout central Europe during the early Bronze Age, especially in the Czech Republic, but also in Poland, eastern Germany and northern Austria. The Únětice Culture appears somewhat later than Bell Beakers, across a similar range. It has been proposed According to Haak (Haak et al., 2015), Únětice ancestry consists of almost 50% Yamnaya, approx. 30% hunter-gatherer and 25% early Neolithic farmers. Like the Bell Beakers, the Únětice Culture has a lesser Yamnaya component than the earlier Corded Ware, but a higher hunter-gatherer ancestry than Bell Beakers.

The defining features of this culture include the typical "hourglass" cups, right-side crouched burials in flat pits as well as specific forms of flanged axes, halberds and metal-hilted daggers (Gimbutas, 1965; Harding, 2000). Although metallurgy had already been practiced by the earlier Corded Ware groups, large hoards of bronze objects indicate that the Únětice Culture produced metal objects on a far larger scale. This culture established far-reaching trade contacts, for example to the Wessex and Brittany Cultures of Great Britain and France, respectively (Cunliffe, 2001). Several metal objects associated with of burials of the Polada Culture of Northern Italy show clear Únětice technology, such as Únětice-type copper ring ingots which attest to a metal trade between the Únětice Culture and the early Bronze Age Polada Culture of the Trentino region (Barfield, 1971).

2.3.14.1.10 Remedello

The site of Remedello di Sotto in Brescia in northern Italy (south of Lake Garda, approx. 120 km from the Trentino sites sampled for this study), which was excavated in the late 19th century, revealed a total of 124 Copper Age graves, whose characteristic grave goods later defined the eponymous

Remedello Culture (Biagi, 1989; De Marinis, 1997). Due to the spatial and chronological proximity of this site to those sampled for this project, a certain affinity is to be expected, especially as typological links between grave goods from the Remedello necropolis and the Iceman's equipment have been proposed. Allentoft et al. (2015), in their genomic investigation of 101 ancient humans, also analysed three samples from Remedello di Sotto. For these samples, therefore, not only mtDNA is available, but also genomic data for the other loci analysed for this project. However, as genetic data is only available from three Remedello individuals, these were not used as a reference group for population genetic analyses due to the small sample size, but regarded individually in each case.

2.3.14.1.11 Neolithic Spain

A study by Hervella et al. (Hervella et al., 2012) comparing Palaeolithic and Neolithic samples from Spain with those of central Europe revealed a clear differentiation between Central European and Spanish Neolithic groups. One notable aspect was the lack of haplogroup N1a in the Spanish samples, whereas this haplogroup is found frequently in the Central European Neolithic groups. This genetic differentiation between the Iberian and Central European Neolithic is confirmed by Gamba et al., who observed a higher frequency of haplotypes that are rare in modern Europe but somewhat higher in the Near East (Gamba et al., 2012), from which an early Neolithic colonisation by groups from the fertile crescent is inferred. The Spanish Neolithic groups show characteristics of the Cardial rather than the LBK, reflecting a Neolithisation route from the Mediterranean rather than through Central Europe via the LBK. These characteristics are also shared by the southernmost Andalusian Neolithic samples studied by Fregel et al. (Fregel et al., 2017) as well as with Neolithic samples from Morocco, which also supports the theory of Neolithic pioneers having reached Spain via the southern seafaring route.

2.3.14.1.12 Bronze Age Sardinia

Genomic sequencing of the Tyrolean Iceman revealed a notable affinity with modern-day Sardinian populations, both on an autosomal as well as on a Y-chromosomal level (Keller et al., 2012; Sikora et al., 2014). Similar observations for other ancient genomes indicate that a genetic structure common in the Neolithic only survived to modern times in geographically isolated regions, a theory that is supported by Sardinia's role as a genetic outlier (Zei et al., 2003). However, patterns of mitochondrial diversity vary greatly in different regions of Sardinia (Morelli et al., 2000). Some genetically homogenous regions show evidence of a Neolithic founder event. On the other hand, the high frequency of a specific Sardinian variant Y-chromosomal haplogroup I in some regions argue for a pre-Neolithic origin of the modern gene pool (Contu et al., 2008), whereas Y-chromosomal haplogroup G2a, which also reaches high frequencies in Corsica and Sardinia, is highly likely to be of Near Eastern origin. Finally, the high level of regional variation as well as variation between the islands of Corsica, Sardinia and Sicily (Francalacci et al., 2003) indicate that the islands may have undergone subsequent demographic events or been affected by genetic drift, especially due to small populations. Although direct comparisons between ancient and modern Sardinian samples revealed a certain continuity (Der Sarkissian, 2011), potential subsequent influencing factors must be borne in mind when comparing ancient and modern populations.

Therefore, a reference group included the earliest available ancient DNA samples from Sardinia, attributed to the Bronze Age Nuraghic culture. Direct archaeological links with the Polada culture of the Trentino region have been reported for one sub-group of the Nuraghic culture, the Bonnanaro culture ((Lilliu, 1988; Moravetti, 1992; Webster, 1996; Turfa, 2014). However, such a connection has not been substantiated in other publications, so this must be regarded with caution.

2.3.14.1.13 Etruscans

The origins of the Iron Age Etruscans have long been a matter of debate, with a local origin being regarded as one possibility and an Anatolian origin (as mentioned by Herodotus) as another. A study by Achilli et al. (Achilli et al., 2007) on DNA from modern inhabitants of region formerly inhabited by the Etruscans revealed high frequencies of Near Eastern haplogroups in certain regions of Tuscany, which were interpreted as an indicator of a Near Eastern Origin of Etruscans. On the other hand, a direct analysis of ancient Etruscan DNA including simulations with Etruscan, Medieval, Modern Tuscan and Anatolian DNA by Ghirotto et al (Ghirotto et al., 2013) showed that the link between the Etruscans and Anatolia is more likely to date back to approximately 3000 BCE, predating the Iron Age by two millennia, in which case the Etruscan culture is more likely to have developed locally. A genomic study of modern Tuscan DNA, on the other hand, also revealed a significant Middle Eastern admixture, but more recent, namely during the Iron Age (Pardo-Seco et al., 2014).

As no Neolithic or Bronze Age material is available for the region of later Etruria, this question cannot be addressed directly. However, similarity between the Etruscans and the Neolithic and Bronze Age groups of Northern Italy would lend weight to the theory of a local origin. The available Etruscan data was therefore included as a reference group to investigate this matter.

2.3.14.2 Modern reference populations

A variety of modern populations throughout Europe, the Caucasus and the Near East are included as reference data sets. These include isolated groups from modern-day South Tyrol and Trentino. Although a certain degree of population continuity may well be the case for these for prehistoric times, a high degree of genetic drift is to be expected due to the small population sizes, as well as the known demographic events that shaped the region after the Bronze Age. However, modern reference groups from Alto Adige-Trentino will not only be compared to the ancient samples according

to geographic location, but also according to language (some of them belong to linguistic isolates) to ascertain whether correlation can be observed.

A map of the following local reference groups, which also includes sampled ancient sites and copper ore resources, can be found online at trento.bioarchaeology.de. Some of these groups represent linguistic isolates, speaking various forms of Romansch, mainly Ladin in the region of the northern Dolomites, as well as Lombard or Venetian in regions west and south of Trento. These groups, which lie in the direct vicinity of the ancient samples in question, are analysed primarily to detect a potential genetic legacy in modern groups reaching back to prehistoric times. With the exception of the sample sets NE Italy and Tyrol, all groups in the following table are relatively small communities subject to a certain amount of isolation and endogamy. A substantial amount of genetic drift is expected due to the small effective population sizes, so that haplotype frequencies are expected to shift over time even without a significant degree of admixture. However, other factors may also reflect possible continuity, such as rare haplotypes seldom observed in other regions.

2.3.14.2.1 Modern reference groups in South Tyrol and Trentino

The first collection of modern reference samples consisted of data from the region of South Tyrol and Trentino, i.e. the vicinity of the ancient settlements. In order to determine whether possible correlations could be linked with region and/or language, these factors were considered when constructing groups for later statistical analysis. In particular, these regions were to be investigated with regard to possible genetic continuity, i.e. potential persistence of prehistoric lineages on a regional scale.

Table 8: Modern reference groups in South Tyrol/Trentino

Group	n	Language group[14]	Region	Reference
Val Badia	91	Romansch (Ladin)	South Tyrol (Northern Dolomites)	Pichler 2006 Thomas 2008
Val di Fassa	47	Romansch (Ladin)	South Tyrol (Western Dolomites)	Coia 2012
Val Gardena	46	Romansch (Ladin)	South Tyrol (Northern Dolomites)	Thomas 2008
Lower Val Venosta	107	German (Tyrolean dialect)	South Tyrol (upper Adige valley, Alpine foothills)	Thomas 2008
Upper Val Venosta	108	German (Tyrolean dialect)	South Tyrol (upper Adige valley, Alpine foothills)	Pichler 2006 Thomas 2008
Val Pusteria	37	German	South Tyrol (Northern Dolomites)	Pichler 2006
Val Isarco	34	German	South Tyrol (Northern Dolomites)	Pichler 2006
Val D'Adige	54	Italian	Trentino (central valley)	Coia 2012
Val di Fiemme	41	Italian	Trentino (Western Dolomites)	Coia 2012
Val di Non	48	Romansch (Nones)	Trentino (plateau west of Adige valley)	Coia 2012
Val di Sole	63	Romansch (Nones)	Trentino (plateau west of Adige valley)	Coia 2012
Val Giudicarie	52	Romansch (Lombard)	Trentino (plateau west of Adige valley)	Coia 2012
Val Primiero	40	Romansch (Venetian)	Trentino (plateau west of Adige valley)	Coia 2012

2.3.14.2.2 Cross-regional modern reference groups

Apart from the modern populations listed above in the direct vicinity of the Trentino samples, several other larger regions of interest were also included in the analysis. These was limited to areas on the Italian peninsula and its surrounding islands, as well as neighbouring countries for which sufficient data were available. Selection was mostly limited to regions/countries with more substantial geographical boundaries that, over the millennia, are likely to have been less changed by demographic events that e.g. mid-continental areas such as Germany and France. However, it should also be borne in

[14] Exact differentiation of language groups is not possible - firstly because most regions are multilingual (speaking variants of German, Italian and Romansch) and secondly because some differences are more a question of different dialects rather than different languages.

mind that such more isolated regions are also more prone to genetic drift/bottlenecks. Therefore, the inferences made from comparisons with this group must be regarded with caution, taking demographic history into account where possible.

Table 9: Cross-regional reference groups

Group	n	Region	Reference[15]
NE Italy	108	Aviano, Treviso, Vicenza	Boattini 2013
NW Italy	197	Bologna, Cuneo, Brescia, Como, Savona, Genova	Boattini 2013
Tuscany	433	Murlo, Volterra, Casentino, Pistioa, Grosseto	Boattini 2013, Varesi 2005, Achilli 2007
Central Italy	99	Terni, Perugia, Macerata	Boattini 2013
Southern Central Italy	97	Benevento, Campobasso, L'Aquila	Boattini 2013
Southern Italy	109	Cosenza, Catanzaro, Crotone, Matera, Lecce	Boattini 2013
Sicily	271	Ragusa, Catania, Agrigento, Castellammare del Golfo, Alia	Cali 2001, Boattini 2013 Vona 2001
Crete	186	Not specified	Villems 1999*
Greece	284	Northern Greece	Irwin 2008
Turkey	73	Not specified	Comas 1996, Schoenberg 2011
Sardinia	367	Gallura, Nuoro, San Antioco Island, San Pietro Island, Olbia Tempo Nuoro, Oristano, Ogliastra, Trexenta	Falchi 2006*, Calo 2005*, Boattini 2013, Fraumene 2006, Varesi 2005*
Corsica	99	Corte, Bonifacio	Varesi 2000, Giovannoni 2005
Switzerland	74	Not specified	Pult 1994
Austria	373	Not specified	Brandstätter 2007[16], Parson 1998
Bosnia	162	Not specified	Harvey 2000*
Croatia	59	Not specified	Harvey 2000*
Serbia	56	Not specified	Harvey 2000*
Dobrudja	54	(Dobrudja, historical province of Romania)	Cocoş 2017
Moldavia	235	(Moldavia, historical province of Romania)	Cocoş 2017
Transylvania	206	(Transylvania, historical province of Romania)	Cocoş 2017
Wallachia	226	(Wallachia, historical province of Romania)	Cocoş 2017
Andalusia	65	Not specified	Via 2005*
Balearic Islands	67	Not specified	Varesi 2005*

** Denotes unpublished data submitted directly to GenBank: see supplement S3 for accession numbers*

[15] For full data on references, see Supplementary Data S3

[16] While the source of the samples is not defined on a more regional level than "Austria" in the publication, the sample set was entered by the authors into the EMPOP database as originating from Tyrol

2.3.15 Statistical analyses (mtDNA)

Haplotype diversity, nucleotide diversity, mean number of pairwise differences, Tajima's D, Fu's F_S and F_{ST} were computed using the Arlequin software, version 3.5.2 (Excoffier and Lischer, 2010).

Parameters were as follows: 10,000 permutations to calculate F_{ST}, significance level 0.05, transition/transversion/deletion weight each = 1, allowed missing level per site: 0.08. The latter parameter was adjusted slightly from the standard setting (0.05) in order to incorporate shorter reference sequences into groups for which not enough full sequences were available, without causing positions of interest (i.e. positions at which the Trentino samples showed polymorphisms) to be disregarded by the Arlequin software.

2.3.15.1 Grouping of samples

For statistics calculations, the mtDNA sequences for the samples analysed in the course of this study were grouped according to archaeological context and age in order to define groups (across different sites) that are likely to be genetically close. As most of the samples are from the same geographical region (the few outliers possibly also belonging to the same groups), the specific site was not taken into account. Instead, the age of the sample was considered, as was site continuity: for example, samples taken from the same site would initially be assumed to belong to the same population if that site exhibited continuous habitation and/or use throughout that time. Conversely, individuals buried centuries apart at a site with long periods without human habitation or use were initially regarded as separate.

2.3.15.1.1 Grouping based on archaeological characteristics

For a more specific indication of possible cultural affinity, archaeological characteristics such as burial type, location (e.g. rock shelter or open space) and typology of the associated artefacts were observed. The F_{ST} between these groups was then computed in order to determine whether the defined group structures are indeed plausible and significant.

2.3.15.1.2 Spatial analysis of molecular variance

In addition to the grouping based on archaeological characteristics, a spatial analysis of molecular variance was performed for the various sites using the software SAMOVA 1.0, a tool to determine geographically homogeneous but genetically differentiated population groups (Dupanloup et al., 2002). Input data includes, alongside genetic sequences, geographical coordinates of populations. The analyst selects the number of groups to be defined and SAMOVA tests all possible grouping options to determine which group formation shows the lowest intra-group variability and highest inter-group variability.

2.3.15.2 Haplotype diversity ($\hat{H}$)

Haplotype diversity (Nei, 1973, 1987) is a measure of variability within a group and denotes the probability of two sequences (haplotypes) selected at random from a given sample set differing from one another. H ranges from 0 (all samples are identical, lowest possible haplotype diversity) to 1 (all samples differ, highest possible haplotype diversity).

It is estimated using the following formula:

$$\hat{H} = \frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^2\right)$$

whereby n denotes the number of gene copies in the sample, k is the number of haplotypes, and p_i is the haplotype sample frequency (Excoffier and Lischer).

2.3.15.3 Nucleotide diversity ($\hat{\pi}$)

Nucleotide diversity (Tajima, 1983; Nei, 1987) is another measure of intra-group variability and is analogous to haplotype diversity, only on a nucleotide level: i.e. the probability of two homologous nucleotides selected at random from a given data set differing from one another.

It is estimated using the following formula (Excoffier and Lischer):

$$\hat{\pi}_n = \frac{\sum_{i=1}^{k} \sum_{j<i} p_i p_j \hat{d}_{ij}}{L}$$

2.3.15.4 Mean number of pairwise differences (π)

This measure indicates the mean number of differences between all pairs of haplotypes in the sample.

It is estimated using the following formula:

$$\hat{\pi} = \frac{n}{n-1} \sum_{i=1}^{k} \sum_{j=1}^{k} p_i p_j \hat{d}_{ij}$$

whereby $\hat{d}_{ij}$ is an estimate of the number of mutations that have occurred since the haplotypes (*i* and *j*) diverged; *k* is the number of haplotypes, p_i is the frequency of haplotype *i*, and *n* is the sample size (Excoffier and Lischer).

2.3.15.5 Test of selective neutrality: Tajima's D

Tajima's D (Tajima, 1989) provides information on the probability of recent population expansion in a given data set by comparing π (mean number of pairwise differences) to S (the number of segregating sites, i.e. polymorphic sites in the data set). In a theoretical population evolving at random (i.e. with no selective pressure, with random mating, constant population size, no migration or recombination), Tajima's D would be 0, representing neutrality. A negative Tajima's D value in an actual population indicates a recent population expansion, for example after a bottleneck or a founder event; a value greater than zero indicates a reduction in population size, e.g. during a bottleneck and a value of 0 indicates a balance between mutation and drift, with no evidence of mutation.

2.3.15.6 Test of selective neutrality: Fu's F_S

Fu's F_S (Fu, 1997) also tests for neutrality in a similar fashion to Tajima's D, including π and S, but also considers singletons (polymorphisms occurring in one sequence only). An excess of singletons leads to a negative value for Fu's F_S, indicating recent population expansion. Although Tajima's D and Fu's F_S tend to correlate, the latter is often more sensitive and more suited to short DNA sequences (Excoffier and Lischer). However, it is also dependent on sample size. To be considered significant, the p-value for this measure should be under 0.02 rather than 0.05 (Excoffier and Lischer; Fu, 1997).

2.3.15.7 Haplotype sharing

Haplotype sharing is defined as the likelihood of two samples selected at random from two populations having identical haplotypes. Haplotype sharing analysis between the study population and reference populations was initially performed incorporating all haplotypes.

However, in the case of comparisons between ancient Trentino samples and modern reference groups, one particular aspect had to be taken into account. One haplotype – H2a2a1, the revised Cambridge reference sequence – is very common across the modern reference groups used for this study (frequency=0.153), occurring six times as frequently as the next most common haplotype[17]. In initial calculations, this uneven distribution shifted the pattern in favour of this particular haplotype, with the value determined for haplotype sharing corresponding directly with the relative frequency of H2a2a1 in the respective reference population, as demonstrated later on the basis of the results of this study in Figure 4.

In other words, the "haplotype sharing" values calculated for each reference population corresponded largely to the frequency of H2a2a1 in that population, with all other haplotypes having a negligible impact. This skewed the obtained results, as the high frequencies of H2a2a1 in modern

[17] Based on the stretch of the HVR1 taken into account by Arlequin, 16033-16382

populations are not directly dependent on this haplotype's frequency in their prehistoric ancestral population, but developed over millennia due to various demographic events. Therefore, to obtain a more representative haplotype sharing analysis reflecting all haplotypes, haplotype sharing analyses between ancient Trentino and modern reference groups were excluded this specific haplotype. Comparisons between ancient Trentino and other ancient reference groups, on the other hand, took all haplotypes into account, as relative frequencies of any particular haplotype do indeed play a role when comparing (more or less) contemporary populations, especially with regard to determining ancestry/admixture.

2.3.15.8 Fixation index (F_{ST})

The ancient Trentino groups studied in this manner were then compared to the other ancient reference sample groups listed in Section 2.3.14.1, to determine potential genetic closeness between other prehistoric groups from specific cultures, subsistence patterns (e.g. hunter-gatherers versus farmers) or other geographic regions. Finally, the Trentino samples were compared to modern data sets. This was first carried out on a local level, with contemporary populations from South Tyrol and Trentino. Comparisons were not only performed according to region, but also according to language, to investigate any potential correlation. Comparison with modern data sets were then conducted on a larger scale, with other regions of Italy and others of potential relevance for Neolithic and Bronze Age population shifts, such as the Mediterranean islands of Crete, Corsica, Sardinia and Sicily as well as the Balkans (see Table 9).

To visualise the overall inter-population structure, a multidimensional scaling plot was used to depict genetic differences, based on Reynold's F_{ST} values previously calculated with the Arlequin software and plotted in R using RStudio (RStudio Team, 2016). The isoMDS function of the MASS package was used to test Kruskal's goodness-of-fit and the stress value (which is stated in percent by isoMDS) was evaluated according to Kruskal (1964):

Stress value	Goodness-of-fit
20%	poor
10%	fair
5%	good
2.5%	excellent
0%	perfect

3 RESULTS

3.1 Amplification and sequencing success

To assess the rate amplification success, PCRs performed on samples listed in Section 2.1.3 are taken into account. Although the Tyrolean Iceman is, in a broader sense, also a subject of this study, PCRs performed on Iceman DNA using loci of interest for this study are not taken into account for calculating the amplification rate as these were performed solely to confirm the authenticity of the SOLiD4 genomic data. As many amplification attempts (using different approaches) were performed on the poorly-preserved Iceman DNA due to the individual's relevance, this would cause a bias toward poorer amplification rates than is true for the Trentino individuals as a whole. However, a similar bias still applies to the sample group in general: For extracts that could not be amplified in a standard approach, PCR was attempted more often (e.g. using different primer pairs for shorter target fragments, larger extract amounts, dilution series to combat potential inhibiting substances etc.). As many of these attempts were still unsuccessful despite the different approaches, this would also indicate a lower success rate if only the amplifications per attempt are taken into account without observing the samples/extracts individually. On the other hand, removing poorly preserved samples early on from the analytical workflow due to negligible chances of success could also cause a bias, although in the opposite direction. For this reason, both calculation types are given and compared below.

Table 10: Overall amplification rates according to PCR attempts

DNA type Locus	Amplification attempts	amplified	Sequencing attempts	% sequenced[18]
mtDNA	1026	852 (83%)	611	472 (77.4%)
ncDNA -13.910*T/C HERC2 Amelogenin	279 131 76 72	143 (51.2%) 56 (42.7%) 54 (71%) 33 (45.8%)	102 52 50 Not sequenced	73 (71.6%) 30 (57.7%) 43 (86%) Not sequenced
Y-DNA L91 M201 P287	82 11 56 5	46 (56.1%) 10 (90.9%) 22 (39.3) 5 (100%)	35 10 20 5	32 (91.4%) 9 (90%) 18 (90%) 5 (100%)

Table 11: Amplification rates according to number of individuals/samples

	mtDNA	ncDNA[19]
Number of individuals tested	76	
Number yielding amplicons	59 (77.6%)	36 (47.4%)
Number of samples tested[20]	127	
Number yielding amplicons	84 (66.1%)	48 (37.8%)
Individuals sequenced & meeting scoring requirements	36	-13.910*T/C: 7 (9.2%) HERC2: 16 (21.1%)

Amplification rates for mtDNA are consistent with many other studies on ancient material. While amplification rates for nuclear DNA initially (when observed according to PCR attempts) appear higher than in many other studies, this is due to the fact that only samples yielding good mtDNA preservation were tested for ncDNA. For this reason, the amplification rates shown in table 2 (according to individuals) are lower and concur with other PCR-based ancient DNA studies.

[18] "Sequenced" is defined as obtaining a clearly legible sequence without double bands/excessive background noise.

[19] Amplicon obtained for any of the autosomal loci. Y-chromosomal loci are not included in the table, as this would distort the result: PCRs for x-chromosomal loci also included female samples as controls; these would not yield an amplicon even if well-preserved.

[20] Amplicon obtained for at least one HVR1 fragment

3.2 Authentication & reproducibility

3.2.1 Discrepancies due to sampling errors

One of the main methods for evaluating authenticity is attempting to reproduce sequencing results from a second extract from the same individual. Three options are available:

- The second aliquot of the first sample may be used. This ensures that both samples are derived from the same individuals. However, contamination incurred up to or during the milling step could be reproduced in two extracts.
- A second sample may be taken from the same bone. This also ensures that both samples are derived from the same individual. As the sample preparation steps are done at different times, there is less chance that the same endogenous contamination incurred during sample preparation would be present in both extracts. However, there is a certain possibility that the bone in question contains the same endogenous contamination, e.g. from the archaeologists or museum personnel who originally handled the bone (and which would be difficult to extinguish from ancient DNA, as some degradation would be expected for decade-old genetic material). Although steps may be taken to minimize contamination from previous handlers, it cannot be excluded.
- Sampling two different bones/teeth from the same individual further reduces the probability of reproducing a surface contaminant in both extracts. All laboratory steps from sampling to PCR are separate for both extracts. This method would therefore be the most reliable way of documenting authenticity.

This third method was therefore selected wherever possible. Samples were taken from separate bones/teeth of collectives labelled as one individual. However, in the course of genetic analysis, some discrepancies became

apparent. In 11 cases, the first and second extracts yielded different sequence results which could be reproduced for each respective extract in multiple PCRs. Contamination incurred during extraction were unlikely, as strict anticontamination measures were observed and the extraction controls were negative for the extractions in question. Later osteological analyses of the skeletal material confirmed that some bones and teeth originally labelled as belonging to one individual did, in fact, comprise several individuals (Paladin, 2013). All three sites for which this was observed (Moletta Patone, Romagnano and Solteri) revealed collective and/or disturbed burials, in which it was not possible to differentiate individuals with certainty.

If two extracts obtained from the same bone hat yielded different sequences, this would have been a clear indicator of exogenous contamination. However, in all cases, the differing extracts had been obtained from different bones/teeth. Taking into account the low contamination rates for extraction controls (see next chapter), this clearly indicates that the discrepancies are due to samples being obtained from different individuals rather than to any exogenous contaminations incurred during the analysis. Under ideal conditions, further extracts would have been taken from the bones/teeth already sampled. However, further extractions were no longer possible due to time constraints and other circumstances. For this reason, a scoring system was established to evaluate whether results can be regarded as meeting the required authenticity criteria (see Section 3.2.2).

Table 12: Samples with differing A and B extracts

Putative individual (orig.)	Extract No.	Extracted bone/ tooth	Mutations	Grave
Moletta Patone 1	A (XVI), 1st sampling	Lower molar (adult)	16037G 16126C 16294T 16296T 16304C	Scatolino 1 MNI=2 individuals (Paladin 2013) Scatolini 1-9 are all within the same sector
	B (XXXVIII), 3rd sampling	Caninus (adult)	16270T 16311C 16355T	
Moletta Patone 2	A (X), 1st sampling	Molar	16069T 16126C	Scatolino 2 MNI=3 individuals (Paladin 2013)
	B (XXXVIII), 3rd sampling	Premolar (adult)	16093C 16224C 16311C	
Moletta Patone 7	A (XXI), 1st sampling	Caninus (adult)	16270T 16311C 16355T	Scatolino 7 MNI=1 (Paladin 2013)
	B (XXXIX), 3rd sampling	Incisor (adult)	16189C	
Romagnano III 91	A (XIV), 2nd sampling	Molar	16069T 16126C 16261T	Three of the individuals analysed in the course of this study are from Tomb 2: Rom89, 90 and 91. However, this tomb was described as containing the remains of four individuals: Two adults and two children. Rom 89 and 90 have identical haplotypes: Unless the same individual was sampled twice by mistake, this may indicate mother and child or siblings whereas 91a and 91b may therefore be from the other two individuals.
	B (XXXIV), 3rd sampling	Pars petrosa	16189C 16223T 16278T	
Romagnano III 100	A (XIV), 2nd sampling	Molar	CRS	Tomb 5 MNI=3 individuals (Paladin 2013)
	B (XXXXI), 3rd sampling	Caninus	16182C 16183C 16189C 16234T 16324C 12308G	

Table 12 cont.

Putative individual (orig.)	Extract No.	Extracted bone/ tooth	Mutations	Grave
Romagnano III 118	A (XXV), 2nd sampling	Pars petrosa	16294T 16304C 16320T	Tomb 9-10 MNI=6 individuals
	B (XXXIV), 3rd sampling	Femur diaphys.	16294T 16304C	
Romagnano III 120	A (XV), 2nd sampling	Molar	CRS	Tomb 9-10 MNI=6 individuals
	B (XXXVII), 3rd sampling	Caninus	16182C 16183C 16189C 16234T 16324C 12308G	
Romagnano III T11A	A (XVII), 2nd sampling (XXXXI), 3rd sampling	Molar M1 Molar M2	16294T 16304C 16320T	Tomb 11 MNI=4 individuals
	B (XXV) 2nd sampling	Molar (crown only)	16294T 16304C	
Romagnano III T12E	A (XVIII), 2nd sampling	Molar M1	16126C 16292T 16294T 16296T	Tomb 12 MNI=5 individuals
	B (XXII), 2nd sampling	Pars petrosa	16069T 16126C	
	C (XXXIV + XXXV), 3rd sampling	Humerus + ulna	16294T 16304C 16320T	
Solteri 64	A (XIV), 2nd sampling	Molar M1 (child)	16093C 16224C 16311C	Scattered bones in two sectors, 26 (MNI =4) and 27 (MNI = 9). These two individuals were among the larger collective (which comprised Numbers 59-67). Eight different haplotypes were sequenced from the 11 samples taken from sector 27.
	B (XXXX), 3rd sampling	Incisor I2 (7-8 years)	16300G	
Solteri 65	A (XV), 2nd sampling	Molar M3 (child or small adult)	16294T 16304C 16320T	
	B (XXXX), 3rd sampling	Premolar P2	16311C	

Samples identified as belonging to separate individuals were subsequently labelled accordingly, e.g. RomIII118a, RomIII118b, RomIIIT12e-a, etc. Conversely, the observation that bones could not always be allocated to specific individuals within a collective or disturbed burial could also lead to the opposite case, i.e. that bones from assumed separate individuals sharing the same haplotype could possibly be from the same individual, unless this can be excluded on an osteological basis (e.g. age of the individual or if the same bone was sampled for each), causing an over-representation of actual haplotype frequencies. If samples derived from the same documented grave/sector yielded identical sequences, these can only be identified with certainty as separate individuals on the basis of an osteological analysis.

This applies to the following:

Romagnano Tomb 6

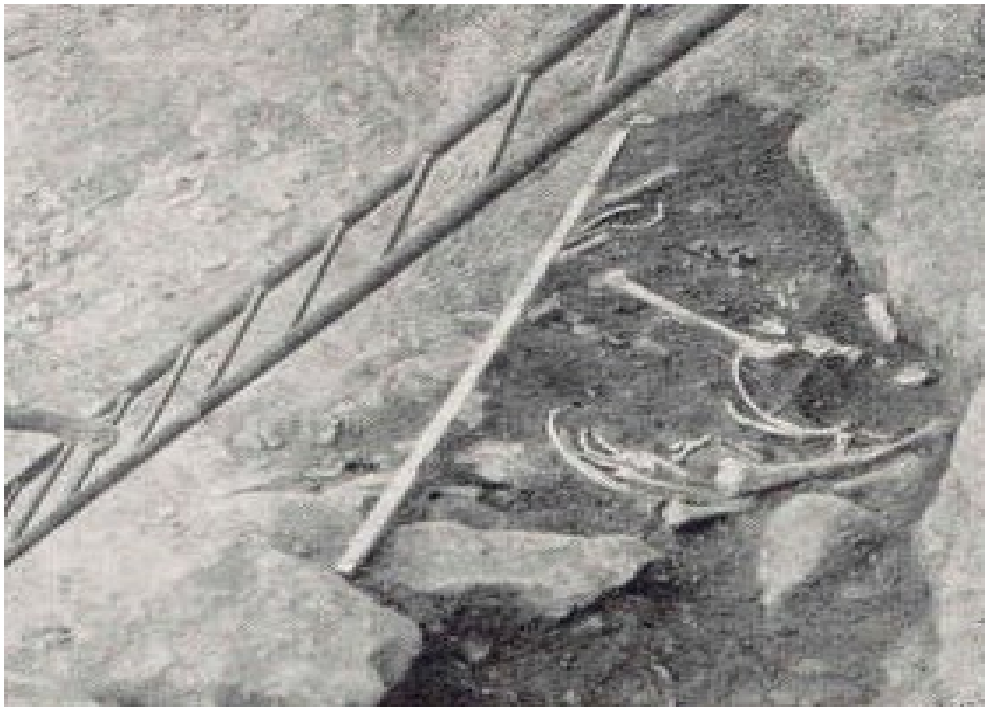
Grave No.	Shared haplotype	Collective[21]	Individual	Osteological analysis by A. Paladin
Romagnano III, Tomb 6	rCRS	46	Romagnano 103	Female, 17-20 years
		47	Romagnano 105	Female, 6-7 years

Tomb 6 also contains the remains of two infants for whom no DNA could be obtained. As the samples used for DNA analysis could be clearly differentiated with regard to age (Romagnano 103 was an adult incisor, Romagnano 105 was a child's molar), inadvertent sampling from the same individual can be excluded. While both samples were extracted in the same extraction run, all extraction and PCR blank controls were negative, rendering cross-contamination highly unlikely. While it seems likely that two individuals sharing the same haplotype might be maternally related, the

[21] Although not entirely certain, "collective" seems to refer to anatomical collection, i.e. what was thought at excavation to belong to one individual (rather than burial collective of several individuals)

high general frequency of this haplotype in ancient and modern populations does not permit a significant observation in this case.

Figure 3: Romagnano III, Tomb 6 (Perini, 1975)



Romagnano Tomb 2

Grave No.	Shared haplotype	Collective	Individual	Osteological analysis by A. Paladin
Romagnano III, Tomb 2	16093C 16179T 16189C 16223T 16278T 16362C	39	Romagnano 89	Female, 40-50 years
		40	Romagnano 90	Female, 30-40 years

Romagnano Tomb 2 contained the remains of four individuals: two adult females (30-40 years and 40-50 years, respectively), one child of 5-7 years and one infant of 38-40 weeks (Paladin, 2013). The samples used for DNA analysis were derived from the two adult females: For Romagnano 89 from the femur diaphysis, for Romagnano 90 from a molar (i.e. differentiation

cannot be made on the basis of the bone type, i.e. if separate left femurs had been sampled).

If these sequences are indeed derived from the same individual, then the unique haplotype would certainly indicate that the two individuals were maternally related (and possibly buried together for this reason). However, it cannot be ruled out that both samples were derived from the same individual.

Figure 4: Romagnano III, Tombs 2, 3, 4 and 5 (Perini, 1975)

Solteri collective 26/27

Grave No.	Shared haplotype	Collective	Individual	Osteological analysis by A. Paladin
Solteri (no specific grave no. available	16093C 16224C 16311C	26	Solteri 56	Female, 45-50 years
		27	Solteri 64a	Female, 7-8 years

In this case, the samples could be differentiated on the basis of age: osteological analysis permitted clear differentiation of individuals, and the

molar from Solteri 64a that were used for the study were significantly smaller and lighter than those from Solteri 56 (Solteri 64a: 16x11 mm, 1.23 g, Solteri 56: 21x12 mm, 1.69 g). Therefore, these sequences belong to two separate individuals from the same maternal lineage.

The samples that shared the same haplotype cannot be allocated with certainty to two separate individuals on the basis of burial position, age/sex or bone type (i.e. Romagnano 89 and 90) were regarded as one individual in the subsequent population genetic analyses to avoid potentially false overrepresentation of a particular haplotype.

It must be borne in mind, in any case, that the samples analysed here do not represent a completely random cross-section of the actual prehistoric populations: individuals buried together or in close proximity are more likely to be closely related (leading to a certain sampling bias). The frequencies of the various haplotypes observed here do not necessarily reflect their actual frequency during the lifetimes of those populations.

Therefore, inference of intra- and inter-population relationships are based more on the general occurrence of particular haplotypes (especially rare variants) than on their frequency in a given sampling group.

3.2.2 Scoring system results

The results of the scoring system developed for this study and described in section 2.3.13 were as follows:

In practice, samples were seldom excluded solely due to a poor score, but usually also because of failure to reproduce each amplicon at least twice. This is because poor-quality sequences were usually evaluated as "unusable" directly after sequencing, and not considered further.

Table 13: Samples achieving minimum score of 8

These samples were used for statistical analysis as full sequences (413 bp) as all four HVR1 amplicons were reproduced in at least 2 PCRs.

Sample	No. of	Reproduced	Sequence quality	Overall score
NogII69	2	yes	5	11
AcqA	2	yes	5	11
RomIII89	2	yes	4	10
RomIVT2a	2	yes	4	10
RomIII82	2	yes	4	10
RomIIIT11A-a	2	yes	4	10
Solt67	2	yes	4	10
NogIII75	2	yes	3	9
RomIII85	2	yes	3	9
RomIII90	2	yes	3	9
RomIIT12E-c	2	yes	3	9
Schla203	2	yes	3	9
Solt59	2	yes	3	9
MP2b	1	yes	5	9
RomIII118a	1	yes	5	9
RomIII91b	1	yes	5	9
Solt64a	1	yes	5	9
PdVT2	1	yes	5	9
PdVT3	2	no	4	8
RomIII114	2	no	4	8
RomIII97	2	no	4	8
RomIII103	2	no	4	8
RomIII118b	1	yes	4	8
MP1a	1	yes	4	8
RomIII100a	1	yes	4	8
RomIII100b	1	yes	4	8
RomIIIT11A-b	1	yes	4	8
Solt63	1	yes	4	8
RomIII105	2	no	4	8
Solt56	2	no	4	8
Solt61	1	yes	4	8

Table 14: Samples achieving minimum score of 8 for incomplete sequences

These samples were used for statistical analysis as partial sequences (>413 bp) as three of four HVR1 amplicons were reproduced in at least 2 PCRs.

Sample	No. of extracts	Reproduced in 2nd lab	Sequence quality (5= very good, 1=very poor)	Section used	Overall score
MP6	2	No	5	15997-16347	9
MP1b	1	Yes	5	16117-16409	9
MP2a	1	Yes	5	15997-16347	9
RomIII120b	1	Yes	5	15997-16347	9
RomIII91a	1	Yes	5	15997-16347	9
RomIIT12E-a	1	Yes	4	15997-16221; 16287-16409	8

Table 15: Samples not used for statistical analysis

The following samples were not used for statistical analysis due to poor score and/or as less than three of four HVR1 amplicons were reproduced in at least 2 PCRs

Sample	No. of extracts	Reproduced in 2nd lab	Sequence quality (5= very good, 1=very poor)	Overall score	Reason for exclusion • Poor = poor score • > 3 = Les than 3 fragments covered twice
LV60/1	1	no	3	5	Poor score, < 3
LV60/2	1	no	4	6	Poor score, < 3
LV60/4	1	no	4	6	Poor score, < 3
LV75/2	1	no	2	6	Poor score
LV76/3A	1	yes	2	6	Poor score, < 3
LV76/3B	1	no	3	5	Poor score, < 3
MP3	1	no	5	7	Poor score, < 3
MP7a	1	no	2	4	Poor score, < 3
MP7b	1	no	3	5	Poor score, < 3
MP8	1	no	1	3	Poor score, < 3
Mezzo2	1	yes	4	8	Poor score, < 3

Table 12 cont.

Sample	No. of extracts	Reproduced in 2nd lab	Sequence quality (5= very good, 1=very poor)	Overall score	Reason for exclusion Poor = poor score < 3 = Les than 3 fragments covered twice
RomIII117	1	no	3	5	Poor score
RomIII120a	1	yes	4	8	< 3
RomIII121	1	no	2	4	Poor score, < 3
RomIII83	1	no	3	5	Poor score, < 3
RomIV58	1	no	5	7	Poor score, < 3
RomIVT2b	2	no	2	6	Poor score
RomT12E-b	1	yes	5	9	< 3
Solt55	1	no	3	5	Poor score, < 3
Solt60	1	no	3	5	Poor score
Solt64b	1	yes	4	8	< 3
Solt65a	1	no	5	7	Poor score, < 3
Solt65b	1	no	3	5	Poor score, < 3

More detailed scoring results (i.e. providing individual scores for each of the 4 HVS1 fragments) are provided in Supplement S6 on the accompanying CD.

3.3 Contamination rate

To assess the contamination rate, PCR and extraction controls from all PCRs run during the analytical period of this study are taken into account. This includes not only PCRs involving samples from this study, but PCR controls from primer set-ups, reagent tests, spike tests etc. Some of the PCR blanks/extraction controls showed a positive band blank during electrophoresis. This does not automatically indicate contamination, but can have several other reasons, e.g. primer dimers, bacterial contamination, artefacts etc. For this reason, sequencing was attempted for positive controls to determine whether a sequence could be obtained.

Table 16: Positive control rates

	Number of controls	Positive electrophoresis band (%)	Positive yielding sequence
mtDNA PCR controls	419	57 (13.6%)	18 (4.3%)
mtDNA extraction controls	109	5 (4.6%)	0 (0%)
mtDNA all controls	528	61 (11.6%)	18 (3.4%)
ncDNA	118	3 (2.5%)	0 (0%)

A detailed list of all positive control results, including comparison of obtained sequences with other samples and laboratory personnel DNA, and action taken regarding contamination, is provided in Supplement S8 on the accompanying CD. Anonymised HVS1 haplogroups of personnel at the Bolzano laboratory are provided in Supplement S9 on the accompanying CD.

3.4 Sequence data

This section contains an overview of the SNPs determined for each individual. Full sequence alignments and raw data are provided in the supplement.

3.4.1 Mitochondrial DNA sequences

Table 17: HVR1 mutations and scoring results

Site	Sample	Region covered[22]	Meets score requirements	Mutations
Acquaviva	AcqA	15996-16409	Yes	16126C 16153A 16290T 16294T 16296T
La Vela	LV60/1	16119-16217	No	16193T
	LV60/2	16221-16409	No	16356C
	LV60/4	16118-16409	No	Non-reproducible
	LV75/2	15997-16409	No	16126Y 16189Y 16356Y 16360Y 16362Y
	LV76/3A	16105-16185; 16210-16409	No[23]	16114A 16147A 16172C 16316G
	LV76/3B	16118-16301	No	16184T
Mezzocorona	Mezzo2	16287-16409	No*	16292T 16294T
Moletta Patone	MP1a	15997-16409	Yes	16037G 16126C 16294T 16296T 16304C
	MP1b	15997-16409	Yes (partial sequence 16118-16409)	16270T 16311C 16355T
Moletta Patone	MP2a	15997-16409	Yes (partial sequence 15997-16347)	16069T 16126C
	MP2b	15997-16409	Yes	16093C 16224C 16311C
	MP3	16288-16409	No	16311C 16355T
	MP6	15997-16409	Yes (partial sequence 15997-16346)	16298C

[22] This denotes the stretch covered at least once. For some samples, only a partial section of the regio covered met score requirements, so that only this partial sequence was used for statistical analyses. The partial section in question is provided in the next column.

[23] Although the La Vela 76/3A and Mezzocorona samples do not meet scoring requirements, the obtained sequences were still regarded as reliable: in the case of Mezzocorona, because results concur with those published previously by Di Benedetto et al. (2000). In the case of La Vela, because the haplotype found in this individual is typically Neolithic and not found in modern populations.

Site	Sample	Region covered[22]	Meets score requirements	Mutations
	MP7a	16212-16409	No	16270T 16311C 16355T
	MP7b	15997-16209, 16218-16409	No	16189C
	MP8	16211-16398	No	16234T 16298Y
Nogarole II	NogII69	15997-16409	Yes	16294T 16304C 16320T
Nogarole III	NogIII75	15997-16409	Yes	16069T 16126C 16189C
Paludei di Volano	PdVT2	15997-16409	Yes	16189C 16223T 16278T
	PdVT3	15997-16409	Yes	16298C
Romagnano III	RomIII82	15997-16409	Yes	16266T 16270T 16304C 12308G
	RomIII83	15997-16141, 16288-16370	No	16290T
	RomIII85	15997-16409	Yes	16069T 16126C 16261T
	RomIII89	15997-16409	Yes	16093C 16179T 16189C 16278T 16362C
Romagnano III	RomIII90	15997-16409	Yes	16093C 16179T 16189C 16278T 16362C
	RomIII91a	15997-16409	Yes (partial sequence 15997-16346)	16069T 16126C 16261T
	RomIII91b	15997-16409	Yes	16189C 16223T 16278T
	RomIII97	15997-16409	Yes	16311C
	RomIII100a	15997-16409	Yes	CRS
	RomIII100b	15997-16409	Yes	16182C 16183C 16189C 16234T 16324C 12308G
	RomIII103	15997-16409	Yes	CRS
	RomIII105	15997-16409	No	CRS

Site	Sample	Region covered[22]	Meets score requirements	Mutations
	RomIII114	15997-16409	Yes	16304C
	RomIII117	15997-16409	No	16069T 16126C 16261T
	RomIII118a	15997-16409	Yes	16294T 16304C 16320T
	RomIII118b	15997-16409	Yes	16294T 16304C
	RomIII120a	15997-16409	No	CRS
	RomIII120b	15997-16409, 12308	Yes (partial sequence 15997-16346)	16182C 16183C 16189C 16234T 16324C 12308G
	RomIII121	15997-16409	No	16294T 16304C 16320T
	RomIIIT11A-a	15997-16409	Yes	16294T 16304C 16320T
Romagnano III	RomIIIT11A-b	15997-16409	Yes	16294T 16304C
	RomIIIT12E-a	15997-16409	Yes (partial sequence 15997- 16232; 16289-16409)	16126C 16292T 16294T 16296T
	RomIIIT12E-b	15997-16409	No	16069T 16126C
	RomIIIT12E-c	15997-16409	Yes	16294T 16304C 16320T
Romagnano IV	RomIVT2A	15997-16409	Yes	CRS
	RomIVT2B	15997-16409	No	16215G 16263A
	RomIV58	16288-16409	No	16294T 16296T 16304C
Schlanders	Schla203	15997-16409	Yes	16126C 16294T 16296T 16324C
Solteri	Solt55	16123-16232	No	CRS
	Solt56	15997-16409	Yes	16093C 16224C 16311C
	Solt59	15997-16409	Yes	16069T 16126C
	Solt60	15997-16409	No	16069T 16126C
	Solt61	15997-16409	Yes (partial sequence)	16240G 16311C

Site	Sample	Region covered[22]	Meets score requirements	Mutations
	Solt63	15997-16409	Yes	CRS
	Solt64a	15997-16409	Yes	16093C 16224C 16311C
	Solt64b	15997-16409	No	16300G
	Solt65a	16210-16409	No	16294T 16304C 16320T
	Solt65b	15997-16409	No	16311C
	Solt67	15997-16409	Yes	16069T 16126C 16193T 16278T 16290T

3.4.1.1 Haplotype allocation

The tables below list the haplotypes determined for those samples that met scoring requirements (and the two Neolithic samples that were taken into account for reasons specified above).

Table 18: Mitochondrial haplotypes determined for ancient Trentino samples

HT = Haplotype (numerical value assigned to each haplotype determined in the course of this study
HG = Haplogroup allocation according to Phylotree build 17

Individual	Mutations (HVR1 + 12308 if tested)	HT	HG
NEOLITHIC			
Mezzo2	(16126C)[24] 16292T 16294T	22	T2
LV76/3A	16114A 16147A 16172C 16316G	23	N1a
COPPER AGE			
Solt63	none (rCRS)	1	H2a2a1
Solt61	16240G 16311C	2	H1
NogII69	16294T 16304C 16320T	6	H5a4a
MP1b	16270T 16311C 16355T	7	U5b1c
MP6	16298C	10	HV0
Solt56	16093C 16224C 16311C	13	K1a
Solt64a			
MP2b			
Solt59	16069T 16126C	14	J
MP2a			
Solt67	16069T 16126C 16193T 16278T 16290T	16	J2
MP1a	16037G 16126C 16294T 16296T 16304C	19	T2b
AcqA	16126C 16153A 16290T 16294T 16296T	21	T2e
BRONZE AGE			
RomIVT2A	None (rCRS)	1	H2a2a1
RomIII100a			
RomIII103			
RomIII105			
RomIII97	16311C	3	H2a
RomIII114	16304C	4	H5
RomIII118b	16294T 16304C	5	H5a4
RomIIIT11A-b			
RomIII118a	16294T 16304C 16320T	6	H5a4a
RomIIIT11A-a			
RomIIIT12E-c			

[24] Only a partial sequence for Mezzocorona was obtained in this study. However, as the other two mutations (which are exceedingly rare in this combination in modern groups) match the results for the same sample (other half of sampled molar) determined by Di Benedetto et al. in 2000,

Table 14 cont.

Individual	Mutations (HVR1 + 12308 if tested)	HT	HG
BRONZE AGE			
RomIII82	16266T 16270T 16304C 12308G	8	U5b3h
RomIII100b	16182C 16183C 16189C 16234T 16324C 12308G	9	U8
RomIII120b			
PdVT3	16298C	10	HV0
RomIII91b	16189C 16223T 16278T	11	X
PdVT2			
RomIII89	16093C 16179T 16189C 16223T 16278T 16362C	12	X2j
RomIII90			
NogIII75	16069T 16126C 16189C	15	J1c
RomIII85	16069T 16126C 16261T	17	J1c
RomIII91a			
RomIIIT12E-a	16126C 16292T 16294T 16296T	20	T2c1
Schla203	16126C 16294T 16296T 16324C	18	T2

Table 19: Frequency of ancient Trentino haplotypes in other ancient groups

95% Confidence intervals are specified in brackets. To preserve clarity, only the most relevant reference groups are shown (Starčevo and LBK as "Neolithic" groups, and Corded Ware, Bell Beakers and Únětice as groups with a certain proportion of Steppe ancestry.

Individual	HT	HG	Starčevo (n=31)	LBK (n=73)	Corded Ware (n=29)	Bell Beaker (n=36)	Únětice (n=28)
NEOLITHIC							
Mezzo2	22	T2	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
LV76/3A	23	N1a	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
COPPER AGE							
Solt63	1	H2a2a1	0.065 [0.018-0.207]	0.137 [0.076-0.234]	0.103 [0.036-0.264]	0.278 [0.158-0.440]	0.071 [0.020-0.226]
Solt61	2	H1	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
NogII69	6	H5a4a	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
MP1b	7	U5b1c	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.034 [0.006-0.172]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
MP6	10	HV0	0.000 [0.000-0.110]	0.041 [0.014-0.114]	0.034 [0.006-0.172	0.000 [0.000-0.096]	0.071 [0.020-0.226]
Solt56	13	K1a	0.097 [0.033-0.249]	0.081 [0.038-0.168]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
Solt64a							
MP2b							
Solt59	14	J	0.032 [0.006-0.162]	0.096 [0.047-0.185]	0.103 [0.036-0.264]	0.028 [0.005-0.142]	0.000 [0.000-0.121]
MP2a							
Solt67	16	J2	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
MP1a	19	T2b	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
AcqA	21	T2e	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
BRONZE AGE							
RomIVT2A	1	H2a2a1	0.065 [0.018-0.207]	0.137 [0.076-0.234]	0.103 [0.036-0.264]	0.278 [0.158-0.440]	0.071 [0.020-0.226]
RomIII100a							
RomIII103							
RomIII105							
RomIII97	3	H2a	0.000 [0.000-0.110]	0.041 [0.014-0.114]	0.034 [0.006-0.172	0.000 [0.000-0.096]	0.036 [0.006-0.177]
RomIII114	4	H5	0.032 [0.006-0.162]	0.000 [0.000-0.050]	0.069 [0.019-0.220]	0.083 [0.029-0.218]	0.000 [0.000-0.121]
RomIII118b	5	H5a4	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
RomIIIT11A-b							

Table 15 cont.

Individual	HT	HG	Starčevo (n=31)	LBK (n=73)	Corded Ware (n=29)	Bell Beaker (n=36)	Únětice (n=28)
BRONZE AGE							
RomIII118a	6	H5a4a	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
RomIIIT11A-a							
RomIIIT12E-c							
RomIII82	8	U5b3h	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
RomIII100b	9	U8	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
RomIII120b							
PdVT3	10	HV0	0.000 [0.000-0.110]	0.041 [0.014-0.114]	0.034 [0.006-0.172	0.000 [0.000-0.096]	0.071 [0.020-0.226]
RomIII91b	11	X	0.032 [0.006-0.162]	0.014 [0.002-0.074]	0.103 [0.036-0.264]	0.000 [0.000-0.096]	0.071 [0.020-0.226]
PdVT2							
RomIII89	12	X2j	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
RomIII90							
NogIII75	15	J1c	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
RomIII85	17	J1c	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
RomIII91a							
RomIIIT12E-a	20	T2c1	0.000 [0.000-0.110]	0.027 [0.008-0.095]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]
Schla203	18	T2	0.000 [0.000-0.110]	0.000 [0.000-0.050]	0.000 [0.000-0.096]	0.000 [0.000-0.096]	0.000 [0.000-0.121]

Likelihood of haplotype sharing according to group (rather than on an individual basis) is shown in Fig. 4.

3.4.2 Nuclear DNA sequences

Sequence results were regarded as reliable if obtained from at least 2 PCRs from 2 extracts. Results obtained from one extract were taken into account if obtained in at least 2 PCRs of good quality (or at least moderate quality but replicated in a separate lab). Results obtained in only one PCR are not regarded as reliable but are listed here for sake of completeness.

3.4.2.1 Lactase persistence (-13.910*T/C)

Table 20: Lactase persistence (-13.910*T/C) genotypes

Sample CA=Copper Age BA=Bronze Age	LCT genotype	PCRs	Extracts	Reproduced in separate lab	Sequence quality
NogII69 (CA)	C/C	2	2	yes	good
PdVT2 (BA)	C/C	2	1	yes	good
PdVT3 (BA)	C/C	4*	2	no	moderate
RomIII89 (BA)	C/C	3	2	yes	good
RomIVT2A (BA)	C/C	3	1	yes	good
Solt63 (CA)	C/C	2	1	yes	good
Solt67 (CA)	C/C	3	2	yes	moderate

Samples not meeting authentication requirements

Sample	LCT genotype	PCRs	Extracts	Reproduced in separate lab	Sequence quality
RomIII90 (BA)	C/C	2	1	no	moderate
RomIII114 (BA)	C/C	2	1	no	moderate
Solt56 (CA)	C/C	2	1	no	moderate
Solt59 (CA)	C/C	2	1	no	moderate
MP2a (CA)	C/C	1	1	no	moderate
RomIII91b (BA)	C/C	1	1	no	good
RomIII97 (BA)	C/C	1	1	no	moderate
RomIII100a (BA)	T/T	1	1	no	moderate
RomIII103 (BA)	C/C	1	1	no	good
RomIII105 (BA)	C/C	1	1	no	moderate
RomIII120b (BA)	C/C	1	1	no	moderate
RomIIIT11A-b (BA)	C/C	1	1	no	poor
RomIIIT12E-b (BA)	C/C	1	1	no	moderate
RomIIIT12E-c (BA)	C/C	1	1	no	poor
Schla203 (LBA)	C/C	1	1	no	poor
Solt60 (CA)	C/C	1	1	no	poor
Solt61 (CA)	C/C	1	1	no	good
MP1a (CA)	C/C	1	1	no	poor
RomIVT2B (BA)	C/C	1	1	no	poor
NogIII75 (BA)	C/C	1	1	no	poor

All 7 samples providing reliable results have the ancestral C/C allele, denoting lactase nonpersistence. Although the other 20 samples from which samples were obtained cannot strictly be regarded as reliable, the overall prevalence of C/C (obtained in 23 of 24 of PCRs from these samples) indicates that this genotype is likely to have been commonplace in this population. No reproducible sequence with the derived allele T was obtained – the one sample from which T/T was sequenced could not be amplified a second time, so that the obtained sequence may represent exogenous contamination.

3.4.2.2 Eye colour (HERC2)

Table 21: Eye colour (HERC2) genotypes

Sample	HERC2 genotype	PCRs	Extracts	Reproduced in separate lab	Sequence quality
MP1a (CA)	A/G	3	1	yes	good
NogIII75 (BA)	A/A	2	1	yes	good
PdVT2 (BA)	A/A	2	1	yes	good
RomIII89 (BA)	A/G	4	2	yes	good
RomIII90 (BA)	A/G	3	2	yes	good
RomIII91b (BA)	A/G	2	1	yes	good
RomIII97 (BA)	A/A	2	2	yes	good
RomIII105 (BA)	A/A	2	2	no	moderate
RomIII114 (BA)	A/A	2	2	no	moderate
RomIVT2A (BA)	A/G	2	1	yes	good
Rom IVT2B (BA)	A/A	2	1	yes	good
Solt56 (CA)	A/G	2	2	no	good
Solt59 (CA)	A/G	2	2	no	moderate
Solt60 (CA)	A/G	2	1	yes	good
Solt61 (CA)	A/A	2	1	yes	good
Solt67 (CA)	G/G	3	2	yes	good

Samples not meeting authentication requirements

Sample	HERC2 genotype	PCRs	Extracts	Reproduced in separate lab	Sequence quality
MP1b (CA)	A/A	1	1	no	moderate
MP6 (CA)	Ambiguous (1A, 1G)	1/1	1	Results from separate labs	moderate
MP7b (CA)	A/A	1	1	no	moderate
NogII69 (CA)	A/G or A/A	2/1	2	A/G obtained in separate labs	good
PdVT3 (BA)	A/A or A/G	2/1	2	A/A obtained in separate labs	good
RomIII91a (BA)	G/G	1	1	no	good
RomIII120a (BA)	G/G	1	1	no	good
RomIIIT11A-b (BA)	A/A	1	1	no	good
RomIIIT12E-b (BA)	A/G	1	1	no	good
RomIIIT12E-c (BA)	G/G	1	1	no	moderate
Solt63 (CA)	A/A or A/G	1/1	1	Results from separate labs	good
Solt64a (CA)	A/G	1	1	no	moderate

Ambiguous results were shown in some cases: this is likely to be the result of G→A deamination as a result of DNA degradation. In the case of Nogarole II 69, the one PCR which did not show a G peak was of poor general quality and low amplitude (in other words, this PCR did not document the absence of a G allele and therefore does not necessarily contradict the other results obtained from this sample).

3.4.2.3 Amelogenin (sex determination)

Table 22: Amelogenin genotypes

Sample	Sex determination Amelogenin)	M201 amplification (sequence results in next section)	Osteological analysis by A. Paladin 2013
MP1a (CA)	Female	Negative (175)	N.D.
MP2b (CA)	Female		N.D.
MP6 (CA)	Male	Positive (295)	N.D.
NogII69 (CA)	Male (2P2E)	Positive (175, 201**, 321**)	Male
PdVT2 (CA)	Male	Negative (295)	Female
PdVT3 (CA)	Male	Positive (295, 196, 321**)	Male
RomIII83 (BA)	Female	Negative (175, 201)	Female
RomIII89 (BA)	Female (2P2E)	Negative (201**)	Female
RomIII90 (BA)	Female (2P2E)	Negative (175)	Female
RomIII91a (BA)	Female		N.D.
RomIII91b (BA)	Female	Negative (295)	N.D.
RomIII103 (BA)	Female	Negative (295, 296)	Female
RomIII105 (BA)	Female	Negative (201)	Female
RomIII114 (BA)	Female	Negative (175, 201**)	Female (infans I)
RomIII117 (BA)	Female		N.D.
RomIIIT11A-b (BA)	Female	Negative (295)	Female
RomIIIT12E-c (BA)	Male	Negative (295, 296)	Male
RomIVT2A (BA)	Female	Negative (175)	Female
RomIVT2B (BA)	Female		N.D.
RomIV58 (BA)	Male	Positive (201)	N.D.
Schla203 (BA)	Female	Negative (201)	N.D.
Solt56 (CA)	Female	Negative (175)	Female
Solt59 (CA)	Female	Negative (175)	Male (adult)
Solt63 (CA)	Male	Positive (296, 321)	Female (adult)
Solt64a (CA)	Female	Negative (295)	N.D.
Solt65b (CA)	Male	Positive (296)	N.D.
Solt67 (CA)	Male	Negative (295_XV), positive (296_XXXIII)	N.D.

Of the 15 cases where both osteological and genetic sex determination data are available, 12 concur. Only in three cases are the genetic findings inconclusive with the osteological analysis: Paludei di Volano T2 was determined as female on the basis of bone morphology. However, the individual died at 1.5-2 years of age, and sex determination in such a young individual is difficult to state with certainty. On the other hand, the genetic findings are not wholly conclusive either – although the amelogenin band appears to be double, no Y-chromosomal DNA was amplified for M201, so

that this individual may plausibly be female and the "double" amelogenin band may indicate that the agarose concentration was not high enough to permit clear separation. The other inconsistent result is Solteri 59, which yielded no male DNA in the M201 PCR and only showed a single PCR band. The osteological analysis tentatively determined male sex for this individual. Both genetic and osteological analyses are limited in this case: the genetic findings are based on one PCR only, and osteological determination was based on fragments of the occipital bone, limiting the possibility of making a specific determination. Lastly, Solteri 63 was identified as female during osteological analysis, whereas genetic analysis showed not only a double band for amelogenin, but also one amplification for the Y-chromosomal marker M201. Regarded together, these results increase the likelihood of the genetic sex determination as a male. In this case too, osteological sex determination was hampered as only a fragment of the jaw was available (Paladin, 2013).

3.4.3 Y-chromosomal SNPs

3.4.3.1 M201

Table 23: M201 (haplogroup G) haplotypes

Sample	M201 allele	PCRs	Extracts	Repro-duced in separate lab	Sequence quality
NogII69 (CA)	T	7	2	yes	good
PdVT3 (BA)	T	6	2	yes	good
RomIIIT12b (BA)	T	2	1	yes	good

Samples not meeting authentication requirements

Sample	M201 allele	PCRs	Extracts	Repro-duced in separate lab	Sequence quality
MP6 (CA)	T	1	1	no	moderate
RomIV58 (BA)	T	1	1	no	good
Solt63 (CA)	T	1	1	no	good
Solt65b (CA)	T	1	1	no	good
Solt67 (CA)	T	1	1	no	moderate

All 3 samples that complied with criteria of authenticity showed the T allele, which is indicative of Y-chromosomal haplogroup G, as did the 5 other samples which yielded sequences but could not be authenticated. Male staff of the Bolzano ancient DNA facilities had been tested for Y-chromosomal haplogroup G previously in connection with analysis of the Iceman's genome; none belonged to this haplogroup.

3.4.3.1.1 P287

Table 24: P287 (haplogroup G2) haplotypes

Sample	P287 allele	PCRs	Extracts	Reproduced in separate lab	Sequence quality
NogII69 (CA)	T	2	2	no	Good
PdVT3 (CA)	T	2	2	no	Good

Samples not meeting authentication requirements:

Sample	MP287 allele	PCRs	Extracts	Reproduced in separate lab	Sequence quality
RomIIIT12B (BA)	T	1	1	no	Good

The derived allele (T) for P287 (which was found in all of the above) denotes haplogroup G2.

3.4.3.1.2 L91

Table 25: L91 (haplogroup G2a-L91) haplotypes

Sample	L91 allele	PCRs	Extracts	Reproduced in separate lab	Sequence quality
NogII69 (CA)	G	4	2	no	good
PdVT3 (CA)	G	3	2	no	good

Samples not meeting authentication requirements:

Sample	L91 allele	PCRs	Extracts	Reproduced in separate lab	Sequence quality
RomIIIT12B	G	1	1	no	moderate

The derived allele C at the L91 locus denotes haplogroup G2a-L91. Whereas the Iceman has the derived allele for L91, the individuals for which this position could be typed all show the ancestral allele, indicating that they do not belong to the same subgroup as the Iceman.

3.5 Population genetic analyses

3.5.1 Group structuring

To compute the summary statistics between the Trentino samples and other ancient and modern reference populations, definition of group structures was attempted according to spatial analysis of genetic variance (SAMOVA) as well as on the basis of archaeological characteristics as described in Section 2.3.14.4.

3.5.1.1 SAMOVA

Differentiation by means of SAMOVA analysis was only applied to Copper and Bronze Age samples. On the one hand, the Neolithic samples (La Vela and Mezzocorona) must clearly be regarded as a separate group simply on the basis of their chronological and typological placement. On the other hand, the data obtained from these samples were poor and incomplete. Therefore, SAMOVA calculations were aimed at investigating a possible grouping of Copper and Bronze Age samples.

Table 26: SAMOVA analysis including all Copper and Bronze Age sites

K denotes the number of pre-defined groups.

K	F_{SC}	P (F_{SC})	F_{CT}	P (F_{CT})	Groups
2	0.00122	0.00782	0.30680	0.00000	1. Moletta Patone (CA), Nogarole (CA/BA), Romagnano (BA), Paludei di Volano (BA), Solteri (CA) 2. Acquaviva (CA)
3	-0.08161	0.00880	0.17454	0.00000	1. Nogarole (CA/BA), Romagnano (BA), Paludei di Volano (BA) 2. Acquaviva (CA) 3. Moletta Patone (CA), Solteri (CA)
4	-0.10855	0.01466	0.19444	0.00000	1. Moletta Patone (CA) 2. Nogarole (CA/BA), Romagnano (BA), Paludei di Volano (BA) 3. Acquaviva (CA) 4. Solteri (CA)
5	-0.13744	0.00782	0.18777	0.00000	1. Acquaviva (CA) 2. Moletta Patone (CA) 3. Nogarole (CA/BA), Romagnano (BA), 4. Solteri (CA) 5. Paludei di Volano (BA)

As the highest F_{CT} value is obtained for the lowest number of groups, this analysis therefore does not indicate any genetically separate groups within the overall sample population, with the Acquaviva sample being the only one that is set apart despite spatial closeness (in all probability due to the fact that is a single sample – SAMOVA does not take group sizes into account). In summary, the SAMOVA results give no indication of a

particular grouping. Therefore, analyses were run in duplicate: once with all Trentino individuals in one group, and once with groups constructed on the basis of chronology and archaeological context.

3.5.1.2 Grouping according to archaeological characteristics

On the basis of chronology alone, the samples fall into the following groups[25]:

Neolithic (5300-4200 BCE): Mezzocorona, La Vela

Early Copper Age (3300-3000 BCE): Acquaviva, Solteri, Tyrolean Iceman

Mid-late Copper Age (2900-2600 BCE): Nogarole, Moletta Patone

Early Bronze Age (2300-2100 BCE): Nogarole, Paludei di Volano, Romagnano

Late Bronze Age (1800-1500 BCE): Schlanders

However, grouping purely on the basis of chronology makes little sense, if groups show typological continuity over a longer period of time. Therefore, archaeological characteristics are also taken into account. The following general typological groups can be differentiated:

[25] Dates given denote the age ranges of the samples analysed here, not necessarily the entire span of the era in question

Table 27: Sub-groups of sample population according to archaeol. characteristics

Era	**Neolithic (5300-4200 BCE)**	**Copper Age (3300-2500 BCE)**	**Early Bronze Age (2400-2100 BCE)**
Burials & artefacts	Early Neolithic (Gaban): persistence of Mesolithic traditions, impressed ware, Castelnovian blades VBQ typology: - *Vasi di bocca quadrata* (VBQ) style pottery - Left crouched burials in cist graves	Remedello typology: - Crouched burials - Characteristic dagger and arrowhead form; flint daggers, stone arrowheads and polished stone axes, first use of copper artefacts	Polada typology: - Babies and children buried in urns - Separate skull burials - Inhumations under stone piles - Traces of (possibly) "ritualistic" fires
Site characteristics	Early Neolithic: Rock shelters Mid Neolithic: Open sites, no rock shelters	Rock shelters	Rock shelters; also, open-air lake sites (Fiavè)
Subsistence	Although domesticates (caprines, bovines, pig) are found in faunal remains, hunting (red & roe deer) represents major subsistence strategy Barley, spelt and emmer finds document agriculture, but also gathering (hazelnuts, elderberry, raspberry)	Proportion of remains from domesticates increases. At some sites, domesticates represent the majority (e.g. Moletta Patone) while wild animals are more numerous at others (Acquaviva). Stomach contents and equipment of the Tyrolean Iceman reflects combination of subsistence strategies: hunting (bow & arrows, ibex and red deer in stomach) and agriculture (einkorn wheat in stomach)	Domesticates represent large majority: caprines[26], pigs and bovines. Faunal remains from wild animals (red deer, roe deer, wild boar) decrease in frequency: small percentage of wild animals, mainly red deer, but also roe deer, wild boar and hare, indicate occasional hunting[27], Plant remains include emmer and barley.

[26] The ages at death indicate use for milk as well as for wool in the case of sheep (Fontana 2009)

[27] Fontana (2009) has suggested that this may not be solely for subsistence purposes, but also to maintain pastures for domesticates.

Table 23 cont.

Era	Neolithic (5300-4200 BCE)	Copper Age (3300-2500 BCE)	Early Bronze Age (2400-2100 BCE)
Other	By the middle Neolithic, cultural contact with LBK cultures as well as Sardinia (obsidian trade)	While earliest documented Bell Beaker contact occurs at the end of the Copper Age, it occurs later than the Copper Age samples analysed in the course of this study. However, other contacts (e.g. Corded Ware) occurred prior to the large-scale Bell Beaker dispersal	After earliest documented Bell Beaker contact (2500 BCE). First appearance of domesticated horse remains (although faunal remains are not extensive enough to infer information on either possible slaughter or use as draught or riding animal.
Sampled sites[28]	La Vela Mezzocorona	Acquaviva Moletta Patone Nogarole II Solteri* Tyrolean Iceman	Nogarole III Paludei di Volano* Romagnano

* Sites marked with an asterisk lacked archaeological context and were allocated on the basis of chronology.

The middle Neolithic sites of Mezzocorona and La Vela were clearly separate from the later groups: not only due to the time gap of over a millennium, but also due to the archaeological characteristics. The La Vela burials were characterised by burial types (cist graves) not observed during the Copper or Bronze ages, and contained pottery characteristic of the VBQ culture. However, due to the poor overall quality of the Neolithic sequences, these were not used for calculating summary statistics; but were simply defined as a separate group for general observations and comparisons.

Those sites for which comprehensive archaeological information is available fall into two groups: The Copper Age burials (approx. 3200-2700 BCE, although the latest possible dates for Moletta Patone are around 2500 BCE), some of them including implements of "Remedello" typology, and the

[28] Refers only to samples used for this study – most sites span several eras. All known sites were taken into consideration regarding characteristics.

Bronze Age sites (approx. 2300-2100 BCE) showing the characteristics typical of the Polada Culture: babies and children interred in vases/urns, separate skull burials (interpreted by some authors as a possible "skull cult"), inhumations covered by rock piles and traces of fires (possibly ritualistic in nature). This differentiation is particularly apparent in the two phases of Nogarole: While the later Nogarole III sector shows the same infant vase burials, rock piles, skull inhumations and fireplaces as Romagnano, the Nogarole II sector shows simple crouched burials with no vases, although rock piles are also observed here, indicating that this practice started in the Copper Age. As the sites of Solteri and Paludei di Volano lacked sufficient archaeological context (i.e. disturbed burials, grave goods either not reported or not found) attributed to either group, they were allocated on the basis of the C_{14} dates.

Grouping into Copper and Early Bronze age makes sense from another point of view: The earliest evidence of the Bell Beakers, who had a huge impact on populations throughout Europe during the Copper/Bronze Age, dates to approximately 2500 BCE (on the basis of a pitcher of characteristic Bell Beaker form found on the Pigloner Kopf in South Tyrol). Thus, differentiation between a Copper Age group dating until approx. 2700 BCE and a Bronze Age group dating approx. 2300-2100 BCE (and showing novel cultural attributes) divides the sample collective into a pre-Bell Beaker and a post-Bell Beaker group. A comparison of these two groups with one another and with contemporary groups, enables an assessment of whether an (and if so, which) extraneous group had a notable impact on the population of Trentino toward the end of the Copper Age.

Due to its separate chronological placement (approx. 500 years after the Polada Culture) and lack of archaeological context, the Schlanders individual is not included in summary statistic calculations, but is regarded individually in the discussion.

3.5.2 Statistical analyses

3.5.2.1 Overview

Table 28: Diversity/expansion indices for Trentino samples and ancient reference groups

	Haplotype diversity	**Nucleotide diversity**	**Mean number of pairwise differences**	**Tajima's D**	**Fu's FS** (bold type= significant p- value)
Trentino CA (n=13)	0.9615 ± 0.0496	0.012967 ± 0.007660	4.551282 ± 2.391883	-0.71460 (p=0.26100)	-3.35889 (p=0.03600)
Trentino BA (n=22)	0.9437 ± 0.0282	0.010241 ± 0.005893	4.229437 ± 2.181417	-1.12165 (p=0.13400)	-4.00408 (p=0.03300)
WHG (n=30)	0.9172 ± 0.0375	0.006370 ± 0.003922	2.528736 ± 1.399379	-1.40520 (p=0.06500)	-6.61489 **(p=0.00100)**
PPN (n=18)	0.9673 ± 0.0298	0.014652 ± 0.008727	3.575163 ± 1.904176	-1.36949 (p=0.08200)	-8.38953 **(p=0.00000**)
AAN (n=32)	0.9073 ± 0.0391	0.011804 ± 0.006572	4.875000 ± 2.440299	-0.86459 (p=0.21100)	-2.30877 (p=0.18400)
Starčevo (n=31)	0.9849 ± 0.0124	0.014199 ± 0.007836	5.324731 ± 2.641158	-1.04507 (p=0.15400)	-18.14930 **(p=0.00000)**
LBK (n=72)	0.9562 ± 0.0105	0.012492 ± 0.006785	5.159233 ± 2.528245	-0.77376 (p=0.21900)	-14.16323 **(p=0.00000)**
Yamnaya (n=39)	0.9717 ± 0.0143	0.012064 ± 0.006689	4.801619 ± 2.396335	-1.63825 **(p=0.02900)**	-18.66861 **(p=0.00000)**
Corded Ware (n=29)	0.9704 ± 0.0182	0.010633 ± 0.006034	4.295567 ± 2.190115	-1.43763 (p=0.05300)	-9.97639 **(p=0.00000)**
Bell Beaker (n=36)	0.9921 ± 0.0092	0.008560 ± 0.005005	3.312698 ± 1.742488	-2.05883 **(p=0.00700)**	-15.54637 **(p=0.00000)**
Únětice (n=28)	0.9921 ± 0.0118	0.013451 ± 0.007436	5.420635 ± 2.691546	1.40120 (p=0.06500)	-15.85908 **(p=0.00000)**
Neolithic France (n=29)	0.9557 ± 0.0286	0.009040 ± 0.005289	3.453202 ± 1.815222	-0.69678 (p=0.27900)	-3.61353 (p=0.04400)
Neolithic Spain (n=58)	0.9952 ± 0.0050	0.007256 ± 0.004324	2.764670 ± 1.485178	-1.48694 **(p=0.04500)**	-17.68329 (p=0.00000)
Bronze Age Sardinia (n=32)	0.9980 ± 0.0085	0.006424 ± 0.00401	2.312500 ± 1.299464	-1.52764 **(p=0.04100)**	-5.56462 **(p=0.00600)**
Etruscans (n=42)	0.9942 ± 0.0071	0.010359 ± 0.005926	3.729384 ± 1.920774	-1.58856 **(p=0.02700)**	-25.97233 **(p=0.00000)**

Copper Age Trentino shows a haplotype diversity index corresponding to approximately the mean of all reference groups, and a nucleotide diversity index slightly above the mean. Bronze Age Trentino shows lower values in both cases, with nucleotide diversity slightly below the and haplotype diversity below the mean by a slightly larger margin.

Although the values obtained for Tajima's D and Fu's FS are negative (although to a lesser extent than most ancient reference groups), which would generally indicate a minor degree of population expansion, all values fall short of the significance limit (a p-value of 0.02 is required for Fu's FS rather than the standard 0.05 (Excoffier and Lischer, 2010)).

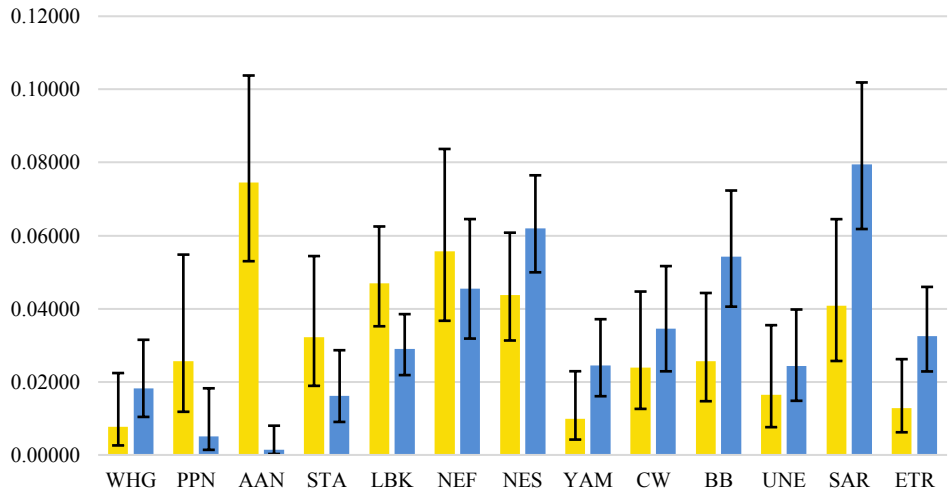
3.5.2.2 Haplotype sharing

Haplotype sharing in the context of this study is defined as the likelihood of single samples selected at random from the ancient study population and a reference population sharing the same haplotype. Identification of haplotype matches were generated using the Arlequin software. The results provided here are based on the mitochondrial HVR1, positions 16033-16382 (other positions initially analysed in the ancient Trentino samples were disregarded in the course of the Arlequin calculation due to excess missing data across all reference sample sequences at those positions; missing data level was adjusted to 0.08 to include all polymorphic sites in the Trentino sample set[29]).

[29] For this reason, certain reference samples reported by Arlequin as "matching" a given ancient Trento sequence could be shorter and lack data on certain defining polymorphic sites. Due to the substantial amount of reference samples, it was not possible to check all sequences manually. However, as this applies to a maximum of 8% of samples for any given position, this "standard error" is the same as for the F_{ST} calculations and is not expected to have a significant impact on the overall results, especially as reference sequences far shorter than the Trento sequences or lacking significant positions were not included in the statistical analyses.

3.5.2.2.1 Haplotype sharing likelihood between ancient Trentino and other ancient populations

Figure 5: Haplotype sharing likelihood between ancient Trentino samples and other ancient reference groups



Yellow bars=Copper Age Trentino; blue bars=Bronze Age Trentino.
Black lines indicate 95% confidence intervals.
WHG=Western Hunter-Gatherers; PPN=Pre-Pottery Neolithic; AAN=Aegean & Anatolian Neolithic; STA=Starčevo; LBK=Linearbandkeramik; NEF=Neolithic France (Treilles); NES=Neolithic Spain; YAM=Yamnaya; CW=Corded Ware; BB=Bell Beaker; UNE=Únětice; SAR=Bronze Age Sardinia; ETR=Iron Age Etruscans

Haplotype sharing analysis etween Copper Age Trentino and ancient reference populations shows the highest levels of haplotype sharing likelihood with Neolithic groups. All five Neolithic groups[30] show values above the mean – Starčevo minimally, the other four (Anatolian & Aegean Neolithic, LBK, Neolithic France, Neolithic Spain) by a substantial amount. The Steppe-influenced groups Yamnaya, Corded Ware, Bell Beaker and Únětice show, in most cases, far lower likelihoods of haplotype sharing with Copper Age Trentino. The one Bronze Age group constituting an exception is Bronze Age Sardinia, representing the only non-Neolithic group with an above-mean likelihood of haplotype sharing with CA Trentino.

[30] Despite the name, the Pre-Pottery Neolithic is deemed separate from those Neolithic groups representing the expansion from the Fertile Crescent, and has been shown in various studies to be separate from these, see 2.3.14.0.3 Pre-pottery Neolithic.

Haplotype sharing analysis between Bronze Age Trentino and other ancient reference populations, however, shows a different picture: The Neolithic groups, which showed high likelihoods of haplotype sharing with CA Trentino, now show far lower values for BA Trentino. The one exception is Neolithic Spain, which shows higher values. The Anatolian & Aegean Neolithic group, which showed the highest likelihood of haplotype sharing with CA Trentino, is down to almost zero with BA Trentino. The Bronze Age groups (Yamnaya, Corded Ware, Bell Beaker, Únětice and Bronze Age Sardinia, all show a higher likelihood of haplotype sharing with BA Trentino than with CA Trentino, Bronze Age Sardinia now showing the highest value.

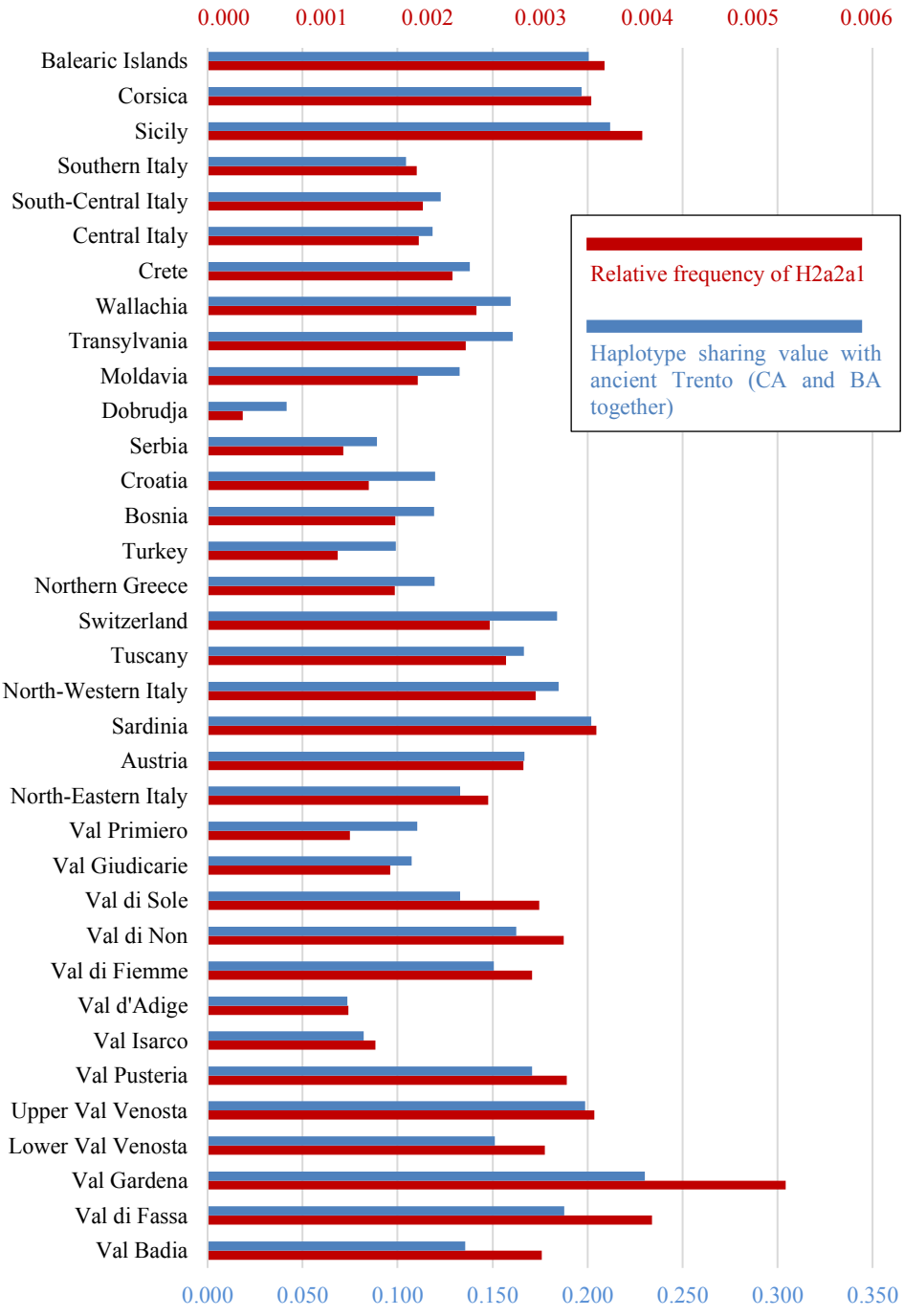
The Western Hunter-Gatherers show generally low values, rising slightly for BA Trentino. The same applies to the Iron Age Etruscans. The Pre-Pottery Neolithic group already shows low values as compared to CA Trentino, going down further for BA Trentino.

3.5.2.2.2 Haplotype sharing between ancient Trentino and modern reference populations

Haplotype sharing analysis between the study population and modern reference populations was initially performed incorporating all haplotypes.

However, one haplotype – H2a2a1, the revised Cambridge reference sequence – is very common across all modern reference population samples (frequency=0.153), occurring six times as frequently as the next most common haplotype. This uneven distribution shifts the pattern in favour of this particular haplotype, with the calculated value for haplotype sharing corresponding directly with the relative frequency of H2a2a1 in the respective reference population, as shown in the figure below.

Figure 6: Comparison between haplotype sharing values (blue) and relative frequencies of Haplotype H2a2a1 (red)

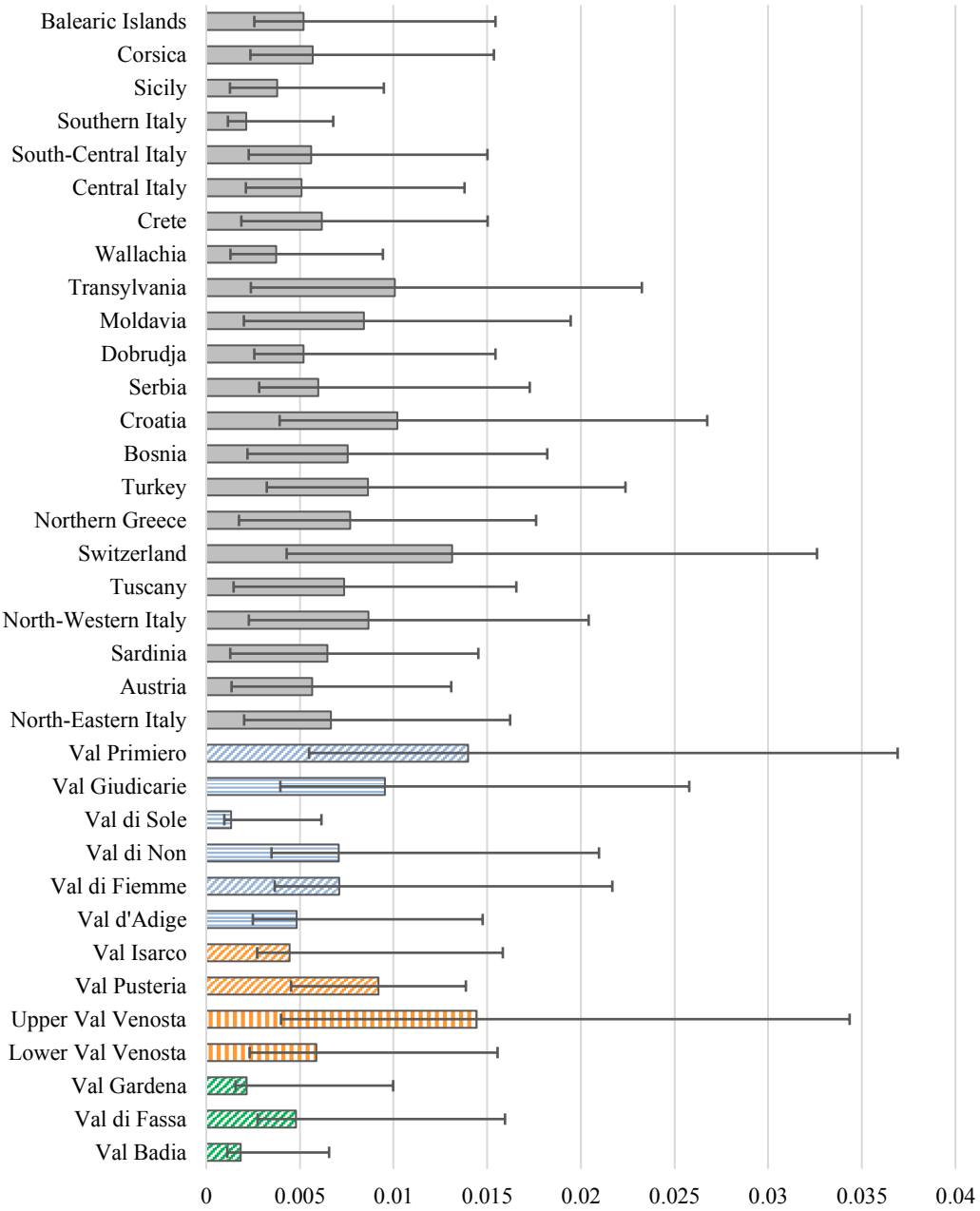


As the purpose of the diagram is to demonstrate the correlation between the two values, representation of confidence intervals is dispensed with in order to preserve clarity.

In other words, the "haplotype sharing" values calculated for each reference population correspond largely to the frequency of H2a2a1 in that population, with all other haplotypes having a negligible impact. This skews the obtained results, as the high frequencies of H2a2a1 in modern populations are not directly dependent on this haplotype's frequency in their prehistoric ancestral population[31]. Therefore, to obtain a more representative haplotype sharing analysis reflecting all haplotypes, and to avoid rarer haplotypes being "overshadowed" by H2a2a1, additional analyses were performed, excluding this particular haplotype (see below).

[31] This only applies to comparisons between ancient and modern populations, where the relative frequencies have shifted over millennia and been shaped by numerous subsequent demographic events. Although H2a2a1 is also the most common haplotype amongst the ancient reference groups, it was taken into account in the previous analysis as relative frequencies of any particular haplotype do indeed play a role when comparing (more or less) contemporary populations, especially with regard to determining ancestry/admixture.

Figure 7: Haplotype sharing likelihood between ancient Trentino samples (CA and BA) and modern reference groups



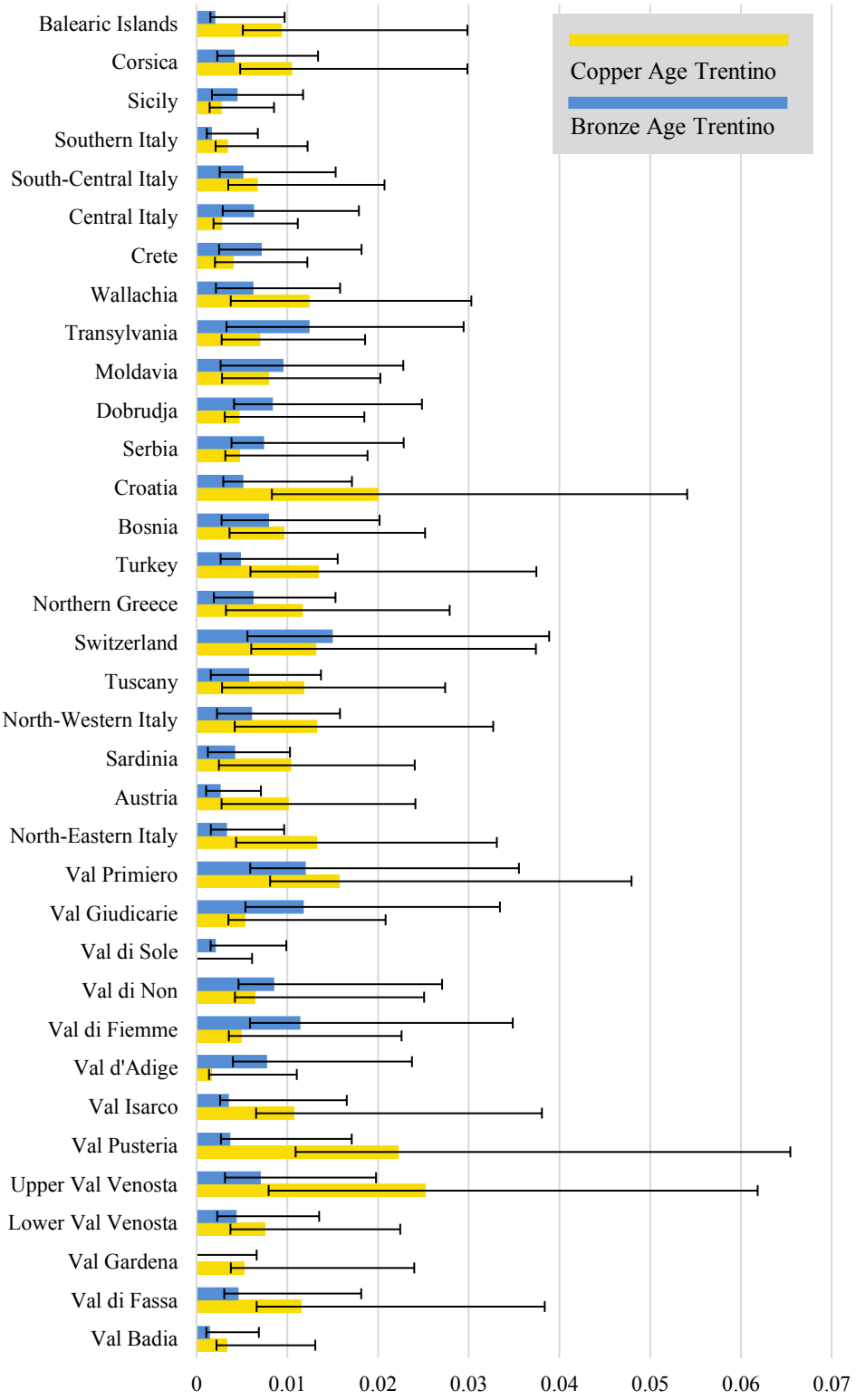
Grey lines represent 95% confidence intervals.

Colour indicates language distribution of groups in South Tyrol-Trentino:
Green: Ladin-speaking groups
Orange: German-speaking groups
Blue: Italian/Romansch-speaking groups.

Shading indicates geographical distribution of groups in South Tyrol-Trentino: Diagonal stripes: Northern and Western Dolomites
Horizontal stripes: Central Adige Valley and western plateau
Vertical stripes: Upper Adige valley (Alpine foothills)

Populations with the highest likelihood of sharing a haplotype with the ancient Trentino group (Copper and Bronze Age together) were Upper Val Venosta (German-speaking South Tyrol, Upper Adige Valley), Val Primiero (Romansch-speaking Trentino, Belluno Dolomites) and Switzerland. No particular pattern was observed either within language groups in South Tyrol-Trentino nor according to geographical location: Both Italian and German-speaking groups show both high and low values, as do the various geographical areas within South Tyrol-Trentino. The Ladin-speaking groups are the only ones with a uniform tendency, namely a low likelihood of haplotype sharing with ancient Trentino groups. On a larger scale, Northern Italy, Tuscany, Switzerland, Northern Greece and Turkey showed values above the mean, as did 4 of the 7 Balkan groups observed (Bosnia, Croatia, Moldavia and Transylvania). Populations with values below the mean (i.e. less than average likelihood of sharing a haplotype with ancient Trentino) were Central to Southern Italy, Sicily, Sardinia and Corsica, Crete, the Balearic Islands and Austria.

Figure 8: Haplotype sharing likelihood between ancient Trentino samples (CA and BA separated) and modern reference groups.
Bars lines denote CI_{95}.



A certain trend was observed when Copper Age and Bronze Age samples were analysed separately. The populations located in the vicinity of the central Adige Valley (Val D'Adige; Val Giudicarie, Val di Sole, Val di Non on the plateau west of the Adige valley and Val di Fiemme in the main valley stretching east from the central Adige Valley via Cal di Cembra) all show higher haplotype sharing values for Bronze Age Trentino than for Copper Age Trentino. All other groups in South Tyrol-Trentino (German-speaking upper Adige Valley, Northern and Central Dolomites, South-Eastern Trentino/Belluno Dolomites) have a higher likelihood of sharing with the Copper Age than the Bronze Age.

On a broader scale, Northern Italy (NE Italy, NW Italy, Tuscany) and Austria show a higher likelihood of sharing with Copper Age Trentino, whereas Switzerland shows similar values for both. Corsica, Sardinia, Southern-central Italy and Southern Italy show higher likelihood of sharing with Copper Age Trentino (the difference is particularly large for Corsica and Sardinia), whereas Sicily and Central Italy have higher haplotype sharing values for Bronze Age Trentino. In the Balkan region, Croatia, Wallachia and Bosnia are closer to the Copper Age, whereas the other Balkan groups show higher haplotype sharing values to Bronze Age Trentino. Northern Greece and Turkey show a higher likelihood of sharing with Copper Age Trentino.

Of all cross-regional modern reference populations, the German-speaking groups Val Pusteria (Northern Dolomites) and Upper Val Venosta show the highest overall haplotype sharing values to Copper Age Trentino, followed by Croatia and Val Primiero (Belluno Dolomites). Regarding the likelihood of haplotype sharing with Bronze Age Trentino, Switzerland and Transylvania returned the highest values, followed by Val Primiero (Trentino, Belluno Dolomites), Val Giudicarie (Plateau west of Adige Valley) and Val di Fiemme (Western Dolomites, side valley of Adige).

It must be borne in mind that haplotype sharing analysis is limited to identical sequences, providing no information on sequences that are (highly)

similar but differ in at least one position. Information on the latter is provided by F_{ST} analyses.

3.5.2.3 F_{ST} Values

3.5.2.3.1 Trentino samples with ancient reference groups

Table 29: F_{ST} values between ancient Trentino and other ancient reference groups

Significant F_{ST} values are denoted in bold type.

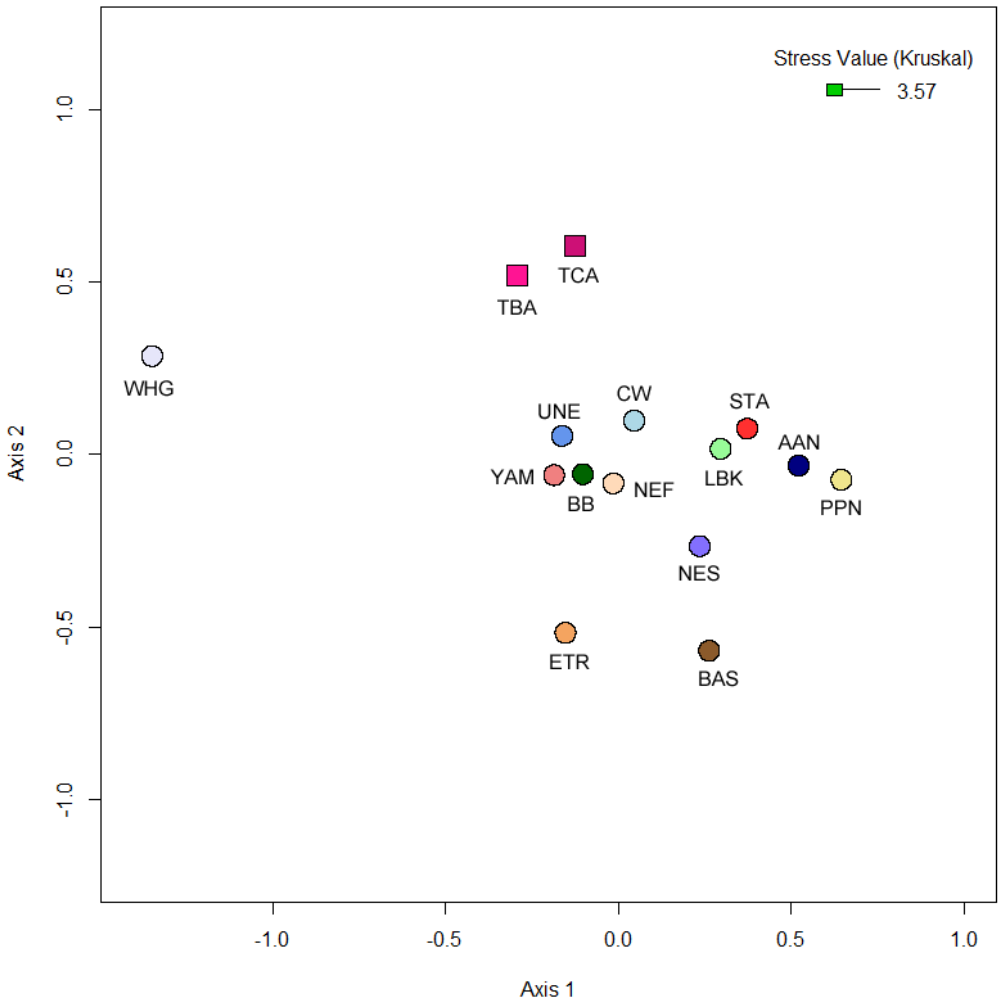
Reference group/ Abbreviation	Trentino Copper Age (n=13)	Trentino Bronze Age (n=22)
	Trentino Copper Age vs. Trentino Bronze Age F_{ST} = 0.**05782** (p=0.029)	
Western Hunter-Gatherers (WHG)	**0.24776** (p=0.00)	**0.19269** (p=0.00)
Pre-Pottery Neolithic (PPN)	-0.00751 (p=0.53)	**0.10902** (p=0.00)
Anatolian/Aegean Neolithic (AAN)	0.02481 (p=0.16)	**0.09152** (p=0.00)
Starčevo (STA)	-0.00329 (p=0.46)	**0.04014** (p=0.02)
Linearbandkeramik (LBK)	0.00081 (p=0.39)	**0.04300** (p=0.02)
Yamnaya (YAM)	**0.05200** (p=0.02)	**0.04251** (p=0.01)
Corded Ware (CW)	0.02658 (p=0.13)	**0.04751** (p=0.01)
Bell Beaker (BB)	**0.06163** (p=0.01)	**0.03851** (p=0.01)
Únětice (UNE)	**0.04754** (p=0.03)	0.01859 (p=0.11)
Neolithic France (NEF)	0.03551 (p=0.10)	0.02424 (p=0.10)
Neolithic Spain (NES)	0.03041 (p=0.10)	**0.07130** (p=0.00)
Bronze Age Sardinia (BAS)	**0.06337** (p=0.02)	**0.05357** (p=0.01)
Etruscans (ETR)	**0.07213** (p=0.00)	**0.06053** (p=0.00)

Table 30: F_{ST} values between all ancient groups

	TCA	TBA	WHG	PPN	AAN	STA	LBK	YAM	CW	BB	UNE	NEF	NES	BAS	ETR
TCA		0.030	0.000	0.535	0.159	0.466	0.385	0.019	0.127	0.009	0.028	0.104	0.096	0.023	0.003
TBA	**0.058**		0.000	0.000	0.001	0.020	0.015	0.007	0.010	0.009	0.109	0.101	0.001	0.007	0.001
WHG	**0.248**	**0.193**		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PPN	0.008	**0.109**	**0.255**		0.617	0.357	0.075	0.001	0.123	0.005	0.008	0.007	0.024	0.001	0.000
AAN	0.025	**0.092**	**0.231**	-0.011		0.544	0.041	0.000	0.116	0.001	0.002	0.006	0.002	0.000	0.000
STA	0.003	**0.040**	**0.190**	0.003	-0.005		0.342	0.003	0.231	0.004	0.015	0.035	0.011	0.006	0.000
LBK	0.001	**0.043**	**0.185**	0.028	**0.026**	0.002		0.003	0.012	0.004	0.007	0.013	0.002	0.017	0.000
YAM	**0.052**	**0.043**	**0.105**	**0.070**	**0.078**	**0.044**	**0.039**		0.084	0.615	0.388	0.062	0.003	0.085	0.003
CW	0.027	**0.048**	**0.128**	0.022	0.017	0.008	**0.038**	0.017		0.276	0.413	0.261	0.052	0.006	0.001
BB	**0.062**	**0.039**	**0.129**	**0.053**	**0.066**	**0.039**	**0.041**	-0.004	0.005		0.673	0.129	0.133	0.13	0.002
UNE	**0.048**	0.019	**0.094**	**0.050**	**0.059**	**0.034**	**0.042**	0.001	0.001	-0.007		0.415	0.005	0.012	0.003
NEF	0.036	0.024	**0.146**	**0.066**	**0.061**	**0.031**	**0.039**	0.021	0.008	0.013	0.000		0.067	0.14	0.012
NES	0.030	**0.071**	**0.201**	**0.042**	**0.055**	**0.035**	**0.038**	**0.038**	0.021	0.009	**0.040**	0.021		0.147	0.000
BAS	**0.063**	**0.054**	**0.241**	**0.101**	**0.091**	**0.050**	**0.034**	0.017	**0.047**	0.011	**0.037**	0.017	0.011		0.009
ETR	**0.072**	**0.061**	**0.170**	**0.087**	**0.111**	**0.074**	**0.076**	**0.033**	**0.041**	**0.029**	**0.037**	**0.030**	**0.054**	**0.031**	
	TCA	**TBA**	**WHG**	**PPN**	**AAN**	**STA**	**LBK**	**YAM**	**CW**	**BB**	**UNE**	**NEF**	**NES**	**BAS**	**ETR**

F_{ST} values are shown below the diagonal. Significant values are highlighted in bold type and a colour range (green = lowest F_{ST}, red = highest F_{ST}) to illustrate comparative values. P-values are shown above the diagonal, with significant values highlighted in blue.

Figure 9: MDS plot of Trentino samples vs. ancient reference groups



TCA: Copper Age Trentino, TBA: Bronze Age Trentino, WHG: Western Hunter-Gatherers, PPN: Pre-Pottery Neolithic, AAN: Anatolian & Aegean Neolithic, STA: Starčevo, LBK: Linearbandkeramik, YAM: Yamnaya, CW: Corded Ware, BB: Bell Beaker, UNE: Únětice, NEF: Neolithic France, NES: Neolithic Spain, BAS: Bronze Age Sardinia, ETR: Etruscans

In the comparison between Copper Age Trentino and the other ancient samples, several Neolithic groups (Starčevo, LBK, PPN, Anatolian & Aegean Neolithic) initially show very low F_{ST} values. However, the p-values for these results fall short of the significance value by a large extent. The lowest significant values are that of the Únětice and Yamnaya groups, but are not low enough to indicate a close relationship. Bell Beakers, Bronze Age Sardinia and Etruscans show higher F_{ST} values, and the F_{ST} between Copper Age Trentino and Western-Hunter Gatherers is very

high. Regarding comparison of the same reference groups with Bronze Age Trentino, the Neolithic groups of LBK and Starčevo show higher F_{ST} values than before, as do PPN and Anatolian/Aegean Neolithic to an even greater extent (although direct comparison with Copper Age Trentino is not possible due to lack of significance). One notable observation is that the F_{ST} between BA Trentino and Bell Beakers is far lower than for CA Trentino and Bell Beakers (and, to a lesser extent, in the Yamnaya and Western Hunter-Gatherers, who all show relatively low F_{ST} values to one another).

Regarding the MDS plot of Reynold's F_{ST} data, the two Trentino groups cluster some distance away from the other reference groups. The "Neolithic" groups (PPN, AAN, STA, LBK) form one cluster, the "Steppe-influenced" groups (YAM, BB, UNE) form another (together with Neolithic France), and Corded Ware in between. The observation from the F_{ST} data that Copper Age Trentino is slightly closer to Neolithic groups, whereas Bronze Age Trentino shows a closer affinity to the Steppe-influenced groups, is reflected to a small extent in the MDS plot, although the differentiation is not striking.

No particular similarity is observed between either of the Trentino groups with Neolithic Spain, Bronze Age Sardinia or the Etruscans.

3.5.2.3.2 Ancient Trentino vs. modern populations (regional)

Table 31: F_{ST} values between ancient Trentino and modern reference groups in South Tyrol-Trentino

Reference group/ Abbreviation	Region	Language	F_{ST} to Trentino Copper Age (n=13)	F_{ST} to Trentino Bronze Age (n=22)
Val Badia (BAD)	N/W Dolomites	Ladin	**0.03141** (p=0.046)	**0.04305** (p=0.001)
Val Gardena (GAR)	N/W Dolomites	Ladin	**0.04697** (p=0.041)	**0.04068** (p=0.016)
Val di Fassa (FAS)	N/W Dolomites	Ladin	**0.04442** (p=0.022)	**0.03370** (p=0.009)
Lower Val Venosta (LVV)	Upper Adige Valley	German	0.02479 (p=0.071)	**0.01826** (p=0.047)
Upper Val Venosta (UVV)	Upper Adige Valley	German	**0.03466** (p=0.038)	**0.05780** (p=0.000)
Val Pusteria (PUS)	N/W Dolomites	German	**0.06395** (p=0.014)	**0.07631** (p=0.000)
Val d'Adige (ADI)	Central Adige Valley & West	Italian	**0.03366** (p=0.037)	0.01341 (p=0.098)
Val di Fiemme (FIE)	N/W Dolomites	Romansch	0.02663 (p=0.095)	0.01256 (p=0.165)
Val di Non (NON)	Central Adige Valley & West	Romansch	0.02040 (p=0.132)	0.00973 (p=0.178)
Val di Sole (SOL)	Central Adige Valley & West	Romansch	**0.04853** (p=0.011)	**0.04008** (p=0.002)
Val Giudicarie (GIU)	Central Adige Valley & West	Romansch	0.01880 (p=0.136)	0.01390 (p=0.117)
Val Primiero (PRI)	N/W Dolomites	Romansch	0.03192 (p=0.071)	0.01326 (p=0.151)
Val Isarco[32] (ISA)	N/W Dolomites	German	**0.10162** (p=0.001)	**0.09658** (p=0.000)

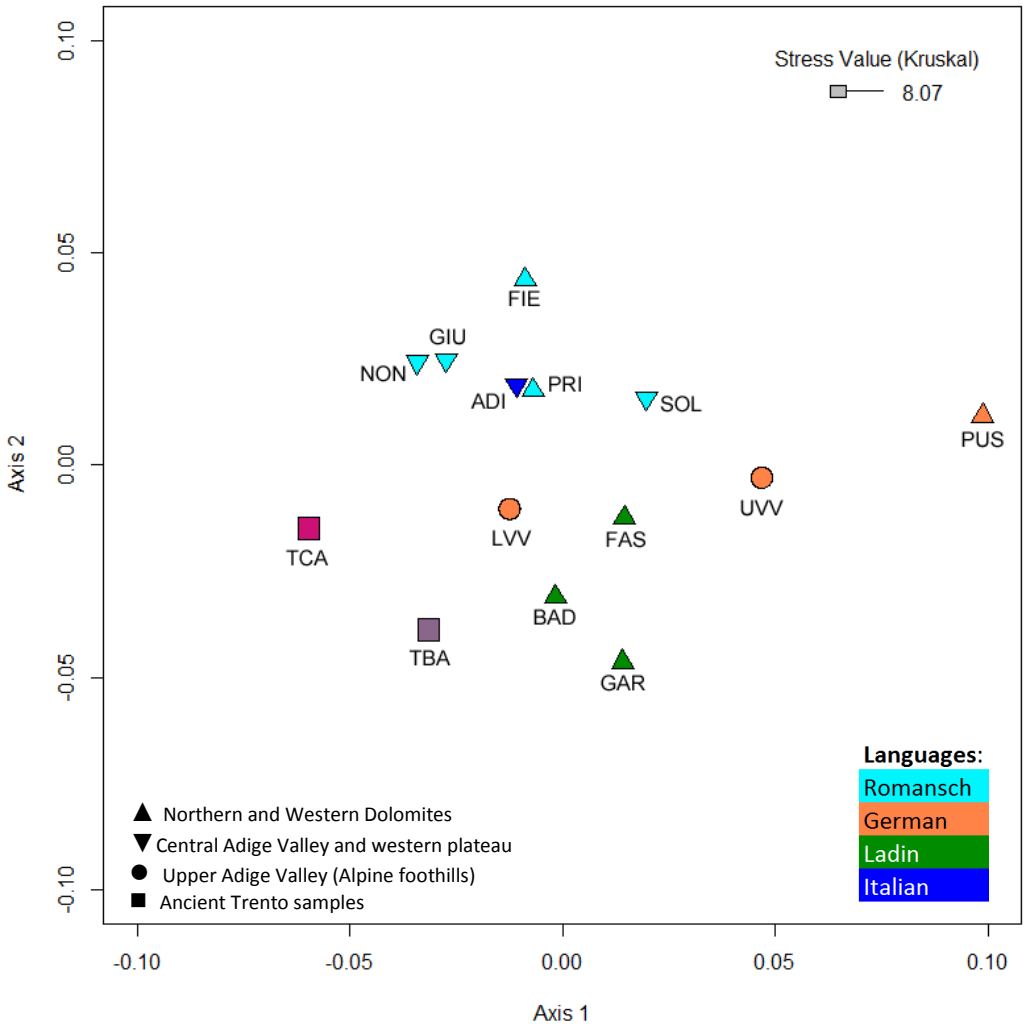
[32] The modern reference group Val Isarco was removed from the subsequent MDS plot as it appeared as an extreme outlier (probably due to genetic drift as a result of population bottlenecks), distorting the plot.

Table 32: F_{ST} values between ancient Trentino and modern reference groups in South Tyrol-Trentino (chart)

	TCA	TBA	BAD	GAR	FAS	LVV	UVV	PUS	ADI	FIE	NON	SOL	GIU	PRI	ISA
TCA		0.030	0.051	0.038	0.022	0.065	0.040	0.015	0.041	0.101	0.128	0.011	0.126	0.071	0.001
TBA	**0.058**		0.002	0.017	0.009	0.046	0.001	0.000	0.105	0.165	0.182	0.002	0.115	0.146	0.000
BAD	0.031	**0.043**		0.000	0.038	0.038	0.111	0.006	0.010	0.006	0.012	0.001	0.001	0.028	0.001
GAR	**0.047**	**0.041**	**0.044**		0.008	0.005	0.000	0.001	0.008	0.001	0.293	0.003	0.054	0.010	0.000
FAS	**0.044**	**0.034**	**0.011**	**0.031**		0.020	0.024	0.000	0.043	0.083	0.047	0.039	0.032	0.136	0.001
LVV	0.025	**0.018**	**0.007**	**0.022**	**0.013**		0.048	0.016	0.162	0.035	0.389	0.011	0.086	0.131	0.000
UVV	**0.035**	**0.058**	0.004	**0.048**	**0.012**	**0.006**		0.060	0.001	0.001	0.001	0.003	0.001	0.036	0.001
PUS	**0.064**	**0.076**	**0.022**	**0.065**	**0.039**	**0.018**	0.011		0.003	0.002	0.005	0.007	0.003	0.013	0.011
ADI	**0.034**	0.013	**0.014**	**0.027**	**0.012**	0.004	**0.022**	**0.032**		0.267	0.187	0.063	0.090	0.245	0.009
FIE	**0.027**	0.013	**0.021**	**0.042**	0.013	**0.012**	**0.026**	**0.040**	0.004		0.095	0.055	0.167	0.223	0.006
NON	0.020	0.010	**0.016**	0.003	**0.013**	0.000	**0.023**	**0.036**	0.005	0.011		0.021	0.388	0.180	0.000
SOL	**0.049**	**0.040**	**0.019**	**0.031**	**0.012**	**0.012**	**0.015**	**0.023**	0.009	0.012	**0.017**		0.065	0.050	0.006
GIU	0.019	0.014	**0.024**	0.014	**0.014**	0.007	**0.022**	**0.033**	0.008	0.007	0.001	0.010		0.131	0.000
PRI	0.032	0.013	**0.014**	**0.032**	0.008	0.006	**0.012**	**0.028**	0.004	0.006	0.007	0.012	0.008		0.000
ISA	**0.102**	**0.097**	**0.038**	**0.099**	**0.040**	**0.044**	**0.040**	**0.042**	**0.027**	**0.040**	**0.071**	**0.027**	**0.056**	**0.054**	

F_{ST} values are shown below the diagonal. Significant values are highlighted in bold type and a colour range (green = lowest F_{ST}, red = highest F_{ST}) to illustrate comparative values. P-values are shown above the diagonal, with significant values highlighted in blue.

Figure 10: Trentino samples vs. modern populations in South Tyrol & Trentino



TCA: Copper Age Trentino, TBA: Bronze Age Trentino, BAD: Val Badia, GAR: Val Gardena, FAS: Val di Fassa, LVV: Lower Val Venosta, UVV: Upper Val Venosta, PUS: Val Pusteria, ADI: Val d'Adige, FIE: Val di Fiemme, NON: Val di Non, SOL: Val di Sole, GIU: Val Giudicarie, PRI: Val Primiero

Correlation appears more marked with regard to language than to exact geographic location. A certain cluster formation can be observed for Italian & Italian Romansch language groups, irrespective of region (both N/W Dolomites and central Adige valley and west thereof). Ladin-speaking groups form a cluster, whereas the three German groups show no particular grouping. While the ancient Copper Age samples appear somewhat closer to the Italian/Romansch group, the Bronze Age samples cluster closer to the Ladins and Upper/Lower Val Venosta, although this pattern is not particularly marked. Low (significant) F_{ST} values are shared between the

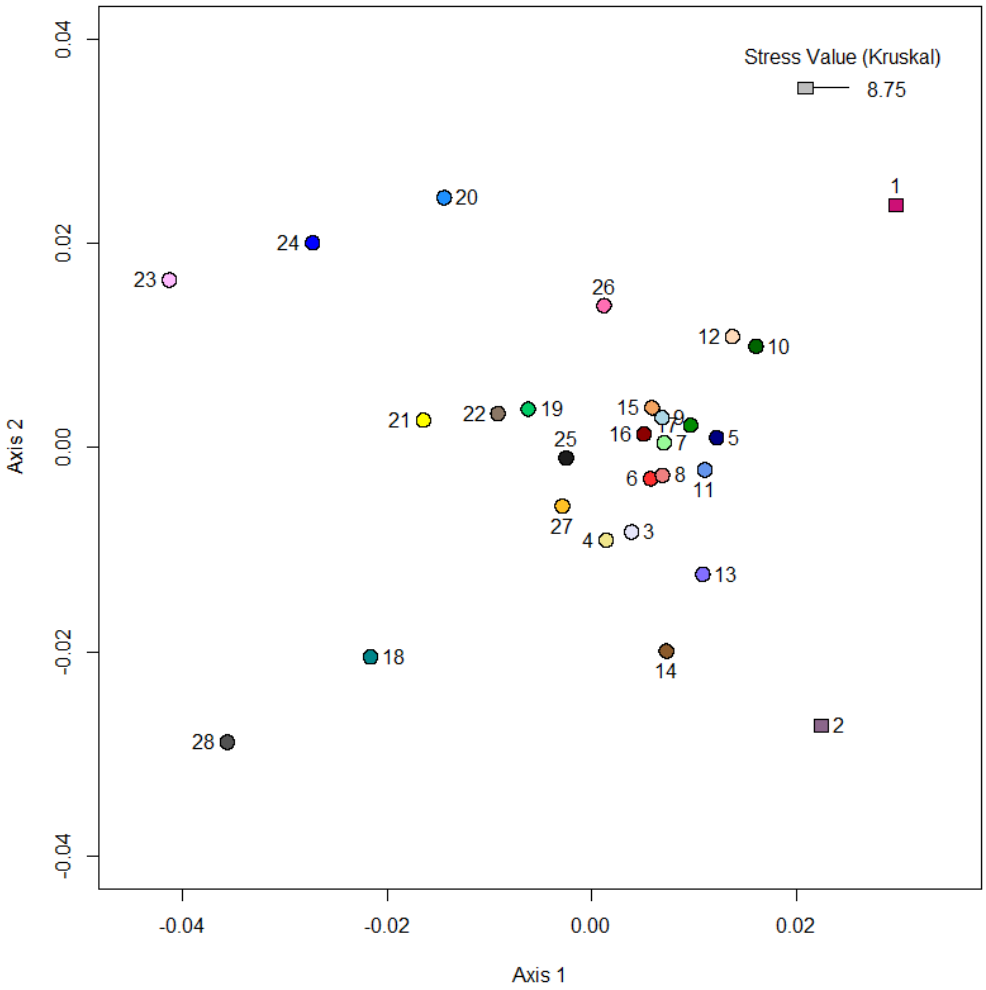
Copper Age group and Val di Fiemme; as well as between the Bronze Age group and Lower Val Venosta.

3.5.2.3.3 Ancient Trentino vs. modern populations (all)

Table 33: F_{ST} values between ancient Trentino and modern reference groups

Reference group	Trentino Copper Age (n=13)	Trentino Bronze Age (n=22)
	Trentino Copper Age vs. Trentino Bronze Age F_{ST} = 0.**05782** (p=0.029)	
Ladin Dolomites	**0.0326**	**0.0313**
German-speaking Val Venosta	**0.02971**	**0.0358**
Adige Valley region & West	**0.02862**	0.0140
Sardinia	**0.03068**	**0.0253**
North-western Italy	**0.02586**	**0.0231**
Tuscany	**0.03388**	**0.0220**
Crete	**0.02717**	**0.0216**
Central Italy	0.01417	0.0133
Southern Central Italy	**0.03421**	0.0124
Southern Italy	0.02042	0.0066
Sicily	**0.04648**	0.0138
Corsica	**0.05195**	**0.0236**
North-eastern Italy	**0.02884**	**0.0172**
Austria	**0.02955**	**0.0199**
Baleares	0.0289	0.0137
Switzerland	**0.05478**	**0.0397**
Northern Greece	**0.02796**	**0.0313**
Turkey	0.02037	**0.0197**
Bosnia	**0.03267**	**0.0299**
Croatia	0.02556	**0.0317**
Serbia	0.0204	**0.0503**
Dobrudja	0.02204	**0.0306**
Moldavia	**0.03368**	**0.0202**
Transylvania	0.02095	**0.0160**
Wallachia	**0.03951**	**0.0240**
Andalusia	**0.05982**	**0.0383**

Figure 11: MDS plot of Trentino samples vs. all modern reference groups



1. Trentino Copper Age; 2. Trentino Bronze Age; 3. Ladin Dolomites; 4. German-speaking Val Venosta; 5. Adige Valley & west thereof; 6. Sardinia; 7. North-western Italy; 8. Tuscany; 9. Crete; 10. Central Italy; 11. Southern Central Italy; 12. Southern Italy; 13. Sicily; 14. Corsica; 15. North-eastern Italy; 16. Austria; 17. Balearic Islands; 18. Switzerland; 19. Greece; 20. Turkey; 21. Bosnia; 22. Croatia; 23. Serbia; 24. Dobrudja (Bulgaria/Romania); 25. Moldavia (Romania/Ukraine); 26. Transylvania (Romania); 27. Wallachia (Romania); 28. Andalusia

Regarding the F_{ST} values, it becomes apparent that Bronze Age Trentino is generally closer to modern populations than Copper Age Trentino (mean value of significant F_{ST} values: CA 0.036, BA 0.028). Almost all individual reference groups for whom significant values are available for both Trentino groups show a lower value in comparison with the Bronze Age group – the only exceptions are German-speaking Val Venosta (although the previous analysis described in the previous section shows that this discrepancy is mainly observable in Upper Val Venosta, while Lower Val Venosta shows a relatively low F_{ST} value). Lower F_{ST} values are found between almost all

modern populations and either ancient Trentino group than between the two Trentino groups (F_{ST}=0.058), an observation not least due to the small sample sizes of the ancient groups. The lowest significant values to the Copper Age group are found for North-western Italy (0.026), Crete (0.272), Northern Greece (0.028) and the Adige Valley and Western Plateau of Trentino (0.029). The lowest significant F_{ST} values between Bronze Age Trento and modern groups are found for Transylvania (0.016), North-eastern Italy (0.017). Sicily and the Adige Valley show even lower F_{ST} value (0.041), but fall short of the significance level by a small degree (p=0.059 and p=0.060, respectively). Values of 0.020-0.025 are found in a variety of modern reference groups, including populations from the Balkans, Alpine area and the Mediterranean (including Corsica and Sardinia).

The MDS plot shows some discrepancies to the F_{ST} values. This is, in all likelihood, attributable to the fact that several F_{ST} values (pairwise difference) showed p-values well above the significance limit of 0.05. However, the MDS plot is based on Reynolds F_{ST} values (matrix of coancestry coefficients) and thus takes all values into account, regardless of significance. Therefore, the individual relative distances shown in the MDS should not be over-interpreted. Overall, most of the modern reference populations form a large cluster with a few modern populations as well as both ancient Trentino groups appearing as outliers. Sardinia, however, did not appear as an outlier, although this is the case if genomic DNA is analysed.

3.5.2.4 Discrepancy between haplotype sharing and F_{ST} values

Both haplotype sharing values and F_{ST} values provide a certain measure of similarity. However, as both measures are based on different calculation aspects, the obtained results vary: For example, some populations with a high likelihood of haplotype sharing with ancient Trentino have high F_{ST} values (indicating a larger genetic distance between the two), or vice versa.

Haplotype sharing calculation regards only identical haplotypes in compared populations but disregards highly similar sequences. F_{ST} calculations take haplotype similarity into account, providing a more general overview of genetic similarity. On the other hand, haplotype sharing may highlight exact matches for rare haplotypes.

However, it must be noted that both methods have limited significance when comparing diachronous populations separated by several millennia – not only due to potentially haplotypes undergoing mutations over the course of time, but also due to the many other demographic factors shaping the genetic composition of the reference populations in question. Therefore, the possibility of using this data to infer relationships between ancient and modern populations is limited and must be regarded with caution. In doing so, the data must be regarded in detail, and take other factors into account, e.g. demographic history of the region in question.

4 DISCUSSION

4.1 Population history of ancient Trentino populations

4.1.1 Neolithic

Although the Neolithic individuals analysed in the course of this study (La Vela, Mezzocorona) were, as a group, not sufficiently well preserved to permit reproducible results, the haplotypes derived from two individuals can be regarded as highly probable. Firstly, the Neolithic individual from Mezzocorona: While only part of the HVR1 could be sequenced, the mutations observed in these sequences exactly matched those reported by Benedetto et al. (2000), denoting haplogroup T2. The second is individual 76/3A from the La Vela. This individual yielded the combination of mutations typical of the European branch of haplogroup N1a, which has been found in several LBK individuals (Haak et al., 2005), but which is not observed in modern populations. Although the sequences could not be reproduced due to lack of successful amplification, it is highly unlikely that this DNA could have been derived from any exogenous source, due to its absence in modern populations and the fact that no other individuals tested in the Bolzano ancient DNA facilities up to that point showed these mutations.

Although no in-depth information can be inferred from these two probable haplotypes, the available data indicates that the Neolithic farming communities in the Trentino region must have had at least some Near Eastern ancestry rather than being Mesolithic communities who adopted an agricultural way of life purely by way of acculturation, as some authors have assumed on the basis of ongoing use of Castelnovian-type stone implements. This supports the archaeological hypothesis that the Square Mouth Pottery Culture (VBQ) developed through a Neolithic introgression (of cultures with Adriatic-style pottery) into local communities.

Whether these incoming settlers completely replaced any existing Mesolithic inhabitants, a hypothesis put forward by Perrin (2009) to explain the lack of archaeological evidence between the Castelnovian Mesolithic and the early Neolithic, or whether early farming communities represented a mixture of Mesolithic and Neolithic ancestry, cannot be addressed using the sparse data obtained for the Neolithic samples. Instead, conclusions regarding the most likely Neolithic population structure must be based on the more substantial data available for the Copper Age, taking into account any known or probable demographic shifts in the course of the Neolithic.

4.1.2 Copper Age

Overall mitochondrial haplogroup distribution in the Copper Age samples shows a significant Neolithic component. In the inter-group comparison, Copper Age Trentino shows the lowest F_{ST} values to Pre-Pottery Neolithic, Starčevo and LBK (all < 0.01). However, these values all fall short of the significance level.

Although the F_{ST} values for the ancient Neolithic groups fall short of the significance level, they are supported by the observation of shared haplotypes: Haplogroups T, J and K, which are common in Neolithic groups constitute the majority of Copper Age samples. In particular, haplogroup K1a, which is found at high frequencies in Neolithic groups (Isern et al., 2017), is observed in three Copper Age Trentino individuals across two sites, and haplogroup J, which is also frequent in LBK groups (but less so in other Neolithic reference groups) is found in two Copper Age samples from two sites. Three Copper Age individuals belong to hitherto unique subclades of the typically Neolithic J and T. The same applies to the Neolithic Iceman, who belongs to a unique branch of haplogroup K. These specific haplotypes may have been introduced by Neolithic settlers (their absence in those individuals sampled to date need not reflect a general absence) and since become extinct. On the other hand, similar haplotypes may have been introduced in the course of the Neolithic, but subsequently undergone additional local mutations. The observation that both Trentino groups cluster

some distance away from the other, less localised ancient groups, would support the latter hypothesis.

Three Copper Age individuals belong to various subclades of haplogroup H. Although H has been observed in most ancient populations, including Hunter-Gatherers, these specific Copper Age individuals are unlikely likely to be of autochthonous Mesolithic descent. Haplogroup H2a2a1 (revised Cambridge reference sequence) is highly infrequent in hunter-gatherers[33], but is frequent in the LBK and Starčevo groups used for reference purposes here. Although overall highest levels are observed in the Bell Beaker reference group (0.2778), its presence in Copper Age individuals indicates that this haplotype is more likely to have been introduced, at least initially, through Neolithic introgression (further re-introduction at a later stage via Bell Beakers may also have occurred).

The H5 subclades, however, are more difficult to place. H5 (16304C) has been found in both Neolithic groups (Central/Eastern European as well as Anatolia) and in later steppe-influenced groups (Bell Beakers, Corded Ware). As subclade H5a is defined by a coding region mutation (Phylotree build 17), and much of the available ancient DNA data includes only HVR1 sequences, H5 and H5a cannot always be differentiated. However, a study of Neolithic haplogroup H genomes (Brotherton et al., 2013) found no H5a (16304C + coding region 4336C) whatsoever in Neolithic/LBK groups, but a H5a frequency of 0.143 in Bell Beakers. H5a was also found in a Bronze Age Karasuk individual from Russia (Allentoft et al., 2015) and in a Corded Ware individual from Estonia (Allentoft et al., 2015). Had subclades H5a4 and H5a4a only appeared in Bronze Age Trentino, the most likely explanation would have been that H5a4 had been introduced with the Bell Beakers. However, H5a4a was also found in a Copper Age individual, Nogarole II 69, on whom radiocarbon

[33] Although it should be noted that several individuals with HVR1 sequences corresponding to the Cambridge reference sequence were published by Fu et al (2016) and described as haplogroup U, although not all had the typical 12308A→G transition that defines haplogroup U. In other words, had these only been tested for HVR1 and 12308, they would have been defined as H2a2a1 (rCRS).

dating was performed, with the latest possible date being 2700 BCE, i.e. predating the earliest known Bell Beaker finds in the region by at least 200 years. It therefore seems more likely that H5a or H5a4 was introduced through contact with Corded Ware groups, in whom H5a has been documented (Allentoft et al., 2015). However, earlier contacts with groups of Steppe-related lineages have been observed in other areas; for example, the genomes of two Copper Age individuals from Varna (Bulgaria) reflected a certain affinity with Steppe lineages, while their contemporaries showed largely Neolithic (Anatolian) lineages (Mathieson et al., 2018). While H5a4 (additional mutation 16294T) has been observed in various modern populations (Central/Eastern Europe, Eurasia, Sardinia), albeit infrequently, H5a4a appears to be absent outside Northern Italy (see Section 4.5). Despite the short time frame, a subsequent local mutation of 16320 therefore seems likely.

Analyses revealed the presence of one confirmed and two further probable individuals[34] with a variant of haplogroup U5b (16270T+16311C+16355T) from the Moletta Patone site. Radiocarbon dating for the individual in question placed the sample at 2600 BCE at the latest, predating the first known documentation of Bell Beaker contact. In general, U5 is regarded as being of Palaeolithic origin – however, as the samples are Copper Age rather than (Early) Neolithic, it cannot be determined whether this lineage is a result of continuity in the region since Mesolithic times or was (re)introduced at a later date through other groups bearing a certain proportion of U5 lineages, such as Corded Ware. The specific haplotype in question is not found in any Palaeolithic or Mesolithic (or indeed in any other published) individual sequenced to date. The one Palaeolithic individual from the wider region in question for whom sequence data is available is the late upper Palaeolithic Villabruna 1 male, found in the Belluno Dolomites. As described in the introductory section, the results

[34] MP7a showed all mutations but was excluded from statistical analyses due to poor score, as each fragment was only covered once. MP3 showed 16311+16355T, but was only sequenced from 16288-16409 so that the presence of 16270T could not be verified.

published to date differ, but the 16270T haplotype obtained by (Fu et al., 2016) is highly likely to be the correct one for reasons previously described. The sequence motif 16270T+16311C was observed in a Mesolithic individual from Croatia (Szécsényi-Nagy et al., 2015), documenting its presence in Pre-Neolithic groups from the Adriatic region, is was also observed in a an early Corded Ware individual found in Tiefbrunn, Bavaria (Allentoft et al., 2015). Dating to 2868-2580 cal. BCE, this male individual was contemporaneous with the Moletta Patone 1a individual, was a non-local according to stable isotope analyses (Sjögren et al., 2016) and the burial context (having been interred with a hammer-headed bone pin typical of the Pontic Steppe region) and had been described as belonging to the Corded Ware due to the collective burial characteristics of the other individuals from the same site. As the archaeological record documents probable cultural contacts between the Trentino populations and Copper Age groups from north of the Alps, it is not entirely implausible that a similar U5 haplotype may have been introduced to the latter at some point during the Neolithic/Copper Age, and the residual mutation 16355T may have occurred subsequently, which would explain its scarcity in other populations.

Regarding Neolithic and Copper Age mtDNA data together, it can be deduced that Neolithic (most likely LBK) lineages represent the majority of haplotypes in ancient Trentino by the Copper Age, thereby refuting the possibility of Neolithic technology having been acquired solely by means of acculturation, i.e. local Mesolithic groups adopting new technologies without substantial admixture with those introducing these new subsistence strategies. However, lineages in the Copper Age are not purely Neolithic: admixture with steppe-influenced people already discernible during the Copper Age. Bearing in mind the archaeological evidence, this is most likely to be Corded Ware, as it predates the earliest known contact with the Bell Beakers. While a U5 lineage was identified which could be a Mesolithic remnant, it cannot be regarded as conclusive evidence of such, as similar haplotypes have also been found in Corded Ware and could thus

been introduced after the beginnings of the Neolithic. Various scenarios are therefore conceivable on the basis of the Neolithic and Copper Age data:

- Neolithic pioneers could have **admixed** with local Mesolithic groups
- Neolithic pioneers could have initially **replaced** the existing Mesolithic communities completely, either actively or by settling in areas from which Mesolithic groups had already disappeared, with the "hunter-gatherer" haplotypes observed in the Copper Age group being introduced at a later point, either:
    - through the re-emergence of local hunter-gatherers still existing in the region, as has been observed in other regions (Lipson et al., 2017)
    - and/or as a result of more wide-ranging cultural contacts, e.g. Corded Ware

While the replacement theory would explain the chronological gap observed in well-dated sites (Romagnano and Gaban) between the late Mesolithic and the earliest Neolithic in the archaeological stratigraphy spanning from approximately 5500-5000 BCE (Bagolini and Biagi, 1990; Perrin, 2009), interpretation of this apparent "gap" (which is also observed in various other regions throughout the northern Mediterranean) as evidence of actual population discontinuity is controversial (Mlekuž et al., 2008). Furthermore, such complete replacement would provide no explanation for the persistence of Mesolithic characteristics such as hunting and typical stone tools within the same communities. Although the coexistence of genetically Neolithic and Mesolithic groups over a long period of time is known for other sites (such as the Blätterhöhle cave in Germany, (Bollongino et al., 2013)), which might have led to the gradual exchange of technologies without actual admixture, there is no archaeological evidence for such differentiation (such as conspicuous differences in subsistence strategies between contemporaneous sites).

Bearing in mind the archaeological evidence, particularly the continuity of certain tool typologies and persistence of hunting as a subsistence strategy until well into the Bronze Age, the most likely scenario is therefore that the process of Neolithisation in the South Tyrol-Trentino region involved a

certain amount of admixture – at least initially – between local hunter-gatherers and incoming Neolithic farming groups, and that the chronological gap does not reflect the a complete population discontinuity, but may simply be due to a change in burial customs. However, this theory requires further data from Neolithic individuals (specifically, whether any U5 haplotypes are present) in order to be regarded as conclusive – this information could not be obtained in the course of this study due to the poor preservation of the La Vela individuals. Attempts with whole-genome sequencing approaches particularly suited for the analysis of highly fragmented DNA may in future provide further insights. Although such methods are time-consuming and still too costly for standard screening purposes, the key significance of the Neolithic La Vela individuals for understanding the Neolithisation processes in this region would justify such an approach for these samples.

With regard to Y-chromosomal haplogroups, the few samples from which Y-chromosomal DNA could be sequenced also lend weight to the observation of a significant Neolithic component in the Copper Age Trentino samples. All five Copper Age samples (which included three different sites) that could be sequenced indicated haplogroup G2 – although four of these must be regarded as unconfirmed as only one PCR could be produced for each, the sequence from Nogarole 69 was confirmed in seven PCRs from two extracts and confirmed in a separate lab, documenting with certainty the presence of this haplogroup in Copper Age Trentino. G2 is firmly associated with the spread of the Neolithic to Western Europe; in addition to its presence in several Anatolian samples dating to the 7th century BCE, it was also found in the majority of Western European Neolithic samples from which Y-chromosomal DNA could be sequenced, for example in Neolithic numerous Starčevo, French and Spanish samples, with the high mtDNA variation standing in stark contrast to apparent limitation to one main Y-chromosomal lineage (although several sublineages are present). However, it is almost completely absent from Copper and Bronze Age samples sequenced to date (the only exceptions

being the Tyrolean Iceman and two individuals from El Mirador Cave in Spain (Gomez-Sanchez et al., 2014)), having been largely replaced by haplogroups R1a and R1b, marking the spread of groups with Steppe ancestry. However, as far less Y-chromosomal data is available for the Copper Age than for the Neolithic or Bronze Age, it is difficult to ascertain the general frequency of haplogroup G2 in Copper Age Europe. Analysis of samples from the North Italian Copper Age site of Remedello di Sotto revealed that all three samples belonged to Y-chromosomal haplogroup I, which in all likelihood indicates a pre-Neolithic lineage (Allentoft et al., 2015). Although the Y-chromosomal haplotypes observed in Trentino, in Remedello and in the Iceman all differ (if only with regard to subtype in the case of the Iceman vs. Trentino), they support the general observation that the "Steppe impact" does not yet seem to have reached Northern Italy during the Copper Age.

4.1.3 Bronze Age

The Bronze Age sample set shows somewhat different affinities than the Copper Age group. While the earlier period showed high similarity with the Neolithic groups, in particular PPN, Starčevo and LBK, the F_{ST} values between Trentino and these groups are higher (indicating greater genetic distance) in the Bronze Age (although these data must be regarded with caution, as the F_{ST} values between these groups and Copper Age Trentino are not significant). Copper Age Trentino also shows higher likelihoods of sharing haplotypes with the Neolithic reference groups than Bronze Age Trentino. On the other hand, the distance to certain European Bronze Age groups is reduced, e.g. Únětice (0.048 to 0.019, although the latter is not significant), the Bell Beaker culture (from 0.062 to 0.039, both significant). Furthermore, the distance between Trentino and Hunter-Gatherers is reduced from 0.248 in the Copper Age to 0.193 in the Bronze Age, which is more likely to be a direct consequence of the higher affinity to the Bronze Age groups of (partial) Steppe ancestry, which are genetically closer to hunter-gatherers (F_{ST} values of around 0.100-0.120) than to indicate any

local re-emergence of Mesolithic lineages. However, ongoing admixture with local Mesolithic groups, as has been observed elsewhere (Lipson et al., 2017) is also possible. While the F_{ST} between Corded Ware and ancient Trentino appears to increase (indicating greater genetic distance) in the Bronze Age, the Copper Age value is not significant. Haplotype sharing likelihoods are higher in the Bronze Age than the Copper Age for all Steppe-origin groups: Yamnaya, Corded Ware, Bell Beaker and Únětice, and lower in the Bronze Age than the Copper Age for the Neolithic groups Pre-Pottery Neolithic, Aegean/Anatolian Neolithic, Starčevo and LBK. In the multidimensional scaling plot run on the basis of mitochondrial DNA Reynold's F_{ST} values, the Neolithic groups (PPN, Anatolian-Aegean Neolithic, Starčevo and LBK) form one cluster, the Steppe-origin Copper Bronze Age groups (Yamnaya, Bell Beaker, Únětice) form another, with Corded Ware approximately in between. The Iron Age Etruscans lie some distance away from the Neolithic, CA/BA clusters and Trentino groups. Trentino Copper and Bronze Age clusters together some distance away from the other groups, showing no clear affinity with any of them – although the Copper Age group is slightly closer to the Neolithic cultures and Bronze Age Trentino to the Steppe-influenced groups, the difference is minimal, and must be regarded with caution due to the relatively high stress value (i.e. distances may be slightly distorted due to resolution limitations). Therefore, the extent of the similarities and dissimilarities were not assessed on the basis of the MDS alone, but took the F_{ST} values and shared haplotypes into account.

Haplotypes belonging to haplogroup H5, one of which was already detected in the Copper Age group, become more widespread, as do H2. While these are also found at high frequencies in Bell Beakers, the high frequencies in the Bronze Age Trentino group may also be in part due to admixture with the aforementioned. However, the presence of a H5a4a individual in the early Copper Age, regarded together with the general infrequence of this particular haplotype, indicates that H5a4 is likely to have been introduced before the Bell Beaker introgression and possibly underwent a subsequent

local mutation (see previous section). The frequency of similar haplotypes in Bell Beaker populations supports the theory of these haplogroups having been introduced with the Corded Ware, as Bell Beakers have been shown to have significant Corded Ware ancestry (Haak et al., 2010). Several variants of haplogroup X, which were not observed in the Copper Age group, also appear at different sites. While haplogroup X has been observed in LBK and Starčevo individuals (0.014 and 0.032, respectively) in the reference sample sets), overall frequencies in groups of steppe ancestry are higher: 0.0103 in Corded Ware and 0.071 in Únětice[35]. While no haplogroup X individuals were found in the Bell Beaker reference sample set used for this study, a recently published, large-scale analysis (Olalde et al., 2017) showed a frequency of 0.0305 (CI_{95}: 0.0131-0.0694). With regard to the subclade of haplogroup X found in individuals Romagnano III 89 and 90, this specific sequence was only found in one other ancient or modern individual: Early Bronze Age burial OBKR-81 (GenBank accession number MF498713) from Königsbrunn in Bavaria (Knipper et al., 2017). With a sigma 1 radiocarbon date range of 2013-1923 BCE, the Königsbrunn individual postdates Romagnano III 89 by at least 130 years, and therefore provides no information on the ancestry of this haplotype. While strontium isotope analyses conducted by Knipper et al. gave no indication that this particular individual was nonlocal, this was unsurprising given the young age of the individual (4 years). Several other individuals from Königsbrunn (as well as other sites in the Lech valley), most of them adult females, showed strontium isotope values indicative of nonlocality, from which the authors infer female exogamy in this region in the early Bronze Age. The occurrence of an exact match of this otherwise unique haplotype with Early Bronze Age Romagnano may therefore possibly even denote direct kinship, with the maternal processor of OBKR-81 possibly originating in the Trentino region, or at least having a relatively recent common ancestor, rather than this haplotype being a remnant of the Neolithic.

[35] For confidence intervals, see Supplement S4 (accompanying CD)

On the whole, bearing in mind the absence of haplogroup X in the Copper Age group, this haplogroup is also likely to have been introduced by populations of steppe ancestry.

At the same time, some typically Neolithic lineages still persist, e.g. various occurrences of haplogroup J and T. The specific variant of individual Romagnano III T12E-a, for example, was also found in an early Alföld LBK individual from Füzesabony-Gubakút, Hungary (middle Neolithic, approx. 5630-5560 BCE, Keerl 2015). The variant of U8 found in individuals Romagnano III 100b and 120b, rather than representing Mesolithic continuity (as may often be the case with U5 and U8), was also found in an Alföld LBK burial in Mezőkövesd-Mocsolyás (approx. 5500-5300 BCE; Lipson et al, 2017). Another highly similar sequence (lacking only 16182C) was found on a Sopot II burial in Alsónyék-Elkerülő (approx. 5000-4910 BCE; Szécsényi-Nagy, 2015).

One sublineage of U5 was found, however, which may possibly be a result of Mesolithic continuity. Individual RomIII82 showed HVR1 mutations 16266T 16270T 16304C and the U-defining 12308G, corresponding to U5b3h as per Phylotree 17. U5b3 has been identified as a Palaeolithic lineage that originated in Italy. A particular subclade of U5b3 (not that of RomIII82) is common in modern-day Sardinians (Der Sarkissian, 2011). The age of the U5b3 lineage has been estimated to be at least 8.1 KYA (Pala et al., 2009). The sequence found in RomIII82 was not found in any other ancient or modern individual, with 16266T probably representing a private local mutation. While the placement of the sample within the Bronze Age makes it possible that this lineage was re-introduced by Steppe populations via Central/Northern Europe, this is less likely given the origin of the U53b clade. Another possibility, however, is that it was introduced into the region as a result of (obsidian) trade contacts with Sardinia.

In sum, the Bronze Age lineages observed in this study represent a mixture of Neolithic (LBK-derived) substratum and other lineages likely to have been introduced by groups of steppe ancestry. While admixture with the latter appears to have started in the Copper Age, the increased frequencies of steppe-derived lineages indicate that admixture continued until into the Bronze Age. The lowest F_{ST} values are observed between BA Trentino and the Únětice Culture. While the F_{ST} value between Trentino BA and Bell Beaker is somewhat higher, it is still lower than between BB and Trentino Copper Age. Both Bell Beakers and Únětice had a known archaeological impact on northern Italy (Barfield, 2001). Direct links between these cultures and Bronze Age Trentino are documented by several archaeological finds: Copper ring ingots of Únětice type found in Polada burials bear testimony to a metal trade between the two (Barfield, 1971). As described in detail in Section 1.4, the influence of Bell Beaker groups is apparent in the earliest phase of the Bronze Age Polada Culture (Barfield, 1971). However, as documented by recent genomic analyses (Olalde et al, 2018), the presence of Beaker artefacts does not necessarily indicate steppe-related ancestry, as some groups of individuals (e.g. in Iberia) buried with artefacts of characteristic Beaker typology showed far lower steppe ancestry than other (e.g. Britain).

When interpreting the Bronze Age data, it must be borne in mind that all but three of the Bronze Age samples were obtained from the same site (Romagnano) and may therefore not necessarily reflect the general population structure in the entire region. However, the overall haplotype diversity in the Romagnano samples is relatively high, with a total of 12 haplotypes observed in 20 individuals, arguing against a closely related family group. Furthermore, some of the variants observed in the Romagnano individuals, including some which are exceedingly rare or absent in other ancient or modern populations, are also found in Copper and Bronze Age individuals from other sites, indicating a certain amount of admixture and shared ancestry.

While this new influence is observed in the mtDNA, the same does not apply to the Y-chromosome: Haplotype G was still present in the Bronze Age, as documented by one confirmed sequence each from Paludei di Volano and Romagnano III (as well as another probable G2a from Romagnano IV). This sets Trentino apart from most other European regions, where R1a and R1b had reached high frequencies as a result of the Steppe-driven expansions, with the previously common G2a becoming scarce. This expansion of R1b is closely associated with the spread of the Bell Beaker culture: genomic study by Olalde et al. (2018) found y-chromosomal haplogroup R1b in 84 of 90 of the analysed non-Iberian males associated with Beaker assemblages.

One striking aspect, however, is that the Trentino individuals do not belong to the same subgroup of G2a as the Tyrolean Iceman. While the latter possesses the derived allele for the L91 marker, denoting haplogroup G2a2b, all successfully tested Trentino individuals show the ancestral allele at this site (although no further typing was performed, as the main research focus was to establish similarity to the Iceman). Particularly high frequencies of the G2a2b (the Iceman's) subtype in modern-day Southern Corsica and Northern Sardinia indicates that this sublineage is more likely to have been introduced in the course of the Neolithic and spread to the Mediterranean islands and the Alpine region, rather than developing in either region as a local subsequent mutation (Keller et al., 2012). Although cultural contacts between Sardinia and Northern Italy are documented from the Neolithic with regard to the obsidian trade, it is unclear whether the Sardinian trade route reached Trentino (as the only obsidian found to date in a burial from the Trentino region is from Lipari rather than Sardinia). While indications for a link between the Bronze Age cultures of Polada (Trentino) and Bonnanaro (Sardinia) have been reported (Lilliu, 1988; Moravetti, 1992; Webster, 1996; Turfa, 2014), published evidence is based mainly on typological similarities (i.e. "Polada-style" pottery). While the majority of known ancient G2a samples were not tested for L91 (or at least no such information is provided), no estimation can be made regarding the relative

frequency or distribution of the G2a-L91 and G2a non-L91 subtypes. However, two LBK samples from Halberstadt were tested at this locus and showed G2a non-L91, as did the Trentino samples. The only other positive confirmation of G2a-L91 is from two samples from Barcın in Anatolia, dating to approx. 6500-6200 BCE (Mathieson et al., 2015a). It therefore seems likely that the initial Neolithic introduction of G2a already included several sublineages of G2a. Nevertheless, the Y-chromosomal haplotypes spread to Europe in the course of the Neolithic show a reduced diversity as compared to those observed in Early Neolithic samples from the Near East (Rootsi et al., 2012). This migratory "bottleneck", as it were, indicates that a lower effective male than female population size.

4.2 Phenotypic loci

4.2.1 Lactase persistence (-13.910*T/C)

The lactase persistence locus was amplified in 24 individuals; however, 17 of these were single sequences which could not be reproduced. Reproducible results were obtained for 7 individuals (3 Copper Age, 4 Bronze Age), all of which showed the homozygous C allele, indicating the ancestral state of lactase nonpersistence. All but one of the 17 single sequences also showed C/C, while one single Bronze Age individual yielded T/T in one PCR. However, as no further amplification was achieved, this isolated occurrence does not provide any conclusive evidence for the presence of the derived allele in either Copper Age or Bronze Age Trentino. The origin of the lactase persistence allele is still a matter of debate. Until recently, the spread of this trait was regarded as being associated with the LBK culture and was proposed to have originated in the Balkan region approximately 7,500 years ago (Itan et al., 2009) and co-evolved alongside the spread of agriculture and dairying, gaining rapidly in frequency due to its selective advantage. However, the allele in question was apparently still very rare in LBK individuals (Burger et al., 2007). By the Bronze Age, levels seem to have reached more significant frequencies: Recent genomic

studies by Allentoft et al. (Allentoft et al., 2015) found the derived allele in approx. 10% of the European Bronze Age population, with the highest Central/Western European levels found in Corded Ware. Higher levels still (0.300-0.500) were found in Steppe cultures such as the Yamnaya and Mezhovskaya, from which the authors postulate that the LP allele may have been spread to Central and Western Europe by the Yamnaya, rather than the LBK as previously thought. As the Bronze Age Trentino groups show a certain affinity with the Central/Western European populations of Yamnaya descent (Únětice, Corded Ware, Bell Beaker), the appearance of the LP allele in the later Trentino individuals would not necessarily have been surprising. However, no evidence for the presence of the derived allele at this time could be inferred from this study.

The spread of the lactase persistence variant is a classic example of positive selection – however, the precise nature of the advantage(s) that caused the selective sweep are still subject to debate. While the spread of LP is generally linked to subsistence strategies based on domestic animals, it is unclear whether LP developed as a result of pastoral lifestyles or whether pastoral lifestyles spread due to the presence of the LP allele. Putative advantages include an easily and quickly accessible food source in times on shortage, an alternative source of liquids to potentially contaminated water supplies (e.g. cholera) or potential health benefits (such as increased bone turnover rates and bone mineral density, Fardellone et al., 2017).

Whether or not the ability to digest milk into adulthood would confer a selective advantage in a particular region/population depends on the general settings and availability of (alternative) food sources. Archaezoological studies have documented the presence of domesticated cattle at the Neolithic La Vela site, with domesticates replacing the hunted animals which previously constituted the greater part of faunal remains. While transhumance in an Alpine setting has been shown to change the composition of cow's milk, the precise effect on lactase has not been investigated (Gorlier et al., 2012). While protein and fat content are affected

mainly as a result of the fodder plants available at different altitudes, it seems unlikely that the lactase content (which is, unlike the protein and fat parameters, relatively uniform across different cattle breeds) would be affected to such an extent as to render the milk digestible to lactase non-persistent humans. But would the ability to digest that milk confer a benefit? Many other food sources were available: While the Adige valley remains to date a fertile area with a Mediterranean climate that is extensively used for agriculture (in particular apples, but also grapes and other fruits), the high plateaux in the direct vicinity offer extensive grazing and hunting. Plant remains from Fiavè (Jarman and Gamble, 1975) document the cultivation of emmer, barley, pulses as well as the consumption of a variety of fruit and nuts such as grapes, raspberries, hazelnuts, apples, blackberries and cherries. Remains of wild animals (mainly red and roe deer) at various sites attest the rich hunting grounds on the higher areas. Caprines and bovines were also kept as domesticates. All in all, food sources are widely varied, rendering a dependence on a single food source unlikely – in times of crop failure, for example, hunting or gathering (depending on the season) would still have been a viable option.

It therefore seems plausible that the selective advantage of lactase persistence in prehistoric Trentino was negligible, given the probable availability of alternative food sources. It cannot be determined with certainty whether the -13.910*T allele was completely absent or simply infrequent in the Neolithic to Bronze Age Trentino. This is supported by the observation that levels of lactase persistence are still relatively low (0.240) in modern North-Eastern Italian populations (Anagnostou et al., 2009) (although higher than other regions of Italy).

4.2.2 Eye colour

Reliable results for the HERC2 (rs12913832) eye colour SNP were obtained for 16 individuals (7 Copper Age, 9 Bronze Age). While seven individuals had the ancestral A/A genotype, eight had the A/G genotype (evenly spread across Copper and Bronze Age individuals) and one Copper Age individual

had G/G. Although only the latter was likely to have had a blue eye colour, it is clear that the G allele was present at notable frequencies in both the Copper and Bronze Age in this region. No inferences can be made regarding the origin of the G allele in this region: in has been observed in a Mesolithic individual from La Braña-Arintero in Spain, predating the derived allele for lighter skin pigmentation (Olalde et al., 2014). While blue eye colour would not confer a direct health-related advantage (as is the case with lactase persistence), frequency-dependent sexual selection could have favoured a rare variant such as an unusual eye colour.

4.3 Routes of Neolithisation

The question as to whether the Neolithic forerunners of the Trento individuals are more likely to have arrived in the region of South Tyrol-Trentino via the (Adriatic) coastal route or via the Balkans/Danube route associated with the Starčevo/LBK must take both ancient and modern reference populations into account cannot be sufficiently addressed with the sparse data derived from the Neolithic samples. The two mitochondrial haplogroups determined with certainty were N1a and T2. The former is most common in LBK/Starčevo individuals but has also been found in PPN sites from Anatolia (Kılınç et al., 2016), in an Impressed Ware burial (Zemunica Cave) from Croatia (Mathieson et al., 2018) and from the Spanish Neolithic Els Trocs cave (Mathieson et al., 2015a). Based on this distribution, it can be deduced that this haplotype was present in the Neolithic source population before the various pioneer waves set off on differing routes along the Mediterranean coastline or via the land route through the Balkans. The same applies to haplogroup T2: while the majority of T2 individuals are LBK/Starčevo, this haplotype has also been found in several Spanish individuals from various sites.

The few Neolithic samples for which tentatively reliable results were obtained also show LBK-type haplogroups. These samples date to the VBQ period, in which new pottery styles and other artefact typologies are

observed that document links with the Balkan region. Regarded together with the genetic data, it appears plausible that, at least by the full Neolithic, the region was heavily influenced by LBK settlers arriving from south-eastern Europe and/or north of the Alps. The Gaban culture of the preceding early Neolithic showed a more Mediterranean/ Impressed Ware-style pottery, while the persistence of certain Mesolithic traditions (particularly the type of lithic implements and hunting as a subsistence strategy) during the Gaban phase, but no longer observed in the subsequent VBQ phase of the Neolithic, indicate that a population shift may have taken place at the beginning of the VBQ period (although the observation of a Neolithic-type haplogroup in the early Neolithic Mezzocorona 2 individual documents that a certain amount of gene flow took place in the earliest Neolithic). The latter bears the hallmarks of Balkan/LBK/Starčevo influences, both in pottery decoration as well as in other figurative objects such as pintaderas, figurines (Bagolini & Biagi, 1985). While certain Impressed Ware elements – representing the Mediterranean branch of the spread of the Neolithic – are detectable in the Neolithic of Northern Italy, radiocarbon dates indicate that these originated from the northern Adriatic coastal area and in all likelihood also originated from the Balkan area.

The Copper Age samples show a close affinity with the Starčevo/LBK groups, both in terms of F_{ST} (although these results must be regarded very cautiously as they fall short of the significance level) as well as haplotype sharing. However, the haplotype sharing analysis shows equally comparably high values for Neolithic France and Spain, and higher values yet for the Aegean & Anatolian Neolithic. Y-chromosomal haplogroup G, which was also found in several Copper Age individuals (although only replicated in one), is also found at high frequencies in the LBK, but has also been found in PPN individuals. It must be borne in mind that the Copper Age samples date to the mid-fourth to mid-third millennium BCE, approximately 1600 years after the earliest evidence for Neolithisation in this area.

Some individual haplotypes do, however, indicate a closer link with LBK sources rather than a Neolithic spread via the southern Mediterranean route, for example haplotype matches or at least high similarities with Alföld individuals from Hungary and a Sopot II individual from Croatia.

While the ancient DNA data cannot conclusively prove or disprove the Neolithic initially reached Trentino via the Central European/Balkan route, especially as no sufficient "proxy" exists for the Mediterranean route, it appears to be the more likely scenario, regarded together with the archaeological evidence. It would appear plausible that, at least by the full Neolithic, the region was heavily influenced by LBK settlers arriving from north of the Alps or the Balkan region.

4.4 Autosomal link between Tyrolean Iceman and Sardinia

The analysis of the Tyrolean Iceman revealed high similarities with the modern population of Sardinia with regard to autosomal and Y-chromosomal DNA (Keller et al., 2012; Sikora et al., 2014). From this, it was inferred that the Iceman and ancient Sardinia shared a common genetic ancestry dating back to the early Neolithic, which was once common throughout Europe but was gradually replaced in many regions, persisting only in regions of relative isolation. Sardinia's Neolithic heritage is supported by various metagenomic studies, in which Sardinia clusters closely with early European farmers (Günther et al., 2015; Omrak et al., 2016; Raveane et al. 2019; Marcus et al., 2019). A recent study analysing Mesolithic Sardinian mitogenomes (Modi et al., 2017) came to the conclusion that the Pre-Neolithic inhabitants of Sardinia contributed little to the modern gene pool of the island, the latter being mainly derived from incoming Neolithic settlers. However, in the case of ancient Trentino, the Bronze Age group is genetically somewhat closer to Bronze Age (Nuraghic) Sardinia than is the case between Copper Age Trentino and Sardinia: likelihood of haplotype sharing is higher in the Bronze Age, and the F_{ST} value is lower between TBA and ancient Sardinia (0.054, p=0.01) than

between TCA and ancient Sardinia (0.638, p=0.02). The same is observed for the F_{ST} values of ancient Trentino as compared to modern Sardinia: TCA an F_{ST} of 0.0307 (p= 0.04) and TBA an F_{ST} of 0.0205 (p=0.01). The only contradictory finding is in the haplotype sharing analysis between ancient Trentino and modern Sardinia, where Copper Age Trentino shows somewhat higher likelihood values (although both are still below the mean).

Cultural contacts between Sardinia and Northern Italy have been established for the middle Neolithic, mainly through obsidian finds from Sardinia, for example at Arene Candide (Ammerman and Polglase 1993, 1996) and the VBQ site of Gaione (Tykot, 1996). While obsidian from multiple sources was used during the early and middle Neolithic, Sardinia is the most common source for obsidian in Northern Italy in the Late Neolithic (Williams et al, 1979). While obsidian finds from the Eastern coast appear to be of Dalmatian origin (or at least sourced via the Adriatic), a large part of the obsidian found north of the Po river is from Sardinia (Thorpe et al., 1979; Hallam et al., 1976). Whether or not Sardinian obsidian was used in the Trentino area is unclear – the one obsidian blade documented for Trentino was from Lipari (Pedrotti et al., 1998a), and flint was the far more common material for tools. However, archaeological finds at least document extensive obsidian trade between Northern mainland Italy and Sardinia since the Neolithic. Further archaeological parallels have been documented between the Sardinian Bonnanaro Culture, a precursor of the Bronze Age Nuraghi, and the Polada (Lilliu, 1988; Moravetti, 1992; Webster, 1996; Turfa, 2014) with regard to pottery styles. While Lilliu interprets the similarities as evidence of a direct cultural contact between the two regions, a cultural transfer via the spread of the Bell Beaker culture (which was known to have been present in both areas, and appears to have left its mark on the Trentino region) is also conceivable.

4.5 Site continuity in the Trentino region from the Mesolithic to the Bronze Age

Direct assessment of genetic continuity is not possible on a local basis, lacking direct data from human remains from the Mesolithic layers of those sites with a long settlement continuity, and the poor preservation of the Neolithic samples analysed in this study. Potential continuity must therefore be evaluated by regarding the individual haplogroups as to their probable origin, bearing the archaeological evidence in mind. The largest amount of data is available for the site of Romagnano Loc, for which human use is documented almost without interruption from the Mesolithic to the Iron Age. The 19 individuals from which mtDNA sequences could be obtained yielded 10 different haplotypes. Of these, only two belong to basal groups common amongst hunter-gatherers: Haplogroup U5 (one sample, Romagnano III 82) and haplogroup U8b (two samples, Romagnano III 100b and 120b). However, the specific haplotypes observed in these individuals have not been found in any hunter-gatherer sequenced to date, not even in the comprehensive genomic studies by Fu et al. (2016) and Posth et al. (2016). On the other hand, the U8 lineage found in the Romagnano individuals, while generally very rare, was round in a Neolithic individual from Hungary (Lipson et al., 2017) and could therefore have been introduced from this region with the spread of the Neolithic rather than representing a local Mesolithic lineage. Furthermore, the same restrictions apply as described for the Copper Age specimen bearing U5: Although it is tempting to interpret the occurrence of this haplotype in a site such as Romagnano (i.e. with site continuity reaching back to the Mesolithic) as proof that some hunter-gatherer lineages have persisted, it is just as possible that this haplotype was reintroduced by later contacts. However, due to the particular lineage in question (U53b), the contacts in question would more likely to have been Sardinian than those of steppe ancestry.

One haplotype observed in the Trentino sample set that is very rare in modern populations (H5a4a) was not only seen in Romagnano, but in the

Copper Age Nogarole II sample. Apart from CRS and HV0 (two relatively frequent haplotypes), this was the only haplotype found in both Copper Age and Bronze Age samples. The only site at which diachronous samples were analysed was Nogarole. While the two samples differed, the results contrasted with the general observation: the earlier Nogarole sample had a rare variant of H5 (which, at least from the basal group, would have been more common in the Bronze Age) whereas the Bronze Age sample had a variant of haplotype J. Although J is a typically "Neolithic" haplogroup, the only other observation of this specific haplotype in an ancient sample was from an Únětice individual from Quedlinburg, Germany. It is therefore also plausible that this haplotype was not introduced until the Bronze Age.

Overall, although the observation of rare haplotypes occurring at different sites in both the Bronze and Copper Ages documents a certain degree of continuity from the Copper to the Bronze Age, the genetic distance between the two groups and appearance, in the Bronze Age group, of haplotypes associated with groups of Yamnaya ancestry such as Bell beakers, Únětice and Corded Ware indicates that a significant amount of admixture with new incoming groups occurred during the late Copper Age/Early Bronze Age.

Cucina et al. (1999) determined a high degree of similarity between nonmetric dental traits from Neolithic to Bronze Age samples from Trentino, some of which were also analysed here (the exact individuals are not listed, but sites include Madonna Bianca, Moletta Patone, Paludei di Volano, Nogarole II and III, Mezzocorona, Romagnano and Solteri – i.e. most of the sites sampled here). From these dental traits, the authors infer a certain continuity, which can only partly be confirmed here, as a certain generic distance is discernible between the Copper and Bronze Age sample group. However, it should be noted that the dental data included a higher number of samples (16 Neolithic, 37 Copper Age, 47 Bronze Age samples, with the sites of Solteri and Romagnano represented in both the Neolithic and the Bronze Age). Results were given according to era rather than individual, so than no comparisons could be made to determine whether rare

haplotypes occurring at different sites (e.g. H5a4a) correlate with specific nonmetric traits.

A sample pool of iron age Etruscan ancient DNA was also included as a reference group to establish whether any particular genetic link could be ascertained, as the origins of the Etruscans is still a matter of debate (Gómez-Carballa et al., 2015; Pardo-Seco et al., 2014; Vernesi et al., 2004). No particular affinity was determined in this study, with relatively high F_{ST}s and low likelihoods of haplotype sharing (0, in the case of Copper Age Trentino).

4.6 Links with modern populations

One of aims of this study was to establish whether the lineages observed in the ancient individuals from South Tyrol-Trentino are still found in autochthonous modern populations in the area, and whether these can be attributed to a direct continuity (rather than re-introduction at a later stage or simply because the haplotypes in question may be frequent throughout Europe).

When observing the F_{ST} values between ancient Trentino and modern populations, the first point that becomes apparent is that the Bronze Age group is, on the whole, closer to modern populations than the Copper Age group. The mean of all significant F_{ST} values is 0.360 between Copper Age Trentino and modern populations and 0.280 between Bronze Age Trentino and modern groups. Furthermore, all modern reference groups show more similarity to each other than to either ancient Trentino group (not least due to sample size. The lowest individual significant values (≤ 0.02) are found between BA Trentino and Transylvania (0.1602), BA Trentino and North-eastern Italy (0.0172), BA Trentino and Lower Val Venosta in South Tyrol (0.0183), BA Trentino and Turkey (0.0192) BA Trentino and Austria (0.0199) and BA Trentino and Moldavia (0.0202). While F_{ST} values between BA Trentino and Central to Southern Italy (including Sicily) appear

low, and are reflected thus in the MDS plot, it should be noted that these values fall well short of the significance level.

The haplotype sharing analysis for Copper Age Trentino shows highest likelihoods for Val Pusteria and Upper Val Venosta (South Tyrol), followed by Croatia, then Val Primiero (Trentino). For Bronze Age Trentino, highest sharing likelihoods were observed for Switzerland, Transylvania, Val Primiero (Trentino), Val Giudicarie (Trentino) and Val di Fiemme (Trentino). The discrepancies between F_{ST} and haplotype sharing values observed in some cases (for example, Switzerland shows far higher F_{ST} values to Bronze Age Trentino – indicating greater distance – than Transylvania, but shows the highest likelihood of haplotype sharing) are due to methodical differences: While F_{ST} takes highly similar sequences into account, haplotype sharing only regards identical sequences.

In sum, the most consistent aspect is that various populations in South Tyrol-Trentino (i.e. the modern inhabitants of the area from which the ancient samples were obtained) show higher similarities than most other modern reference groups. Some of these similarities are due to frequencies of common haplotypes that are found throughout Europe in modern populations – in such cases, the significance is minor, as relative frequencies have shifted over time due to genetic drift and bottlenecks. In other cases, groups share haplotypes that are rare in modern populations, so that isolated occurrences (provided that the defining polymorphisms are not restricted to mutational hotspots) may give an indication of possible continuity.

Several of the haplotypes found in the Trentino individuals are exceedingly rare in modern populations; these are addressed individually in the following.

Solteri 61 (CA)
16240G, 16311C, variant of haplogroup H1

This haplotype is not found in the EMPOP database (6189 individuals). The only match found in GenBank is a Near Eastern Druze individual. While it

is tempting to infer Near Eastern Neolithic ancestry from this (as the Druze are regarded as a genetic refugium, harbouring Neolithic lineages that may have become rare elsewhere, Shlush et al., 2008) it should be noted that one of the two defining mutations (16311) represents a mutational hotspot (Stoneking, 2000). The same haplotype with only 16240G is also very rare. Of the 9 matches in GenBank with documented source, four are from Sardinia (the others being Druze, Scandinavian, Dutch and the last derived from the Turin Shroud, which is highly likely to bear DNA from many sources, including Italian; Barcaccia et al., 2015). In the modern reference sample set, sequences containing the 16240G mutation were observed in five Sardinian individuals and one from Bolzano. Two interpretations appear possible: That 16240G + 16311C was indeed introduced during (or after) the Neolithic and has since become almost extinct (only surviving in genetic refugia) or that 16240G was the original haplotype reach Northern Italy and Sardinia, persisted in its original form in Sardinia (where it still exists) but, in the Trentino area, underwent a subsequent mutation at some point between the start of the Neolithic and the Copper Age at hotspot 16311 to the variant seen in individual Solteri 61. However, as no direct matches were found in Northern Italy, no continuity into modern populations can be inferred in this case.

Romagnano III 118b, T11A-b (BA)
16294T, 16304C, haplogroup H5a4

This haplotype is also relatively infrequent in modern populations (3 hits out of n=6189 in EMPOP, 26 exact matches in GenBank). The majority of matches in GenBank were from Central Eastern European and Eurasian populations, including one individual each from 10th century Romania (Rusu et al., 2018) and ancient Hungary (Neparáczki et al, 2016). Additionally, three sequences were from Sardinia and one from Northern Italy. Amongst the reference populations used in this study, only one occurrence was outside South Tyrol-Trentino (Tuscany, frequency = 0.0023, CI_{95} =0.00041-0.01296), with the others found in Lower Val Venosta, South

Tyrol (frequency = 0.0095, CI_{95} = 0.0017-0.0520) and Val di Non, Trentino (frequency = 0.0208, CI_{95} = 0.0037-0.1090). The general distribution of this haplogroup in modern groups, representing 10% of haplogroup mtDNA lineages in the Balkans, but very rare in the Near East (Roostalu et al., 2007), could indicate possible Neolithic (LBK) ancestry. The pattern is, to a certain extent, reminiscent of Y-chromosomal haplogroup G: While apparently more frequent in a certain area up to the Copper Age, it became scarcer from the Bronze Age onwards and is rare in modern populations, but survived in higher frequencies not only in the Alpine region, but also in Corsica and Sardinia. Given the distribution in modern populations, the likelihood that the H5a4 lineage in modern-day groups in South Tyrol-Trentino is a direct result of continuity in the region from prehistoric times is high. This is supported by the highly similar H5a4a haplotype, which is addressed below.

Nogarole II 69 (CA), Romagnano III 118a, T11A-a, T12E-c (BA)
16294T, 16304C, 16320T, haplogroup H5a4a

This haplotype is exceedingly rare in modern populations: Four matches were found in GenBank, and two in the EMPOP database. Of these, only two specified the region: both were from Northern Italy (one region of Bologna, the other not further described, but the study included subjects from Lombardy, Veneto, Tuscany and Marche). Within the reference sample set used for this study, the only match was with an inhabitant of Val Giudicarie, Trentino (frequency = 0.0192, CI_{95} = 0.0034-0.1012). Given these observations, it seems plausible that this haplotype may have developed in the region from the precursor H5a4 (whose presence by the Bronze Age was established in other samples) through a subsequent mutation of position 16320. This would, in turn, indicate that H5a4 was already present in the Copper Age, even though it was not found in any Copper Age samples. This, in turn, would predate the introgression of Bell Beaker groups, with H5a4 more likely having been introduced via contact with Corded Ware groups (See Section 4.1). Bearing in mind the extreme

scarcity of this haplotype in modern populations, with no documented occurrence outside of Northern Italy to be found in the literature, the occurrence of H5a4a in modern-day Trentino is highly likely to represent continuity of this lineage in the region from the Copper Age to modern times, perhaps only surviving due to the relative isolation of these areas.

Nogarole III 75 (BA)
16069T, 16126C, 16189C, haplogroup J1c

While no occurrence of this haplotype was found in the EMPOP database, 13 matches with documented ethnicity were found in the GenBank database. Of these, 10 were from Sardinia (the others being from Calabria/Italy, Denmark and Ukraine). In the reference sample set, three were from Sardinia, one from Lower Val Venosta (South Tyrol), and one individual each from Switzerland and Tuscany. This once again highlights the genetic link between Sardinia and prehistoric South Tyrol/Trentino, which is also apparent, for example, in the modern-day frequency of y-chromosomal haplogroup G (specifically, the Iceman's haplogroup) in Corsica and Sardinia. Rather than any direct link between the two regions in ancient times, these similarities are more likely to be the result of ancient haplotypes (that may have been more frequent throughout the Italian peninsula) persisting in genetic refugia such as South Tyrol and Sardinia. The occurrence in Lower Val Venosta may therefore indeed represent continuity, although other explanations (later introgression) cannot be excluded.

Romagnano III 100b, 120b (BA)
16182C, 16183C, 16189C, 16234T, 16324C, 12308G, variant of U8

No match for this sequence was found in the EMPOP database, and only one exact match was found in GenBank: one individual from Bologna (although the 12308 position was not sequenced in this individual), which was also included in the reference data set (Boattini et al., 2013). Lacking any other documented occurrences of this haplotype in extant individuals (although this and a highly similar sequence have been found in Neolithic Hungarian individuals), no inferences can be made regarding continuity.

Moletta Patone 1b (CA)

16270T 16311C 16355T, haplogroup U5b1c

This haplotype was not found anywhere in either modern or ancient reference groups or the GenBank/EMPOP databases. Only one single match in GenBank was found for the same variant without 16355T, in a Finnish individual (although 16270T+16311C is present in several prehistoric reference individuals). While no continuity can thus be inferred, the apparent absence of this haplotype in any other ancient or modern population strengthens the case for this particular type having evolved through local mutation in the region (and having since become extinct).

Romagnano III 82 (BA)

16266T 16270T 16304C, haplogroup U5b3h

As described in Section 4.1.3, this specific haplotype was not found in any ancient or modern reference group or database. The closest links to subclade U5b3h are to be found in modern-day Sardinia, although these belong to a different sub-lineage. Whether this Bronze Age individual from Trentino is of local Mesolithic origin or whether this haplogroup was introduced to the region via trade contacts (possibly form Sardinia) cannot be determined.

Romagnano III T12E-a (BA)

16126C, 16292T, 16294T, 16296T, haplogroup T2c1

6 matches were found in EMPOP for this sequence (3 West Eurasian, 2 Native American, 1 Hispanic). In GenBank, this haplotype[36] in 18 individuals. Those with documented ethnicity included two from Sardinia, three from Poland, and others from Spain, Germany, Denmark and the Czech Republic. Furthermore, the same haplotype was found in a medieval (6th-8th century) individual from Collegno, North-Western Italy (Vai 2018). In the reference sample group, this haplotype was not found in the region of South Tyrol-Trentino, only infrequent occurrences in Moldavia (frequency: 0.0043, CI_{95}: 0.0008-0.0237), Transylvania (frequency: 0.0049,

[36] This sequence was incomplete, with only sections 15997- 16232 and 16289-16409 sequenced.

CI_{95}: 0.0009-0.0270), Crete (frequency: 0.0054, CI_{95}: 0.001-0.0298), Sicily (frequency: 0.0037, CI_{95}: 0.0007-0.0206) and Central Italy (frequency: 0.0202, CI_{95}: 0.0056-0.0707). Because of the scattered occurrences in modern populations and lack of individuals with this sequence in modern South Tyrol-Trentino, no continuity can be deduced (although it is notable that occurrences in North-Western Italy are documented not only for modern, but also medieval times).

Romagnano III 89/90 (BA)
16093C 16179T 16189C 16223T 16278T 16362C, variant of X2j
As described in section 4.1.3, this haplotype was not found in any other reference sample, with one exception, a Bronze Age individual from Bavaria. No modern individuals were found with this haplotype. While the Bronze Age connection indicates that this haplotype may have spread from Trentino to other regions, it may have since become extinct.

While other haplotypes observed in the ancient Trentino samples do occur at significant levels in modern populations, some of these show higher levels in the region in question. However, as frequencies can be greatly affected by e.g. genetic drift or bottlenecks, such observations are not sufficient to infer continuity.

In total, the occurrence of various infrequent to extremely rare variants in both ancient populations – mainly Bronze Age – and extant linguistic and geographic isolates in South Tyrol-Trentino is likely to indicate direct ancestry. However, frequencies vary greatly from population to population, possibly due to relatively low sample sizes.

From a **Y-chromosome perspective**, the matter is somewhat more ambiguous. Although haplogroup G frequencies are generally higher in the Alpine region than in Europe in general (Berger et al., 2013), levels vary greatly amongst the populations of South Tyrol and Trentino – again, in all likelihood due to drift resulting from small effective population sizes. A

study by Coia et al. (2013) including two South Tyrolean and nine Tridentine valleys found that, although variants of R1b were the most common haplotype in most sampled regions, the levels of haplogroup G-M201 were higher than the European mean, averaging 0.092 across Trentino and South Tyrol, with values ranging from 0 in some smaller populations and as much as 0.488 in Val Primiero (Trentino). Haplogroup R1b began to reach notable frequencies in Europe during the Bronze Age, very possibly as a result of the Bell Beaker expansion, and still remains the most common haplogroup in Europe in modern times (even in most of the isolates in Trentino-South Tyrol), a replacement of G-M201 by R1b in Bronze Age samples would have been unsurprising. Had this been the case, it would have argued against modern G-M201 levels in the region representing continuity of ancient prehistoric lineages in favour of possible re-introduction at a later stage (or reappearance of very rare lineages through drift or bottlenecks). However, this is not the case – the two Bronze Age samples from two different sites to yield Y-chromosomal DNA both showed haplogroup G. Although no general inferences can be made regarding the overall relative frequencies of G and R1b during the Bronze Age due to the small number of individuals from which Y-chromosomal DNA could be obtained, it at least documents that haplogroup G was still present at this time, so that the persistence of haplogroup G-M201 at notable levels in this region may well indicate a direct population continuity from the Neolithic.

4.6.1 Haplotype sharing versus F_{ST} values

Both haplotype sharing values and F_{ST} values provide a certain measure of similarity. However, as both measures are based on different calculation aspects, the obtained results may vary: For example, some populations with a high likelihood of haplotype sharing with ancient Trentino have high F_{ST} values (indicating a larger genetic distance between the two), or vice versa.

Haplotype sharing calculation regards only identical haplotypes in compared populations but disregards highly similar sequences. F_{ST} calculations take

haplotype similarity into account, providing a more general overview of genetic similarity. However, it must be noted that both methods have limited significance when comparing diachronous populations separated by several millennia – not only due to potentially haplotypes undergoing mutations over the course of time, but also due to the many other demographic factors shaping the genetic composition of the reference populations in question. Therefore, the possibility of using this data to infer relationships between ancient and modern populations is limited and must be regarded with caution. In doing so, the data must be regarded in detail, and take other factors into account, e.g. demographic history of the reference population (e.g. geographic isolation) and general frequency of observed haplotypes into account. For example, if certain haplotypes identified in the ancient study samples are highly infrequent in modern populations, then their identification in modern populations may be an indicator of ancestry, provided that this can be supported by demographic and geographical aspects.

4.7 Links between Ancient Trentino and the Tyrolean Iceman

Although the Tyrolean Iceman bears variants of mitochondrial haplogroup K and Y-chromosomal haplogroup G2a, the specific genetic signatures differed from those found in the Trentino groups. While the Iceman belongs to the Y-chromosomal G-L91, all Trentino individual for whom this locus could showed the ancestral allele at the L91 position. The Iceman's mitochondrial haplotype, K1f, has not been found in any other ancient or modern individuals to date. Therefore, no direct kinship can be inferred, despite the geographic and chronological proximity. As the Solteri haplotype individuals bear a K1a, which diverged from the lineage leading to the Iceman's subclade an estimated 27k years ago (Coia et al., 2016), a recent common local ancestor is unlikely. Instead, K1a and K1f were two of many haplotypes to have been introduced to the region of Trentino-South Tyrol during the Neolithic, with K1f apparently later having become extinct,

possibly as a result of populations turnover through incoming groups of steppe ancestry. With regard to the Y-chromosomal haplotype, Rootsi et al. (201) estimated the coalescent time for the L91 subclade of L91 to be approximately 10.9KYA, which also predates the start of the Neolithic in South Tyrol-Trentino by several millennia. Therefore, the same applies here as with the mitochondrial haplotype, with the difference that the L91 lineage still exists at low frequencies in various populations, including in the Alpine region (Berger et al., 2013); although strikingly high frequencies are observed in Corsica and Sardinia (Keller et al., 2012; Francalacci et al., 2013).

5 CONCLUSIONS

With regard to the aims and objectives listed in Section 1.6, the following can be concluded:

Question: ***Does genetic data lend more weight to acculturation or replacement models, i.e. are the haplogroups of Neolithic, Copper or Bronze Age individuals more indicative of hunter-gatherer ancestry, lending weight to the hypothesis of gradual acculturation of local Mesolithic groups, or are they typically "Neolithic" in origin?***

The palaeogenetic data obtained from the samples analysed in the course of this study reflects a predominantly Neolithic composition of the groups that inhabited the Adige Valley during the Copper Age, both from a maternal (mitochondrial) and paternal (Y-chromosomal) point of view. The majority of mitochondrial haplogroups found in Copper Age individuals are most commonly observed in LBK or Starčevo individuals, although some individuals showed specific subtypes not observed in any other ancient samples. These may simply represent rare lineages too infrequent to have been detected to date, or the differing mutations may possibly have occurred at some point after settlement in the region. In any case, these results show that Neolithisation in the region of Trentino was largely the result of demic diffusion, rather than simply a process of acculturation without significant admixture. Due to the lack of sufficient data from Neolithic individuals, as well as sufficient Palaeolithic/Mesolithic reference data from the region, the question of possible continuity of pre-Neolithic lineages could not be answered conclusively. While the one of the Copper Age samples showing mitochondrial haplogroup U5 may represent a persisting Mesolithic lineage, other cultural contacts (e.g. with Corded Ware groups) may also account for the presence of this haplogroup. As observed in other studies (Lipson et al., 2017) admixture with Mesolithic populations went on for long periods following the original Neolithic-Mesolithic contact. Another U5 individual shows a haplotype (U5b3h) that originated in the Italian peninsula, therefore

rendering its introduction by steppe-derived groups less likely. However, as the U53b subclade is common in modern Sardinia, it may have been introduced as a result of trade contacts with the Island rather than representing a local Mesolithic lineage.

Whether existing Mesolithic groups were completely replaced by the incoming Neolithic peoples, or whether local Mesolithic communities persisted and adopted the new technologies, cannot be addressed on the basis of the data obtained in the course of this study. This would require more extensive analysis of individuals from both Gaban and VBQ phases. As the samples from La Vela, the principal VBQ site, showed poor preservation and failed to yield satisfactory results using conventional PCR, as did the Mesolithic individual from Vatte di Zambana in a previous study, any further attempts to obtain usable genetic information should be based on next-generation sequencing approaches. Although these techniques are still costly and time-consuming, they have provided a wealth of new information on prehistoric population genetics over the last few years. Bearing in mind that genomic data from Neolithic or Mesolithic samples would contribute toward a full understanding of the Neolithisation of Northern Italy, such an approach seems justified for future analyses.

Question: ***In case of a likely Neolithic origin: Are these more closely linked with the Mediterranean coastal wave of advance (Adriatic Impressed Ware/later Cardial) or with the Neolithic advance via Transdanubia into Central Europe (mainly LBK)?***

The genetic data obtained from the samples in this study is not sufficient to pinpoint with certainty the origin and/or migration route of the original wave of Neolithic settlers to reach the Trentino region. While the finding of an N1a haplotype in one Neolithic individual might initially suggest links with the LBK (as this haplotype has been found in high frequencies in LBK populations), it has also been observed in Neolithic sites along the Adriatic/Cardial route, for example in Spain.

Neither do the observed Y-chromosomal haplotypes provide information on the direction of the original Neolithic wave of advance: G2a is observed in numerous Starčevo/LBK individuals as well as in Cardial/Impressed Ware individuals. While the specific haplotype observed in the Tyrolean Iceman shows its highest modern frequencies in Corsica and Sardinia, none of the Trentino individuals yielding Y-chromosomal DNA share this particular subgroup. In modern populations, G2a is spread over a wide geographical area, favouring more remote and isolated regions such as the Alps, the Mediterranean islands, as well as mountainous areas of France, Spain and the Balkans. This reflects its status as a Neolithic "remnant" which survived the steppe-driven invasion of the Bronze Age and subsequent population shifts only in more remote regions. However, this aspect cannot address the question as to where the original Neolithic pioneers to reach Trentino originally came from.

Several rare haplotypes found in the Trentino groups have parallels with cultures related to the LBK/Starčevo, mainly Alföld in Hungary. Furthermore, the archaeological record documents clear links between the VBQ with the LBK and the Balkan in general. It must be borne in mind, however, that the Gaban group rather than the VBQ were the first inhabitants of Trentino for which a "Neolithic" lifestyle was documented. Gaban typology differs from that of the VBQ, with certain Cardial elements, but also a notable continuation of Mesolithic traditions with regard to subsistence and blade typology. While little ancient DNA exists for the Gaban group, it is conceivable that the Neolithic reached South Tyrol-Trentino in several waves: an initial wave in the Early Neolithic leading to the formation of the Gaban group (although persistence of Mesolithic traditions initially indicate acculturation, the finding of the typically "Neolithic" haplogroup T2 in the early Neolithic Mezzocorona 2 individual documents a certain amount of demic diffusion). Then another, more substantial wave of advance in the Middle Neolithic, more probably via the LBK and/or related groups via the Alpine/Balkan route, leading to

extensive admixture (if not complete replacement) and the introduction of numerous novel lineages of Near Eastern origin.

Question: ***Is a genetic difference discernible after the migration movements originating from the Pontic steppe, and/or the Bell Beaker settlements in northern Italy?***

In total, the Copper Age samples support the observations made on the basis of the Iceman and the three Remedello samples to date, namely that Copper Age groups in Northern Italy were largely Neolithic in origin. However, another influence starts to become apparent during this time, through haplogroups most likely to have been introduced by new peoples of Pontic steppe ancestry, e.g. Corded Ware.

During the Bronze Age, a slightly different genetic composition becomes apparent, with the distance to Neolithic groups becoming larger. Genomic analysis published over the past years show that the early Bronze Age in Europe in general is characterised by demographic shifts originating from the Steppe region. Other groups spreading across central and western Europe, particularly the Corded Ware, Únětice and Bell Beaker groups, have a significant proportion of Yamnaya ancestry. F_{ST} values between these and Bronze Age Trentino are lower than for Copper Age Trentino, underlining the closer relationship.

Question: ***Which variants are observed for lactase persistence and blue eye colour in the ancient samples, can any inferences be drawn from these observations (especially regarding the higher frequencies of lactase persistence in modern north-eastern Italy)?***

Although reliable sequences for the -13.910 (lactase persistence) locus were obtained in 17 individuals from the Copper and Bronze Ages, the derived allele (indicating the ability to digest milk after early childhood) was not found in any of these. Originally thought to have spread during the Neolithic, more recent studies have shown that the derived allele may still have been rare at the start of the Bronze Age, and gradually spread with the

Steppe-driven expansions. Its apparent absence in South Tyrol-Trentino, therefore, is unsurprising. However, the selective pressure may also have been lower in this region. The archaeological evidence documents a variety of available foodstuffs, and the persistence of hunting as an additional subsistence strategy until well into the Bronze Age, although proportion of wild animals amongst the faunal remains gradually dwindles. The region is a fertile one, combining a Mediterranean climate in the valley with extensive pasture grounds on the adjacent plateaux. The general availability of various food sources may therefore have reduced the specific selective advantage in this region, with the allele spreading to its extant frequencies gradually over time.

Regarding eye colour, the derived allele was found in several individuals, one of them homozygous, indicating blue eye colour. As the homozygous derived allele has been found in a Mesolithic European, no inferences regarding ancestry can be made.

Question: ***What can be observed regarding the intra-group diversity and differentiation between local groups and what are the implications regarding population history and expansion?***

Overall, the Trentino groups show slightly lower diversity than other Neolithic and later ancient reference groups, although the difference is not particularly striking, especially regarding the small sample size. What is noticeable, however, is that Bronze Age Trentino shows lower intra-group diversity indices than Copper Age Trentino, although the latter comprises fewer individuals. This is in all likelihood due to the fact that the majority of individuals in the Bronze Age group are from the same site, namely Romagnano.

Question: ***What similarities can be observed between the ancient Trentino samples and the genetic data from the Tyrolean Iceman?***

Although the Iceman shares the same basal mtDNA and Y-chromosomal haplogroups with various individuals from Copper and Bronze Age

Trentino, the specific lineages differ. In each case, the most recent common ancestor of the Iceman and the respective Trentino line predates the start of the Neolithic in Northern Italy by several millennia, so that the respective haplotypes are likely to have been introduced independently by incoming Neolithic pioneers rather than having developed as a local mutation in the region.

Question: ***Does the chronological gap between the late Mesolithic and the early Neolithic observed in the archaeological stratigraphy reflect actual the population situation at the time?***

As no lineages of certain Mesolithic origin could be found in the Trentino data set, a complete hiatus cannot be ruled out. While some individuals do show Mesolithic-type lineages, their chronological placement makes it difficult to determine whether these lineages represent continuity or were re-introduced during cultural contacts after the start of the Neolithic. However, given the persistence of certain Mesolithic traditions, a certain amount of admixture with local communities seem the more plausible explanation, with the "gap" resulting from other circumstances, such as changing burial customs. This would also explain the long-term persistence of Mesolithic traditions such as hunting or characteristic blade typology.

Question: ***Is any genetic continuity discernible from ancient Trentino to later/modern populations from the greater area?***

While values for F_{ST} and haplotype sharing vary from population to population, distances are generally low for the South Tyrol-Trentino region, with Copper Age Trentino clustering somewhat closer to the Italian/Romansch-speaking Trentino groups, and Bronze Age Trentino nearer to the German/Ladin speaking groups in South Tyrol (although the clusters are not particularly distinct). Analysis of individual haplotypes, however, show that some of the ancient Trentino individuals bear haplotypes that are extremely rare in modern populations, but occur at higher frequencies or – in some cases – exclusively in South Tyrol-Trentino.

Such concurrence is a strong indicator of local continuity of some lineages since prehistoric times, although these occur over several modern populations. Modern frequencies of haplotype Y-chromosomal haplotype G2a are also likely to represent continuity in the region, as levels in the Alpine area are higher than in most other regions of Europe.

In sum, therefore, it can be deducted that some regions of modern-day South Tyrol and Trentino still host ancient genetic lineages from the greater area, dating back to at least the Copper Age. The relative isolation of the various individual communities contributed to the preservation of certain lineages, which became rare or even extinct in many other regions as a result of widespread demographic events that occurred throughout the next millennia.

5.1 Zusammenfassung

Bei der vorliegenden populationsgenetischen Studie wurden 76 prähistorische Individuen (8 Neolithikum, 30 Kupferzeit und 38 Bronzezeit) von 12 Fundorten in Südtirol und Trentino paläogenetisch untersucht. Die untersuchten loci umfassten die HVR1 (hypervariable Region I) sowie Position 12308 des mitochondrialen Genoms, den Amelogenin-locus zur Bestimmung des Geschlechts, die SNPs (single nucleotide polymorphisms) rs12913832 (HERC2 Gen, Unterscheidung zwischen brauner/blauer Augenfarbe) und rs4988235 (MCM6 Gen, -13.910*C/T; Laktasepersistenz), sowie verschiedene SNPs auf dem Y-Chromosom zur Bestimmung väterlicher Linien. Die DNA-Daten wurden mit prähistorischen und modernen Referenzpopulationen verglichen. Ziel der Studie war eine populationsgeschichtliche Analyse der prähistorischen Bevölkerung Südtirol-Trentinos. So wurde der Frage nachgegangen, ob diese eher mesolithische Linien zeigt oder ihre Wurzeln in den eingewanderten neolithischen Ackerbauern und Viehzüchtern hatte. Für die frühe Bronzezeit sollte zudem geklärt werden, in welchem Umfang die kupferzeitliche/bronzezeitliche Expansion, die in der pontischen Steppe ihren Ursprung hatte, in Südtirol genetische Spuren hinterlassen hat. Außerdem wurden die prähistorischen Gruppen aus Südtirol-Trentino mit dem Mann aus dem Eis („Ötzi“) und mit modernen Populationen aus dem gleichen Gebiet verglichen.

Bei der Probenaufbereitung und -analyse wurden strenge Antikontaminationsmaßnahmen beachtet, um eine Verunreinigung mit moderner DNA oder Kreuzkontamination mit anderen Proben zu vermeiden. Insgesamt konnte von 59 Individuen mitochondriale und von 36 nukleäre DNA sequenziert werden. Da Zweitextrakte nicht für alle Proben vorlagen bzw. Replikation der Ergebnisse in einem anderen Labor nicht in jedem Fall möglich war, wurde ein scoring System etabliert (basierend auf Sequenzqualität, Anzahl der PCRs und Extrakte sowie Replikation in einem anderen Labor) um die Reliabilität der jeweiligen

Ergebnisse festzustellen. 37 Individuen zeigten mitochondriale Sequenzen mit ausreichendem score und konnten somit für die statistischen Analysen verwendet werden.

Mitochondriale Linien, die mit der mesolithischen Bevölkerung Europas assoziiert werden, lagen in nur geringer Häufigkeit vor. Insgesamt gleicht das beobachtete Haplotypenspektrum eher demjenigen frühneolithischer Gruppen aus Mittel- bzw. Südosteuropa als aus dem Mittelmeerraum. Dieser Befund ist konsistent mit einer Neolithisierung von Südtirol-Trentino im Zusammenhang mit der danubischen Expansion. Während die kupferzeitliche Gruppe neolithischen Referenzpopulationen ähnelte, glich die bronzezeitliche Stichprobe eher prähistorischen Populationen mit Bezug zur pontischen Steppe. Die Tatsache, dass einige seltene Haplotypen nicht nur in den prähistorischen Proben, sondern auch in modernen Populationen Südtirol-Trentinos gefunden wurden, spricht jedoch auch für einen gewissen Grad an Kontinuität seit der Kupferzeit. Während Y-chromosomale Linien im Laufe der Bronzezeit in Mittel- und Nordeuropa weitegehend ersetzt werden, persistieren neolithische Y-Linien in Südtirol-Trentino bis in die zweite Hälfte des 3. vorchristlichen Jahrtausends. Direkte Verwandtschaft mit dem Mann aus dem Eis konnte nicht nachgewiesen werden.

5.2 Riassunto

In questo studio sulla genetica delle popolazioni del Trentino Alto-Adige sono stati esaminati 76 individui preistorici (8 del Neolitico, 30 dell'Età del Rame e 38 dell'Età del Bronzo) provenienti da 12 siti. Sono stati caratterizzati i seguenti loci e polimorfismi genetici: il locus HVR1 (hypervariable region, regione ipervariabile I) e la posizione 12308 del genoma mitocondriale (mtDNA, determinazione origine materna); il locus amelogenina (determinazione del sesso); Il polimorfismo a singolo nucleotide , SNP_rs12913832 (gene HERC2, per la determinazione del colore occhi); lo SNP rs4988235 (gene MCM6, 13.910*T, persistenza della lattasi; capacità di digerire il lattosio oltre l'infanzia) e vari altri SNPs sul cromosoma Y per la determinazione dell'origine paterna. I risultati sono stati confrontati con delle popolazioni di riferimento, sia preistoriche che moderne. Gli obiettivi del presente studio (sulla base di loci mitocondriali, nucleari e Y-cromosomali) erano di: 1) comprendere, ad esempio, se i gruppi analizzati presentasserodelle linee genetiche di origine Mesolitica (cacciatori-raccoglitori) o Neolitica, quest'ultimi più affini agli agricoltori e allevatori di bestiame del Vicino Oriente; 2) individuare possibili differenze genetiche tra gli individui datati all'Età del Bronzo, forse attribuibili all'espansione demografica dell'Età del Rame/Bronzo, originatasi nella steppa pontica; 3) confrontare i gruppi preistorici del Trentino Alto-Adige sia con l'Uomo Venuto dal Ghiaccio ("Ötzi") che con le popolazioni moderne della stessa area per determinare la loro possibile relazione/continuità genetica.

La preparazione e l'analisi dei campioni antichi sono state effettuate seguendo le procedure per evitare qualsiasi tipo di cross-contaminazione con il DNA esogeno (moderno oppure con il DNA di altri campioni). Dei 76 individui, 59 DNA mitocondriali e 36 DNA nucleari sono stati sequenziati con successo. Considerando che non è stato possibile eseguire un secondo processamento di tutti i campioni o replicare i risultati in un altro laboratorio, è stato stabilito un sistema di punteggio (basato sulla

qualità della sequenza, sul numero di PCR ed estratti e sulla replicazione in un altro laboratorio) al fine di misurare la "qualità" dei rispettivi risultati. Solo i risultati con un punteggio sufficiente sono stati inseriti nelle analisi statistiche. A tale proposito, i campioni neolitici sono stati esclusi poiché non hanno ottenuto un punteggio sufficiente. I campioni rimanenti invece, sono stati distinti in due gruppi, "Età del Rame" e "Età del Bronzo", non solo sulla base della cronologia, ma anche delle differenze tipologiche (archeologiche).

Nel complesso, il gruppo dell'Età del Rame ha mostrato una minore distanza genetica dalle popolazioni neolitiche di riferimento, mentre il gruppo dell'Età del Bronzo ha presentato una minore distanza dalle popolazioni di riferimento che provengono in parte da gruppi originari della steppa pontica (cultura di Jamna). Non sono state trovate prove evidenti di continuità dal Paleolitico (anche se, potenzialmente, alcuni individui potrebbero presentare delle linee paleolitiche). Nel complesso, sono state riscontrate più somiglianze con gruppi ceramici a banda lineare provenienti dai Balcani, rispetto a quelli mediterranei. Pertanto, si ipotizza che la cultura neolitica sia stata introdotta in Trentino Alto-Adige via terra. Il fatto che, in Alto Adige, alcuni aplotipi molto rari siano stati trovati non solo in campioni preistorici ma anche in gruppi moderni, suggerisce una certa continuità o persistenza di linee rare dall'Età del Rame fino ai nostri giorni. Nonostante alcuni campioni abbiano mostrato linee simili a quelle dell'Uomo Venuto dal Ghiaccio, non è stato possibile trovare corrispondenze esatte degli aplogruppi mitocondriali e Y-cromosomali, pertanto una stretta relazione tra l'Uomo Venuto dal Ghiaccio e i gruppi ad esso coevi del Trentino Alto-Adige non può essere affermata.

6 APPENDIX

6.1 Instruments and reagents

Table 34: Instruments

Name	Manufacturer
ABI PRISM® 310 genetic analyzer	Applied Biosystems
ABI PRISM® 3100 genetic analyzer	Applied Biosystems
Column loader	Millipore
Dremel Multi blade	Dremel
Electrophoresis apparatus	Roth
HPLC water purification instrument	Millipore
Laboratory centrifuge (various)	Eppendorf, Thermo Scientific, Hettich
Mixer mill MM 200	Retsch
Pipettes	Eppendorf
Precision balance EMB 2000	Kern
Protective visor	Roth
Sand blaster P-G 400	Harnisch & Rieth
Thermocycler MasterCycler®	Eppendorf
Vortexer	Kisker Biotech GmbH

Table 35: Reagents

Name	Manufacturer
Agarose	Invitrogen™
Alconox	Sigma Aldrich
AmpliTaq® DNA Polymerase	Applied Biosystems/Roche
AmpliTaq® Gold DNA Polymerase	Applied Biosystems/Roche
ATP (10mM)	Fermentas
BigDye® Terminator 5x sequencing buffer	Applied Biosystems
BigDye® Terminator v1.1	Applied Biosystems
BSA (Bovine Serum Albumin)	New England Biolabs, Roche
Buffer for ABI PRISM ®3130 Genetic Analyzer	Applied Biosystems
Chloroform	Roth
DNA-ExitusPlus™	AppliChem
dNTP mix, 10mM	Qiagen, Applied Biosystems, Promega
EDTA	Ambion®
Ethanol	Roth
Ethidium bromide	Roth
Formamide	Applied Biosystems
GeneAmp® 10X PCR Buffer II & $MgCl_2$ Solution	Applied Biosystems
GeneRuler™ 50 bp DNA Ladder	Fermentas
Invisorb Spin Swab Kit	Invitek
Loading dye	Fermentas
$MgCl_2$ Solution	Applied Biosystems/Roche
MSB®Spin PCRapace kit	Invitek
O'GeneRuler™ Ultra	Fermentas
Oligonucleotides	Biospring
POP-6™, Performence Optimized Polymer 6	Applied Biosystems
Proteinase K	Fermentas, Roth, Roche
Roti® Phenol chloroform isoamyl alcohol	Roth
Rotipuran analytical grade chloroform	Carl Roth GmbH & Co. KG, Karlsruhe
Sea sand	Roth
Sephadex™ G-50 fine	GE Healthcare
Shrimp Alkaline Phosphatase	Fermentas
Sodium hypochlorite bleach	DanKlorix
Sodium lauryl sarcosinate	Merck
Taq DNA Polymerase w/Std Taq Buffer	New England Biolabs
Tris buffer	Roth
TWEEN 20	Carl Roth

Table 36: Consumables

Name	Manufacturer
Aluminium oxide corundum (50 µ, 250 µ)	Harnisch & Rieth
Amicon® Ultra-15 (30, 50 kDa)	Millipore
Diamond cutting blades	Dremel
DNA LoBind tubes (o.5 ml, 1.5 ml)	Eppendorf
Falcon tubes (15 ml, 50 ml)	Sarstedt
Pipette tips	Sarstedt, Thermo Fischer
Protective gloves (nitrile, latex, vinyl)	Hansa medical
Protective hoods (astronaut)	Hansa Medical
Protective masks	Hansa Medical
Safe-Lock tubes (0.5 ml, 1.5 ml)	Eppendorf
Tyvek Classic overall	DuPont
Tyvek shoe protectors	DuPont

6.2 Abbreviations

aDNA	Ancient DNA
BA	Bronze Age
BB	Bell Beaker Culture
BCE	Years before Common Era (equivalent to B.C.)
BP	Before present (=before 1950)
CA	Copper Age
cal.	Calibrated radiocarbon age
CE	Years after Common Era (equivalent to A.D.)
CI_{95}	95% confidence interval (Wilson method)
CW	Corded Ware
HG	Hunter-gatherer
HVR	Hypervariable region (of the mitochondrial genome)
KYA	Thousand years ago
LBK	*Linearbandkeramik*
mtDNA	Mitochondrial DNA
ncDNA	Nuclear DNA
N.D.	Not determined
NEO	Neolithic
NGS	Next generation sequencing
rCRS	Revised Cambridge reference sequence
SNP	Single nucleotide polymorphism
VBQ	*Vasi di bocca quadrata* (square-mouthed pottery)
YDNA	Y-chromosomal DNA

6.3 List of figures

6.4 List of tables

6.5 Supplementary information on CD

For reasons of clarity, the following data are provided in Excel format on the accompanying CD. Supplements S1-S9 are to be found in separate tabs of the Excel file.

6.5.1 Supplement S1: List of samples with sequencing results

Full details are provided for all samples included in this study, including site type, excavation year, number of individuals for each site, date (rounded), era, osteological age and sex (according to A. Paladin, 2013), bones/teeth sampled, number of extracts, PCR and sampling success. Greyed out fields indicate that no sequence was obtained, either because amplification was not attempted (e.g. for nuclear DNA after mtDNA had failed, or due to lack of sample material), because no amplification was achieved or because sequences were not legible. Osteological age and sex are reported according to A. Paladin (2013). For samples that failed to meet scoring requirements, haplotype allocation was only attempted if the identified mutations gave a clear indication of the haplotype. The list also includes samples from which no DNA could be sequenced.

6.5.2 Supplement S2: Reference sample sets (ancient)

This sheet contains a list of all ancient samples used for reference purposes in statistical analyses for study, specifying the respective culture/group, site, country, as well as mitochondrial and Y-chromosomal haplogroups as reported by the author. References are provided for each individual sample.

6.5.3 Supplement S3: Reference sample sets (modern)

This sheet lists the modern samples used for reference purposes in statistical analyses for the study. Samples are not listed individually, but according to population/country and respective publication, stating the number of individuals in each group as well as GenBank accession numbers, where

available. Data that were not accessible for download via GenBank, European Nucleotide Archive or other source were reconstructed from the information in the publication.

6.5.4 Supplement S4: Haplotype frequencies (ancient)

This sheet lists the samples that were successfully sequenced in this study according to era (Neolithic, Copper Age, Bronze Age, Late Bronze Age) and mitochondrial haplotypes, reporting the frequency of each haplotype in the respective ancient reference groups (listed in Supplement S2). 95% confidence intervals (Wilson) are specified for each frequency.

6.5.5 Supplement S5: Haplotype frequencies (modern)

This sheet lists the samples that were successfully sequenced in this study according to era (Neolithic, Copper Age, Bronze Age, Late Bronze Age) and mitochondrial haplotypes, reporting the frequency of each haplotype in the respective modern reference groups (listed in Supplement S3). 95% confidence intervals (Wilson) are specified for each frequency.

6.5.6 Supplement S6: Full scoring results

This sheet summarises the method of scoring samples (mtDNA only) to establish whether they are deemed to be of sufficient quality and authenticity to be included in statistical analyses. All samples that yielded at least one sequencable amplicon in the course of this study are listed, providing details on the quality criteria number of extracts, successful PCRs per HVS1 fragment, whether samples were replicated in two separate laboratories, sequence quality and overall sequencing result.

6.5.7 Supplement S7: F_{ST} values

This sheet illustrates F_{ST} (fixation index, pairwise differences) values between all samples analysed in this study and all reference groups (ancient DNA, modern South Tyrol-Trentino, other modern groups). Values below the diagonal represent the F_{ST} values, with significant ($p < 0.05$) values

highlighted in a red-green colour gradient, with highest significant F_{ST} values (= greatest genetic distance) in red and lowest significant F_{ST} values (lowest genetic distance) in green. Values above the diagonal are the respective p-values, with significant values highlighted in turquoise and bold type for clarity.

6.5.8 Supplement S8: Positive controls yielding sequence

All PCR/extraction blanks that showed a positive band in electrophoresis were subjected to sequencing: those yielding a legible sequence are listed in this sheet, specifying the mutations. The table specifies whether the contaminant matches any other samples results a) in the same PCR or b) in general, or matches tested laboratory personnel (Supplement S9). Finally, the resulting action is described (e.g. whole PCR discarded, matching samples in same PCR discarded, samples used, stating justification).

6.5.9 Supplement S9: Samples from lab personnel

The only two haplotypes shared between lab personnel and ancient samples are frequent in both ancient and modern populations. These matches, therefore, are unlikely to represent cross-contamination: the separate occurrence of these haplotypes in modern individuals and in the ancient Trentino samples is plausible and supported by replication of results on the one hand (in separate laboratories in many cases) and general anticontamination measures on the other.

6.5.10 Alignment reports

Alignment reports are provided in PDF format on the accompanying CD. Separate alignment reports are provided for HVS1, mtDNA position 12308, HERC2, L91, -13.910*T/C and M201.

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