

**Molecular phylogeography of the European coastal plants  
*Crithmum maritimum* L., *Halimione portulacoides* (L.) Aellen,  
*Salsola kali* L. and *Calystegia soldanella* (L.) R. Br.**

**Dissertation zur  
Erlangung des Grades  
“Doktor der Naturwissenschaften”**

**Am Fachbereich Biologie  
der Johannes Gutenberg-Universität  
in Mainz**

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**Tag der mündlichen Prüfung: 11. Februar 2005**

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### 1. INTRODUCTION

Organisms in their distributional ranges are living in an environment that keeps changing over time. Ecological factors together with life-history, reproductive biology, dispersal, population size and climatic history, all have influence on present day diversity of species. Since the term phylogeography was introduced by Avise *et al.* (1987), attempts have been focused on studying relationships between the genealogy of lineages and their geographical locations at intra- and interspecific levels and linking this aspect with micro- and macroevolutionary processes (Avise, 2000).

It is proven that the global climate witnessed extreme oscillations during the past 2-3 My known as the Quaternary climatic oscillations or the Pleistocene. During the Pleistocene, the earth experienced several glacial cycles (ice ages), lasting around 100 ky each, interrupted with interglacial periods, each lasting around 10-20 ky. These ice ages are characterized by massive accumulation of ice-sheets (glaciers) during the cold cycles in the northern hemisphere where nearly one third of the earth's total land surface was covered under permanent glaciers. The ice-sheets covered particularly northern Europe (Figure 1.1), northern North America, Siberia, and various mountainous regions (Nilsson, 1983; Brown and Lomolino, 1998).



**Figure 1.1** Ice-covered areas and coast line in the Würm glacial in Europe based on Butzer (1965) and Lange (1994).

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These climatic oscillations are considered to be the most influential factor in earth's history affecting the structuring of present populations and have forced many species to adjust their distributional ranges to suitable conditions (Avice *et al.* 1987). During the last decade, it has been intensively investigated and confirmed by palaeobotanical (Ravazzi, 2002) and molecular studies (Taberlet *et al.* 1998; Comes and Kadereit, 1998; Hewitt, 1999) that many of the present ranges of animal and plant taxa in Europe are the result of post-glacial expansion and recolonization from their refugia during the cold cycles of the Quaternary, particularly from southern regions, the Iberian Peninsula, Italy, the Balkans, Greece and Turkey (Hewitt, 1999; Hewitt, 2000).

By investigating the consequences of the ice ages on the European biota, it has become common knowledge that species differ in their response to the cold and warm cycles. This can be due to geographical barriers to dispersal and differences in refugium geography and recolonization routes during and after each cycle (Brown and Lomolino, 1998; Taberlet *et al.* 1998; Petit *et al.* 2003; Comes and Kadereit, 2003). Furthermore, distinction between the recently recolonized and refugial areas is always under investigation. In general, newly recolonized areas are characterized by having lower genetic diversity than refugial areas (Hewitt, 1996; Widmer and Lexer, 2001). Correspondingly, it becomes widely accepted to distinguish areas of refugial and recolonization and to provide insight into organisms' spatial genetic structure using molecular markers when fossil evidences are lacking or in the case of widely distributed species (Avice, 2000). For example, a comparative review by Taberlet *et al.* (1998) based on molecular data from ten taxa, including mammals, amphibians, insects and plants, concluded that pronounced dissimilarities among European-wide phylogeographic patterns exist. Thus, it seems that each taxon has responded independently to Quaternary climatic cycles, for instance, populations of water voles (*Arvicola sapidus*) and newts (*Triturus marmoratus*) in France came from refugia in the Iberian Peninsula, whereas both a grasshopper (*Chorthippus parallelus*) and common beech (*Fagus sylvaticus*) extended to France from the Balkans.

It is well known that terrestrial organisms respond to climatic changes by adjusting their longitudinal, latitudinal and altitudinal distribution ranges by means of dispersal, seeking climatic conditions that support their survival. This spatial complexity is challenging for researchers who want to study dispersal routes during or following a particular historical climatic event. In contrast, those species which are distributed exclusively in coastal ecosystems lack the altitudinal dimensions of distribution. Thus, they respond in a linear

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manner, either retreating or expanding, depending on climatic conditions. Accordingly, this reduces the complexity of possible dispersal routes into or out of the refugial areas. Studying widely distributed species in linear systems also benefits from their azonality, since their wide longitudinal and latitudinal distribution could contain both refugial and recolonized regions. As a third advantage, it is easier to correlate their distribution to limiting environmental factor like temperature. Some examples are the northern distributional limit of *Halimione portulacoides* which correlates with the 16°C isotherm of July (Chapman, 1950) and the southern limit of the world distribution of *Mertensia maritima* which is controlled more by temperature than any other climatic factor and corresponds to the July 19°C and January 4-5°C isotherms (Scott, 1963). Thus, if the considered distribution limit corresponds directly to temperature (or to other factors linked to temperature), this allows fairly safe assumptions to be made about potential refugial distributions based on historical temperature isotherms.

A steadily increasing number of phylogeographic studies on coastal animal and plant species in Europe have been published (e.g. Borsa *et al.* 1997a,b; Röhner *et al.* 1997; Bianchi and Morri, 2000; Clausen *et al.* 2000; Nikula and Väinölä, 2003; Olsen *et al.* 2004). Efforts have been focused on understanding the historical phylogeography, recognizing refugial and recolonization areas, and testing isolation by distance and gene flow status either within or between marine basins. For example, the study by Clausen *et al.* (2000) revealed an Atlantic-Mediterranean genetic subdivision in the two coastal plant taxa *Eryngium maritimum* and *Cakile maritima* which are co-distributed almost along the entire coast of Europe. In a wider sampled study, more details about genetic breaks between Black Sea, Aegean Sea, Mediterranean basin and Atlantic Ocean populations of the lagoon cockle (*Cerastoderma glaucum*) was found by Nikula and Väinölä (2003). Borsa *et al.* (1997) observed distinct Aegean and Adriatic Sea lineages in flounder, and a major subdivision between the east and west Mediterranean Sea. Genetic divergence between Atlantic and Mediterranean Sea was found in many (Borsa *et al.* 1997a,b; Perez-Losada *et al.* 1999; Olsen *et al.* 2004), but not all (Magoulas *et al.* 1996; Bargelloni *et al.* 2003) marine organisms. The main causes of these divergences were attributed either to historical climatic events (consequences of last ice-age), or to contemporary abiotic factors like sea-currents besides some biological factors like dispersal, breeding system etc.

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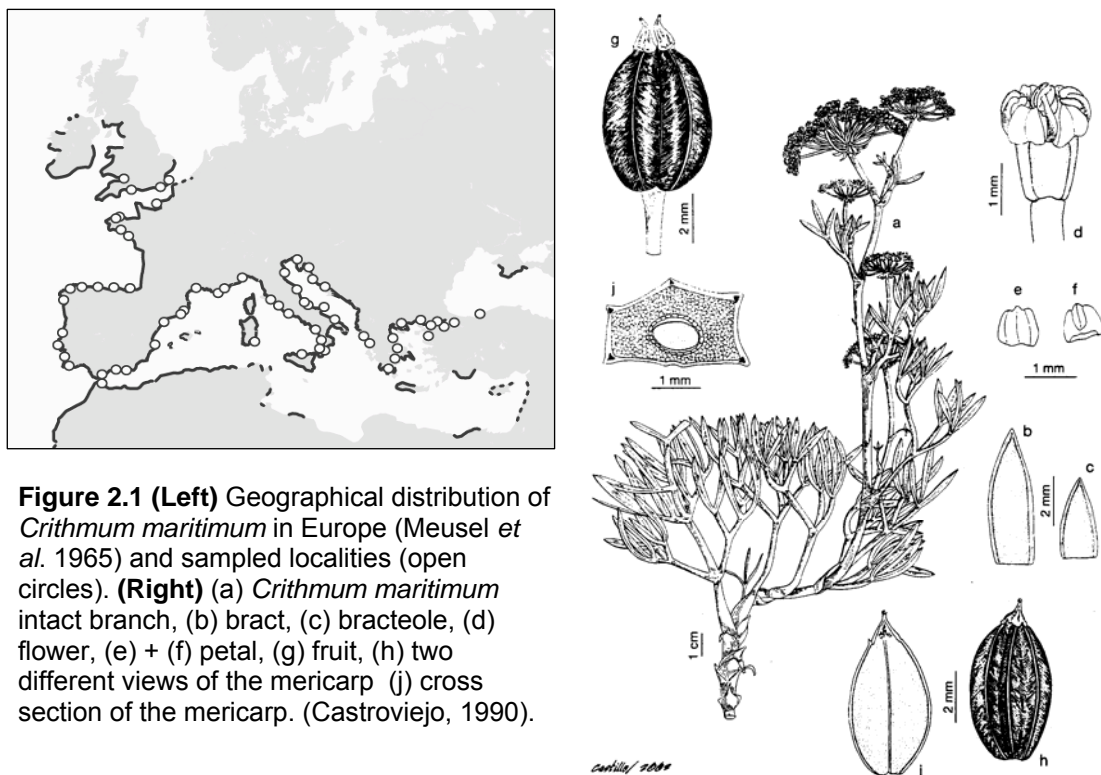
On this background, a comparative phylogeographic study was carried out by investigating seven plant taxa codistributed along the European coasts from the Black Sea to the Baltic Sea. The main aim was to explore the influence of climatic-history, contemporary abiotic and biotic factors on the genetic variation using different molecular markers. The species are the salt marsh plants *Halimione portulacoides* (Chenopodiaceae) and *Triglochin maritimum* (Juncaginaceae), the rocky coast plant *Crithmum maritimum* (Apiaceae), the sandy shore plants *Salsola kali* (Chenopodiaceae) and *Cakile maritima* (Brassicaceae) and the sand dunes plants *Calystegia soldanella* (Convolvulaceae) and *Eryngium maritimum* (Apiaceae). They are taxonomically unrelated and differ in their biology and ecology. In this doctoral work, only four species representing the four coastal ecosystems (*S. kali*, *C. maritimum*, *H. portulacoides* and *C. soldanella*) will be investigated. This study was aimed to answer the following three questions. First, we wanted to know whether any geographical pattern of genetic variation exists in any of the studied species along the distributional range. Second, if we find any pattern, is there any concordance among the species regardless of their biology and ecology. Third, what are the historical, contemporary and biotic factors influencing their phylogeographic pattern?

2. MATERIALS AND METHODS

2.1 Study species:

2.1.1 *Crithmum maritimum* L., (Apiaceae),  $2n = 20$  (Al-Bermani *et al.* 1993), is a perennial plant up to 50cm height with a woody rootstock and 1-2-pinnate succulent leaves which become bluish-green after maturation. The inflorescence (umbell) contains 8 - 36 hermaphrodite, yellowish-green flowers (Tutin *et al.* 1968). Pollination is likely to be done by insects (personal observation) whereas the compatibility system of the species is not yet investigated. The ripe fruits ( $\approx 4 \times 2 - 6 \times 4$ mm) is composed of two mericarps (Figure 2.1; Davis *et al.* 1967) and the fruits are adapted to water dispersal by having aerenchymatous tissue that enables buoyancy. In sea water, fruits were reported to float for around a year (Ridely, 1930).

*Crithmum maritimum* grows on rocky shores and the species occurs at the west European coasts northwards to Scotland, and the Mediterranean and the Black Sea coasts (Tutin *et al.* 1968). The modern distribution of *C. maritimum* has its northern limit at ca.  $54^{\circ}\text{N}$  which corresponds to the  $16^{\circ}\text{C}$  July isotherm. Recently it has been recorded from the island of Helgoland (Germany:  $54^{\circ}10'\text{N}$ ,  $7^{\circ}53'\text{E}$ ) as a newly introduced species (Kremer and Wagner, 2001). No taxonomical subdivision of the species has been proposed. In this study 78 individuals from 64 populations were included in the AFLP analysis (Figure 2.1).

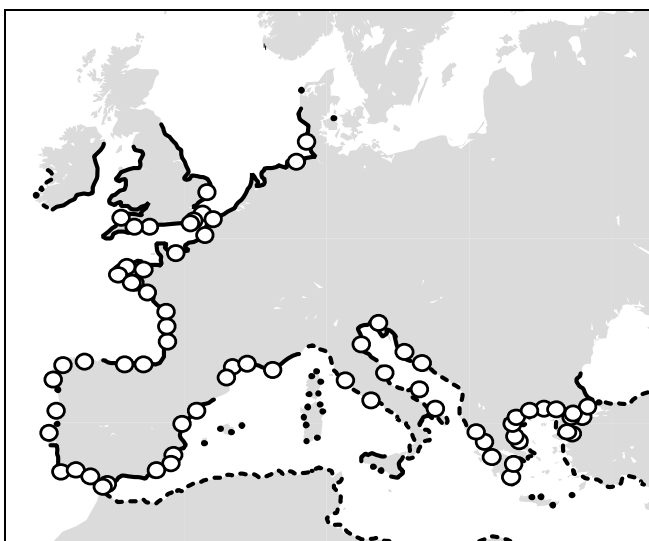


**Figure 2.1 (Left)** Geographical distribution of *Crithmum maritimum* in Europe (Meusel *et al.* 1965) and sampled localities (open circles). **(Right)** (a) *Crithmum maritimum* intact branch, (b) bract, (c) bracteole, (d) flower, (e) + (f) petal, (g) fruit, (h) two different views of the mericarp (i) cross section of the mericarp. (Castroviejo, 1990).

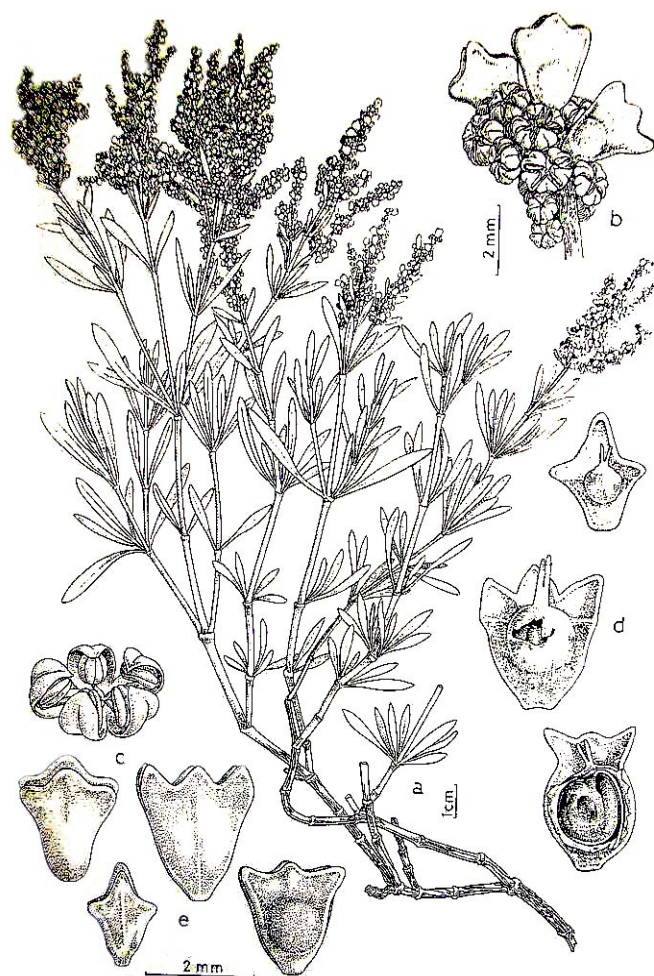


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**2.1.2 *Halimione portulacoides* (L.) Aellen**, partly included in *Atriplex* (Kühn, 1993), (Chenopodiaceae),  $2n = 18, 36$ ; (Castroviejo, 1990). It is a woody perennial, creeping subshrub, between 30 - 80cm height. It occurs in aerated spots in salt marshes and estuaries along the European Mediterranean and Atlantic coasts (Figure 2.2). Its grayish-green leaves (3 - 7cm) make it easily recognizable. The plant has either perfect or unisexual flowers which flower between July and September (Chapman, 1950). Flowers are presumably wind-pollinated and the compatibility system is not investigated. Fruits of *H. portulacoides* are ca. 4.0mm long (Figure 2.3) and have a papery pericarp enclosing the one seed, and the fruits are enclosed by the bracteoles which contribute to their floating ability. The buoyancy of fruits in sea water lasts over a month, and 20% of seeds retain their viability after immersion in sea water (Koutstaal *et al.* 1987). The northern distribution limit of *H. portulacoides* today lies around 55°N, which corresponds to the July 16°C isotherm (Chapman, 1950). In attempts to subdivide the species taxonomically, Chapman (1950) has mentioned three varieties; *latifolia* Gussone, *parvifolia* Rouy and *andangustifolia* Gussone, but this subdivision is limited only to the British Isles and depended on few morphological characters (leaf morphology and flowers abundance) and plant location in the salt marsh. Another subdivision was proposed by Castroviejo *et al.* (1990); they described two varieties in Spain; *laevis* Aellen and *appendiculata* Aellen which differ in bracteole morphology but co-occur in the Spanish coastal salt marshes. Of 71 European Atlantic and Mediterranean populations, 86 accessions were included in the AFLP analysis (Figure 2.2).



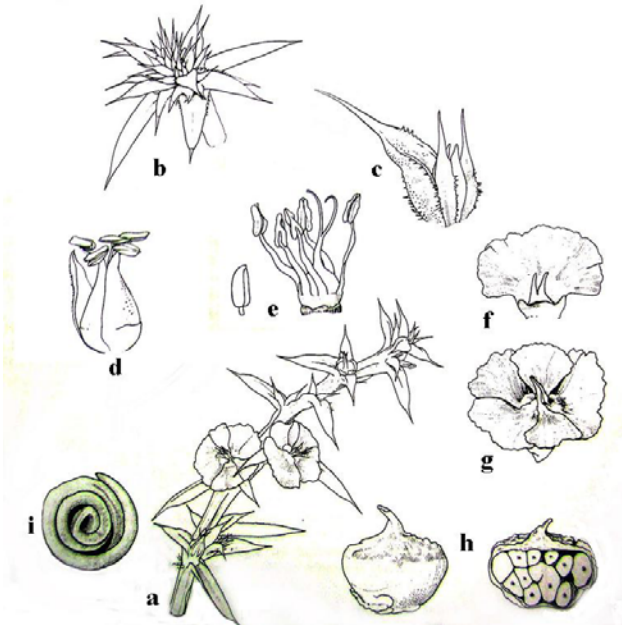
**Figure 2.2** Geographical distribution of *Halimione portulacoides* in Europe (bold coastline; Meusel *et al.* 1965) and sampled populations (open circles).



**Figure 2.3** *Halimione portulacoides* (a) intact plant, (b) flowering cluster, (c) male flower, (d) different perspectives of female flower, (e) bracteoles. (Castroviejo, 1990).

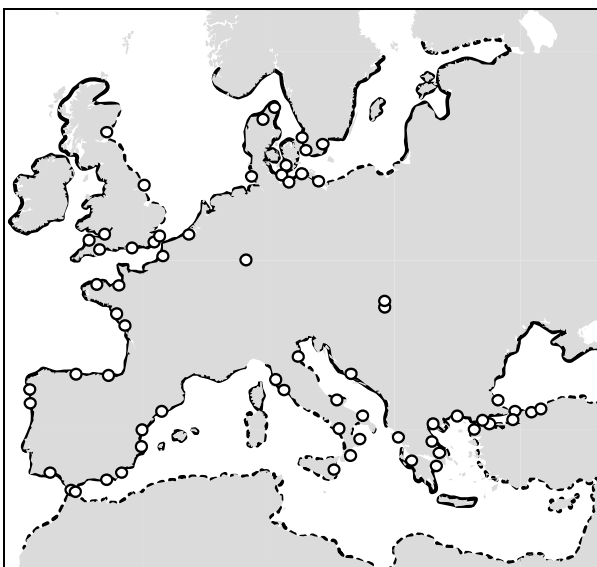
**2.1.3 *Salsola kali* L.** (Chenopodiaceae),  $2n=36$  (Castroviejo *et al.* 1990), is an annual, herbaceous, between 15 - 50cm high shoreline plant. It occurs in various forms of sandy substrates along the coast, and can be found in inland sandy-soil habitats and/or deserts and waste places (Davis *et al.* 1967). Being close to the driftline, it profits from increased nitrogen availability associated with seaweed and macroalgal litter (Pakeman and Lee, 1991a,b). *Salsola kali* can be easily recognized by its spiny, succulent green leaves and bracts. The flower is hermaphrodite (Figure 2.4), presumably wind-pollinated and the plant flowers between May and July. The fruit is a nut, with a diameter of 3 - 5mm, which in the same individual has two different morphotypes (Figure 2.4, 2.6e; personal observation); (i) a winged morphotype which detaches from the mother plant and is adapted to wind dispersal and able to float in sea water. Fruits observed to float in sea water between 5 days and 4 weeks (Ridley, 1930). (ii) an unwinged morphotype that remains attached to the mother plant which either is covered by sand or dispersed as a tumble weed. Dry branches with spiny-leaves may adhere to animals or birds probably in adaptation to epizoochorous dispersal (field observations).

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**Figure 2.4** *Salsola kali* (a) branch, (b) terminal glomerule, (c) bud with bract and bracteoles, (d) flower with and without tepals, (e) anthers, (f) tepal at fruiting stage, (g) perianth at fruiting stage, (h) entire and longitudinal section of the fruit, (i) seed (embryo). (Wilson, 1984).

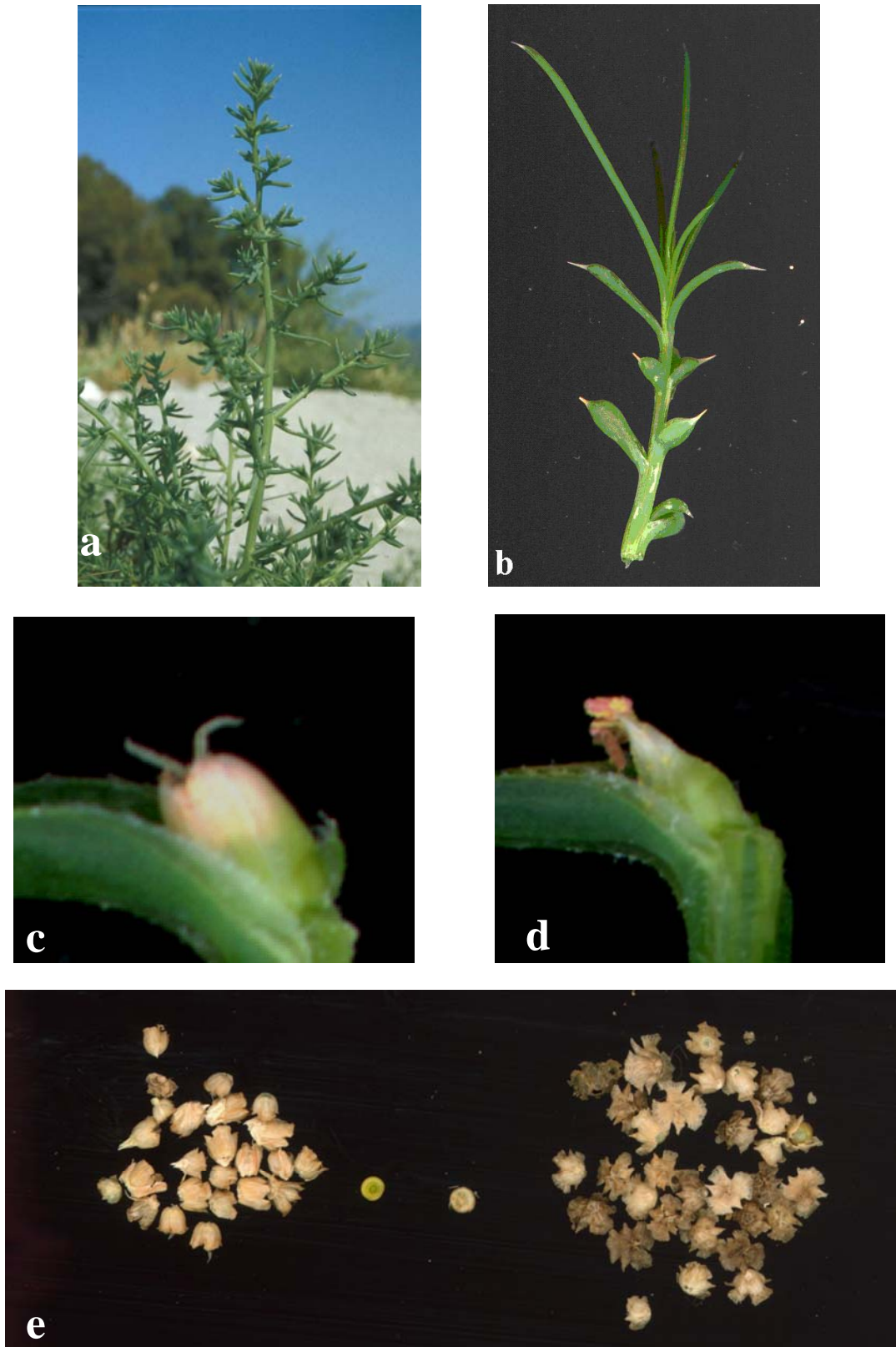
*Salsola kali* shows pronounced morphological variation caused considerable taxonomical difficulties (Rilke and Reimann, 1996; Aellen, 1968; Figure 2.6.b). It has not been determined yet to what extent this variability represents genetic, developmental, or environmental differences. Three subspecies of *Salsola kali* are usually recognized: subsp. *kali* L., *tragus* (L.) Celakovsky, and *ruthenica* (Iljin) Soo (Davis *et al.* 1967). The thick-leaved subsp. *kali* and subsp. *tragus* occur in more or less saline, littoral habitats throughout Europe (subsp. *kali*), and along the Mediterranean Sea (subsp. *tragus*). Subsp. *ruthenica* (= subsp. *iberica*) with slender leaves is known to occur in inland sandy-soil habitats (Rilke and Reimann, 1996). The modern distribution of *S. kali* reaches around 60°N, which corresponds to the July 14°C isotherm. Seventy-nine *S. kali* accessions of 57 populations were included in the AFLP analysis (Figure 2.5).



**Figure 2.5** Geographical distribution of *Salsola kali* in Europe (bold coastline; Meusel *et al.* 1965) and sampled populations (open circles).

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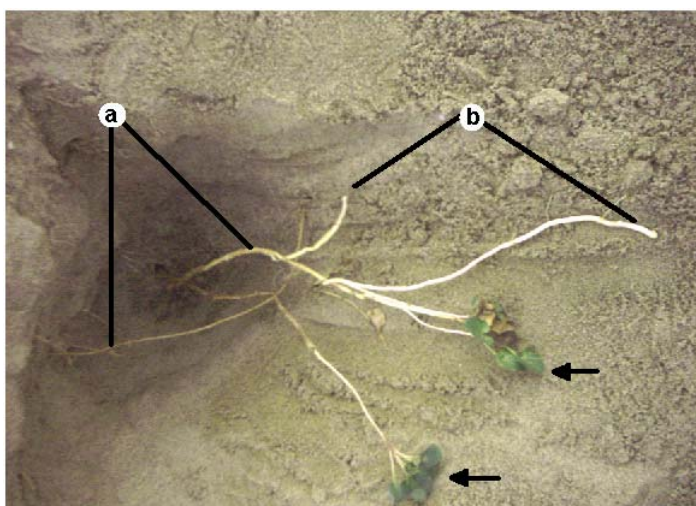


**Figure 2.6** *Salsola kali* (a) intact plant, (b) morphological plasticity of *S. kali*. The plant has been transferred from southern Spanish coast to Germany and substrate has been changed. (c-d) flowering phenology of *S. kali* where stigma lobes appear first then anthers, (e) un-winged fruits (left), seeds (middle), and winged fruits (right) collected from the same plant.

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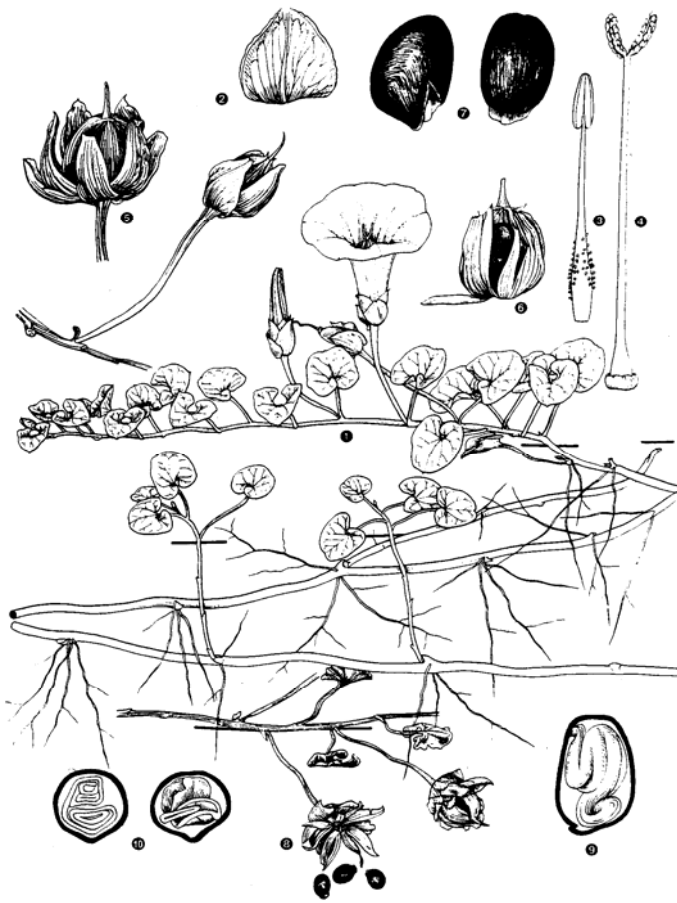
**2.1.4 *Calystegia soldanella* (L.) R. Br., syn. *Convolvulus soldanella* L.,** (Convolvulaceae),  $2n = 22$  (Kitamura *et al.* 1986), is a prostrate herbaceous perennial plant. The above ground shoots are between 10 - 50cm length and the underground rhizomes can reach more than 70cm deep under the sand (Figure 2.7). *Calystegia soldanella* occurs on coastal sand dunes and characterized by dark green, slightly succulent and reniform leaves with whitish veins and contains milky sap (Stefanović *et al.* 2003). The plant flowers between May and July and has up-facing and showy (5 - 7cm) flowers with a rose-pinkish striped corolla. The fruit is a capsule that contains 1 - 4 seeds. The breeding system of *C. soldanella* was investigated by Ushimaru and Kikuzawa (1999). The plant is self-incompatible and depends mainly on insects (bees) as pollen vectors (Ushimaru and Kikuzawa, 1999). Seeds (Figure 2.8) are oval to round in shape ( $\approx$  4 - 6mm diameter) and have a very solid, water-impermeable seed coat. The air space inside makes the seed of *C. soldanella* an excellent floater when it reaches sea water. Seeds are able to float in sea water for long periods of time. Ridley (1930) documented that around 30% germination of *C. soldanella* seeds was achieved after 18 months of floating in sea water and 40 - 50% after 6 months.

*Calystegia soldanella* has a remarkably disjunct global distribution. It has a northern distributional limit in Europe at ca. 54°N, corresponding closely with the 16°C July isotherm. The plant occurs also on the coasts of Japan and the Korean peninsula, south Australia and New Zealand, west North America and South America (Meusel *et al.* 1965; Kitamura *et al.* 1984).

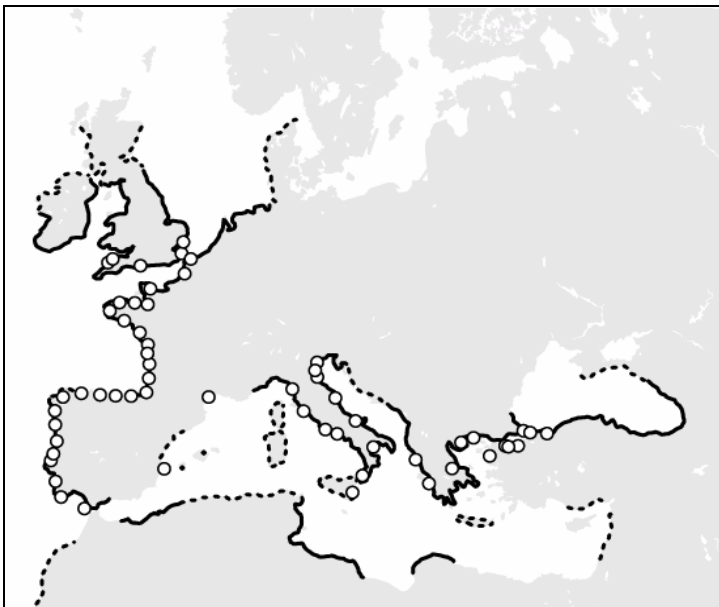


**Figure 2.7** *Calystegia soldanella* (a) underground rhizomes, (b) new shoot tips. Arrows are pointed at the fully developed vegetative parts (arrows). Photo by R. Arafah.

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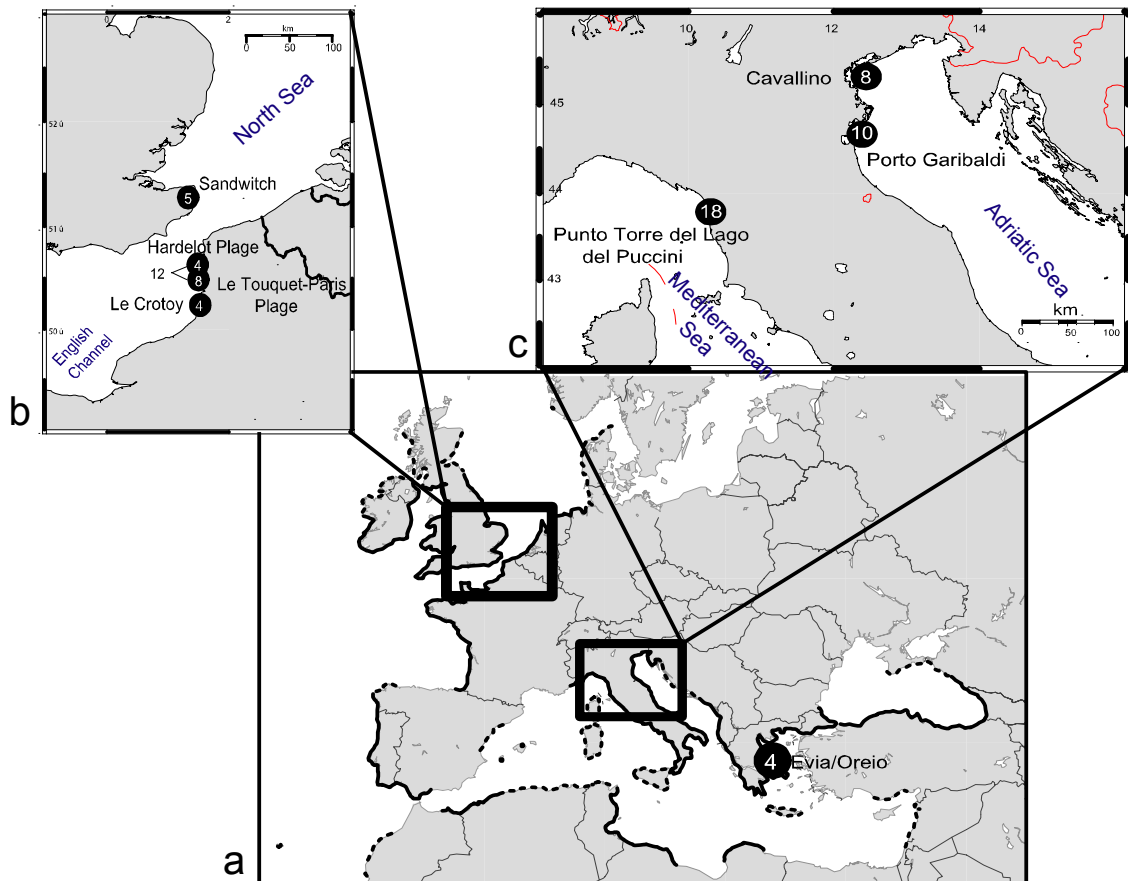
**Figure 2.8** *Calystegia soldanella* (1) whole plant with flowers X 4/5, (2) bracts, (3) stamen, (4) pistil, (5) fruits with persistent calyx, (6) dehiscent fruit, (7) seeds, (8) dissemination of seeds, (9) vertical section of seed, (10) cross section of seed. (Numata and Asano, 1970).



**Figure 2.9** Geographical distribution (dark coastline; Meusel *et al.* 1965), and sampling (open circles) of *C. soldanella* along the European coasts.

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Two approaches were pursued in *C. soldanella* phylogeography. First AFLP analysis for the entire distributional range was performed, including 74 accessions of 53 populations (Figure 2.9). Second, a population level approach including 61 individuals of seven populations was made (Figure 2.10).



**Figure 2.10** Names and number of individuals from *C. soldanella* localities that included in the population level survey. (a) eastern Mediterranean locality Evia - Greece (black circle), (b) Atlantic Ocean/English Channel populations, (c) Adriatic Sea and west Mediterranean populations.

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### 2.2 Sampling of plant material:

Sampling of the plant material took place along the European coastline from the southwestern Black Sea to the western part of the Baltic Sea. Most of the sampled material was collected in summer 2001. Sampling locations were according to availability and accessibility of the plants in roughly 100km intervals between localities. Names, geographical coordinates of localities and collectors of each sample are listed in Appendix I. Since the four species have succulent leaves, the collected material was torn into small pieces and preserved in silica gel beads with moisture indicator (Sigma/Aldrich, Seelze, Germany) to accelerate drying. All samples were kept at room temperature.

### 2.3 Extraction and purification of DNA:

The same DNA extraction and purification procedure was carried out in all species. Approximately 100mg of dry leaf material was ground using mortar and pestle with autoclaved sand (ROTH, GmbH; Karlsruhe, Germany). Total genomic DNA extraction and purification was performed using the DNeasy™ plant Minikit (Qiagen; Hilden, Germany) following the manufacturer's instructions. The only modification was done in *C. soldanella* where more buffer AP1 (600µl) and AP2 (195µl) were used. Quantification of DNA was carried out spectrophotometrically with a GeneQuant RNA/DNA calculator (Pharmacia; Uppsala, Sweden) or estimated visually by ethidiumbromide stained agarose gels (1.4%). Crude DNA was diluted to 30ng/µl with molecular grade H<sub>2</sub>O from ROTH. H<sub>2</sub>O was supplemented with diethylpyrocarbonate (DEPC) to preserve the DNA quality. After dilution, DNA was stored at -20°C.

### 2.4 Amplified fragment length polymorphism (AFLP) fingerprinting:

AFLP is a DNA fingerprinting technique that detects genomic restriction fragments and resembles in that respect the RFLP (restriction fragment length polymorphism) technique, with the major difference that PCR amplification instead of southern hybridization is used for the detection of restriction fragments (Vos *et al.* 1995). The resulting data are treated as dominant markers as for RAPDs and ISSRs.

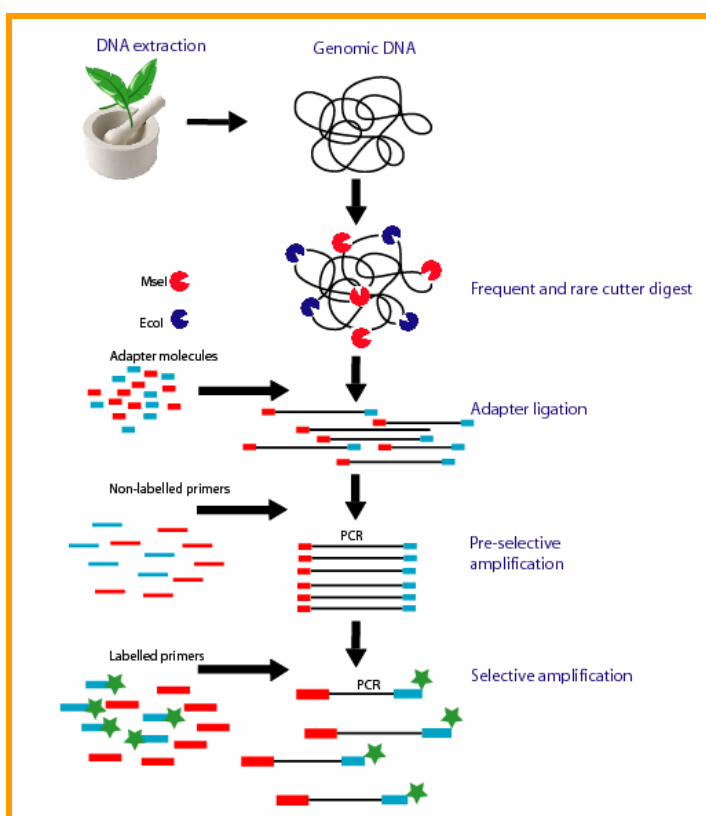
The AFLP technique of Vos *et al.* (1995) is now widely implemented due to several advantages including: (i) no prior knowledge of the genomic sequence is required, (ii) the whole organismal genome (chloroplast, mitochondrial, and nuclear DNA) is involved in the reaction, (iii) it produces a high amount of polymorphic markers in a single reaction (20 - >100 fragments/primer combination) compared to other fingerprinting methods such as RAPDs and ISSRs (usually less than 10 fragments/primer), (iv) small amounts of DNA as



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starting material are needed, (v) it allows distinguishing closely related species or subspecies that may be difficult or impossible to differentiate on the basis of morphological or phenotypic characteristics, (vi) stringent PCR conditions and the primers designed to anneal to specific adapters give high reproducibility (Hansen and Kraft, 1999). On the other hand, AFLPs have some drawbacks including: (i) sensitivity to DNA quality; bad or insufficient DNA quality lead to reaction failure or low reproducibility, (ii) dominant behavior; fragments are scored (1) when present or (0) when absent, and it is not possible in an ordinary AFLP procedure to detect codominant markers (heterozygosity), (iii) compared with other molecular techniques like isozymes, RAPDs and ISSRs, it is laborious, time consuming, and has high a cost.

Technically, AFLP involves essentially three consecutive steps illustrated in figure 2.11. The first is based on digesting the genome (total DNA) with two different restriction enzymes (commonly used enzymes are the rare cutter *EcoRI* and the frequent cutter *MseI*). The resulted fragments are ligated to an end-specific adapter molecule. Second, a pre-selective PCR amplification is performed using primers that are complementary to each of the two adaptor sequences, and has one additional base at the 3'-end to provide selectivity. In the third step, the PCR product of the pre-selective amplification is amplified again using selective primers which are extended by two additional bases. The rare cut primer is usually fluorescent or radioactively labeled.



**Figure 2.11** Illustration of AFLP procedure. The genomic DNA is extracted and purified, then simultaneously restricted with rare-cut (*EcoRI*) and frequent-cut (*MseI*) enzymes. Ligation of adapters is performed using ligase. Preselective amplification is done using primers supplemented with one additional base to provide selectivity. The selective amplification is performed using a fluorescent labelled primer at the rare cut end and supplemented with additional two bases to provide further selectivity.

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In this study, the basic AFLP protocol of Vos *et al.* (1995) and the modifications described in Kropf *et al.* (2002) were implemented taking into account the following precautions: (i) for consistency purposes and to reduce manual errors, all samples of each species were run using one master-mix in each AFLP step, (ii) all samples were loaded and run on one acrylamide gel to avoid “multi-gel effect” or artefactual characters because of background noise and/or other lab errors between two independent gels that may influence the analysis, (iii) all samples have to be studied in one analysis. The full details about AFLP reaction steps, ingredients, primers sequences, and thermocycler settings are listed in Appendix II.

### 2.4.1 AFLP gel processing, evaluation and generating the binary data matrix:

Selective amplification products were separated on 6% polyacrylamide gels with an internal size standard (GeneScan 500 Rox, ABI) on an ABI 377 automated sequencer.

In order to generate electrophoretograms from the acrylamide gel, the lane of every individual was extracted and adjusted separately with the size standard using GENESCAN Software v.3.1 (ABI) for Apple Macintosh.

After generating the electrophoretograms, peaks (characters) were scored automatically with GENOTYPER software v.2.1 (ABI) to assemble a binary present (1), or absent (0) data matrix. The following GENOTYPER options were set: CALCULATE SCALE FACTOR (50–500 bp); FILTER LABELS (0.0–0.9). The generated data matrix was carefully checked in order to correct erroneous scoring. Scoring was done within the range from 75-500 bp. Only reliable and clear peaks were scored and in some cases ambiguous peaks were scored as missing data “?”. Peak intensity was not considered as indication of codominancy, thus peaks were treated as dominant markers.

### 2.4.2 AFLP data analysis:

Pairwise genetic distances ( $D$ ) for all accessions were calculated from the 0/1 matrix using the complementary value of Nei and Li's (1979) similarity coefficient implemented in PAUP v4.010 for Macintosh PPC (Swofford, 2002):

$$D = 1 - SC = 1 - [2n_{xy} / (n_x + n_y)]$$

where SC is the similarity coefficient,  $n_{xy}$  is the number of identical fragments shared between two accessions, and  $n_x$  and  $n_y$  are the total number of fragments in accessions  $x$  and  $y$ , respectively. A Neighbor-joining (NJ) phenogram (Saitou and Nei, 1987) was then calculated from the Nei and Li's (1979) pairwise genetic distance using PAUP. All trees presented in this study were unrooted.

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Furthermore, analysis of principal coordinates (PCO) was carried out using a squared Euclidean distances matrix between all pairs of individuals and projected in two and/or three dimensions using NTSYS-PC software v.2.0 (Rohlf, 2002). The following NTSYS commands were implemented to generate the 2-D and 3-D plots: INTERVAL, choosing Euclidean squared distance; DOUBLE CENTER; EIGENVECTOR, Nr. of dimensions chosen = 8; MINIMUM SPANNING TREE; the GRAPHICS MODEL 3-D and GRAPHICS PLOT 2-D were implemented to generate the graphs using eigenvector and minimum spanning tree output files.

The genetic structure (partition of genetic variation among groups “AMOVA”) and  $F_{ST}$  values (Wright, 1951) were calculated using Arlequin software v.2 (Excoffier *et al.* 1992; Schneider *et al.* 1997). The groups included in the AMOVA analysis were determined according to the previous NJ and PCO clustering pattern.

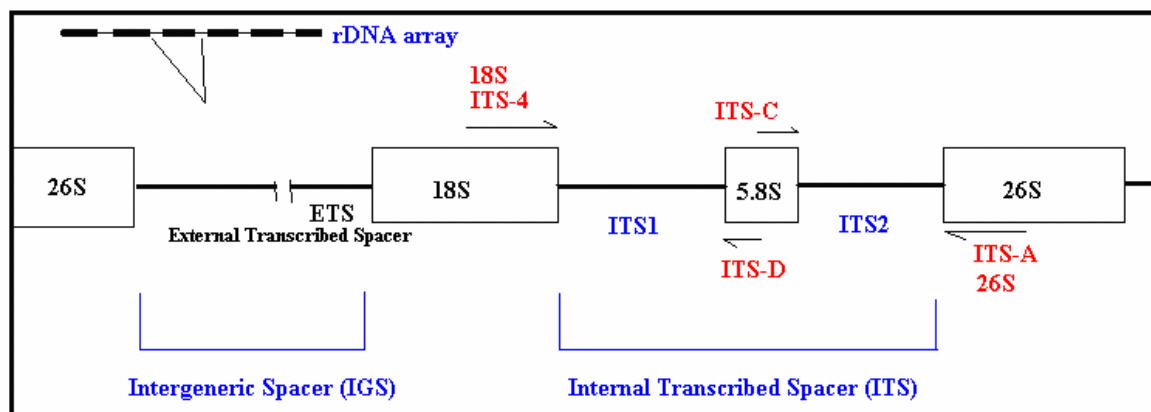
Isolation by distance was evaluated by Mantel’s test (Mantel, 1967) with 9999 random permutations using NTSYS-PC software. This procedure tests whether the matrix of genetic distances of Nei and Li (1979) is correlated with the matrix of geographical distances. Based on the assumption that the four species migrate only along the coast, geographical distance was measured along the coastline between localities.

Because of the single-individual strategy employed for most of the material, no population diversity measures could be compared between postulated refugia and recolonized areas. Instead, all individuals belonging to a region of interest were pooled and the Shannon Index (Shannon and Weaver, 1949) was estimated using the formula  $S = -\sum p_i \log_2(p_i)$  where  $p_i$  is the frequency of  $i$ th band.

### **2.5 Amplification of the Internal Transcribed Spacer (ITS):**

For more than a decade, sequencing of ribosomal DNA (rDNA) has become a commonly used procedure and one of the most widely applied molecular markers for reconstructing plant phylogenies at the intrafamily level (intra- and intergeneric, and interspecific levels) (Soltis *et al.* 1998; Baldwin *et al.* 1995). Nuclear ribosomal DNA is classified as a multi-gene family and is arranged in the Nucleolar Organizing Region (NOR) in arrays of hundreds to thousands of copies. Each unit within this array is composed of specified segments (Figure 2.12). The two internal spacers, ITS1 and ITS2 are located between genes encoding 18S (small subunit), 5.8S and 26S (large subunit) nuclear ribosomal RNA. Both spacers ITS1, ITS2 and 5.8S are referred to as the ITS region.

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**Figure 2.12** Structure of a repeat unit within the nuclear rDNA region. Each repeat consists of External Transcribed Spacer (ETS), followed by the small ribosomal subunit 18S, then the first Internal Transcribed Spacer (ITS1), 5.8S, the second Internal Transcribed Spacer (ITS2), then the large subunit 26S. Every coding repeat is separated from the other by the Intergenic spacer IGS. Arrows indicate orientation of the primers. Primer codes are from Blattner (1999); White *et al.* (1990); Muir and Schlötterer (1999).

Some criteria make the ITS region (18S-5.8S-26S) a powerful marker in inferring phylogenetic relationships including: (i) simple to amplify: the region can be easily amplified from low amounts of DNA because it occurs in arrays of hundreds to thousands of copies (Soltis *et al.* 1998); (ii) universality: the ITS region is bordered by the conserved coding regions 18S and 26S, and thus it has been possible to design universal primers for ITS amplification in plants and fungi (White *et al.* 1990). Furthermore, it is also possible to split the ITS region into two smaller regions benefiting from the conserved 5.8S part, and amplifying ITS1 and ITS2 separately (Blattner, 1999). This alternative is commonly done when encountering low quality DNA, like DNA from old herbarium specimens, (iii) uniformity at the intragenomic level: it is well documented in literature that multi-copy genes (particularly rDNA) tend to have uniform sequences because they are subjected to a phenomenon named 'concerted evolution'. Arising from mechanisms such as unequal crossing over and high-frequency gene conversion, the classical concept of concerted evolution is one whereby inter-repeat sequence variation within an organism is reduced to a negligible level or completely removed, so that the multigene family contains only one unique sequence (Alvarez and Wendel, 2003). Principally, concerted evolution eliminates paralogous sequences, enabling inference of true homology among taxa and accurate phylogenetic reconstruction (Alvarez and Wendel, 2003); (iv) biparental inheritance mode: since it is a part of the nuclear genome, it is biparentally inherited. This make ITS a valuable tool in inferring reticulation, hybrid speciation, and parentage of polyploids (Alvarez and Wendel, 2003).

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However, in certain cases, polymorphism at the intragenomic level in rDNA may occur. The number of publications discussing polymorphic rDNA is increasing recently (e.g. Campbell, *et al.* 1997; Wissemann, 2003; Bailey *et al.* 2003; Alvarez and Wendel, 2003; Buckler IV *et al.* 1997; Aguilar *et al.* 1999; Feliner *et al.* 2004; Hughes *et al.* 2002). In summary, polymorphism in the rDNA at the intraspecific level and disruption of concerted evolution can be attributed to one or more of the following factors: (i) transition stages of concerted evolution, (ii) when mutation rate exceeds the rate of concerted evolution, as in length variants in the intergeneric spacer (IGS), (iii) as a result of interspecific hybridization, (iv) when pseudogenes evolve (silent, non-functional DNA sequence), (v) when rDNA loci is located in nonhomologous chromosomes, accordingly, no crossing over will take place within the loci, (vi) due to polyploidization: allopolyploids would be expected to show more polymorphism than diploids either because of genetic heterogeneity following hybridization or because of a lack in concerted evolution between NORs resulting in divergence over time, (vii) due to agamospermy (asexual formation of embryos and seeds), particularly when the agamospermous taxa are the product of parents that are characterized by having divergent ITS sequences.

For every species in this study, a preliminary survey was carried out involving accessions from different geographical regions to test if any variation in the ITS sequence exist. If any variation was found, the sample size was increased in order to obtain sufficient phylogeographical patterns.

The internal transcribed spacer ITS region (ITS1-5.8S-ITS2; Figure 2.12) was amplified using the primers listed in Table 2.1. The primers 18S and 26S (Muir and Schlötterer, 1999) were first used to amplify the ITS region. In case of unsuccessful amplification (in low quality or degraded DNA), the ITS was split into two smaller segments (ITS1 + part of 5.8S, and part of the 5.8S + ITS2) using primers in the following combination [ITS-A + ITS-C] and [ITS-4 + ITS-D].

**Table 2.1** Primer sequences and references which were used in the ITS amplification.

Primer code	Primer sequence	Reference
ITS-4	5'-TCC TCC GCT TAT TGA TAT GC-3'	White <i>et al.</i> (1990)
ITS-A	5'-GGA AGG AGA AGT CGT AAC AAG G-3'	Blattner (1999)
18S	5'-CCT TNT CAT YTA GAG GAA GGA G-3'	Muir and Schlötterer, (1999)
26S	5'-CCG CTT ATT NAT ATG CTT AAA-3'	Muir and Schlötterer, (1999)
ITS-C	5'-GCA ATT CAC CAA GTA TCG C-3'	Blattner (1999)
ITS-D	5'-CTC TCG GCA ACG GAT ATC TCG-3'	White <i>et al.</i> (1990)

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The reaction was performed in 25.0µl volumes with 10% 10X Biotherm buffer, 5% 50mM MgCl<sub>2</sub> (GeneCraft, Münster, Germany), 2% of 20mM dNTPs mix, 2% from each primer at 25pmol/µl conc., 1.0 unit (0.2µl) BioTherm™ polymerase (GeneCraft), and 1.0µl of template DNA (30-60ng/µl). The rest volume was filled up to 25.0µl with DEPC-H<sub>2</sub>O. The polymerase chain reaction (PCR) mixture was preheated for 1 min. at 94°C, then subjected to 35 cycles of 94°C for 0.3 min., 55°C for 0.5 min, and 72°C for 1 min. and two final incubations of 1.3 min. at 55°C plus 8 min. at 72°C. Amplification was checked by ethidiumbromide staining using 0.7% agarose in 1xTBE buffer and documented with a Polaroid camera.

### 2.5.1 Purification of PCR product:

The PCR amplified products were purified using the QIAquick PCR Purification Kit from Qiagen (Hilden, Germany) and following the manufacturer's instructions. To check product recovery after purification, 5.0µl of the purified product was loaded on 1.4% agarose gel.

### 2.5.2 Sequencing reaction:

The sequencing reaction was performed using 50-100ng of the purified PCR product and ABI-PRISM® BigDye™ Terminators v3.0 Cycle Sequencing Kit following the manufacturer's instructions. Only one of the primers which had been used in the amplification step was used in the sequencing reaction. In case of incomplete and unclear sequence, the reaction was repeated using the second primer. The thermocycler profile was set to 30 cycles of 96°C for 10 sec, then 4 min. at 55°C. Sequences were detected on automated sequencers (ABI 373 or 377).

### 2.5.3 Cloning of PCR product:

In cases where heterogeneous ITS sequences were observed (more than one ITS copy), cloning of the ITS-PCR product was carried out using the p-GEM®-T Vector cloning Kit (Promega; Mannheim, Germany) following the manufacturer's instructions. Positive colonies were picked with a sterile pipette-tip and immersed in PCR tubes each filled with 10.0 µl sterile H<sub>2</sub>O. The PCR tubes were then filled with PCR reaction mixture to 25.0µl. Afterwards the same purification and sequencing reactions as mentioned above were performed.

In *Salsolea kali*, seven individuals from different regions (Table 2.2) were cloned and sequenced.

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**Table 2.2** Geographical origins, names of localities, and number of sequenced ITS-clones in *S. kali* study.

Geographical origin	Names of accession	No. of sequences
Kattegat Sea (N.E. Denmark)	Lyngsa	8
Baltic Sea (N. Germany)	Zinnowitz Strand	9
Baltic Sea (S. Sweden)	Nyehusen	8
Atlantic- Brittany (N. France)	St. Benoit des Ondes	7
Atlantic- N. African coasts (Morocco)	Sale	5
Mediterranean Sea (E. Spain)	Plaja de Olivia	9
Mediterranean Sea (S. Greece)	Lehena	6

### 2.5.4 Sequence editing and alignment:

Sequence data of the ITS region (ITS1-5.8S-ITS2) were manually edited and automatically aligned using SEQUENCHER v.4.0 software package for Macintosh PPC. Boundaries of the ITS sequence were determined by comparison with representatives of Compositae subtribe Madiinae (Baldwin, 1992). Gaps of single nucleotides were treated as single-site or new state. For gaps longer than one nucleotide, the first site in the gap was recoded as a new state and all other sites were coded as 'missing data'. The outgroup taxa in the *Salsola kali* data set were chosen according to a previous phylogenetic study (Pyankov *et al.* 2001) and personal communications from Dr. G. Kadereit (University of Mainz) and Prof. H. Freitag (University of Kassel). The outgroup taxa included *Salsola tragus* L., *S. paulsinii* Litv., *S. collina* Pallas, *S. ikonnikovii* Iljin, and *S. jaquemonte* Moq. Outgroup taxa in the *Halimione portulacoides* ITS data set were the two closely related species *H. verrucifera* (M. Bieb.) Aellen and *H. pedunculata* (L.) Aellen. Edited and aligned sequences were exported as a NEXUS standard file for further analysis.

Complete ITS sequences of the main ribotype and outgroup taxa are listed in Appendix III.

### 2.5.5 Maximum Parsimony (MP) analysis:

Unweighted maximum parsimony (MP) analysis was carried out using PAUP with the following settings: ACCTRAN, full heuristic search, Fitch algorithm, steepest descent=off; MULTREES=on; collapse option in effect=on; addition sequences=random (10 replicates); tree bisection reconnection (TBR) swapping. Robustness of branches was estimated by bootstrap analysis (Felsenstein, 1985) using 100-1000 heuristic replicates depending upon the data set and using the same settings as mentioned above. The parsimony analysis was based on the informative characters only.

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### 2.5.6 Median Joining (MJ) network:

Intraspecific phylogenies for ITS data were also inferred using median-joining (MJ) network analysis (Bandelt *et al.* 1999). MJ networks include all most parsimonious trees supported by the data, and are particularly appropriate for the low resolution encountered in intraspecific data sets (Pitra *et al.* 2000; Posada and Krandall, 2001). The analysis was performed with the software NETWORK version 4.0.0.1, <http://www.fluxus-engineering.com> (Forster *et al.* 2000) assigning equal weights to all variable sites and with default values for the epsilon parameter (epsilon = 0).



## Results

### 3. RESULTS

#### 3.1. *Crithmum maritimum*

##### 3.1.1. AFLP analysis:

Seventy-eight individuals from 64 populations were successfully evaluated and included in the *Crithmum maritimum* AFLP analysis. Markers from two primer combinations (NED, E40-M57; HEX, E45-M57) could be scored within a size range of 70-480 bp. The total number of markers scored for the two primers was 114 (NED, 55; HEX, 59) of which 89 (78%) were polymorphic, eight (7%) were uniform across all accessions and 17 (15%) were autapomorphic (character appears in one individual only). Additional details on AFLP characteristics of *C. maritimum* are presented in Table 3.1.

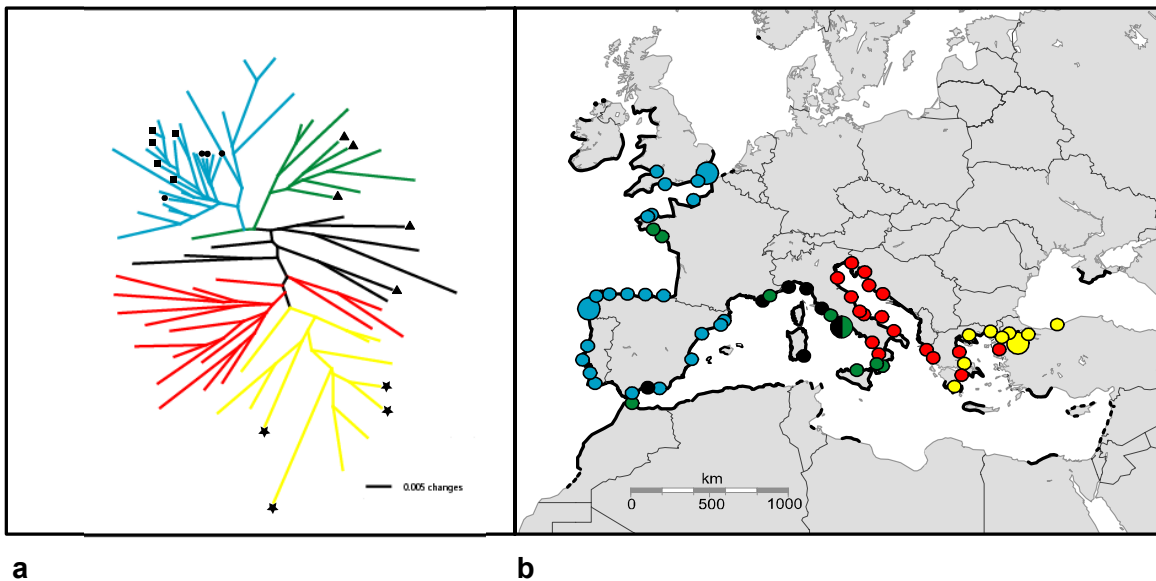
**Table 3.1** Characteristics of AFLP data derived from two primer combinations used on 78 individuals of *Crithmum maritimum*.

	Primer combination		Total	Average
	E40-M57	E45-M57		
No. of characters	55	59	114	57
No. of uniform characters	2	6	8	4
No. of unique characters	12	5	17	8
<b>No. polymorphic characters</b>	<b>41</b>	<b>48</b>	<b>89</b>	<b>45</b>
% of polymorphic characters	74.5	81.3	78	78
Scorable range in bases	70-480	75-472		

The mean value of Nei and Li's (1979) pairwise genetic distances for all AFLP markers between all individuals was  $0.0609 \pm 0.0211$ . The Neighbor Joining (NJ) phenogram based on Nei and Li's (1979) genetic distance is shown in Figure 3.1. The pattern reflects a spatial structure of genetic variation that can be observed along the entire sampling range. Four major clusters (groups of genetically similar plants) could be recognized: (i) individuals from the coasts of the Atlantic Ocean together with those from the whole of east Spain fall together in a separate cluster (named "Atlantic/E Spain" from here on). (ii) all individuals from the Adriatic Sea fall into a separate cluster (Adriatic). It also includes two samples from south Italy, two from west Greece, two from east Greece and one from west Turkey. (iii) in the eastern Mediterranean, individuals from the Black Sea, Marmara Sea and the west Aegean Sea fall into one cluster (Aegean). (iv) the remaining accessions in the west Mediterranean (from south Italy to south France) fall into small clusters intermediate in position between the Atlantic/E Spain and the Aegean and Adriatic clusters (W Mediterranean). When more than one sample from the same locality was included (marked with symbols in the tree), individuals did not fall into an own cluster. However, they all fall within the cluster corresponding to their geographical region. Outliers were two individuals in west France that fell into the W Mediterranean cluster, and

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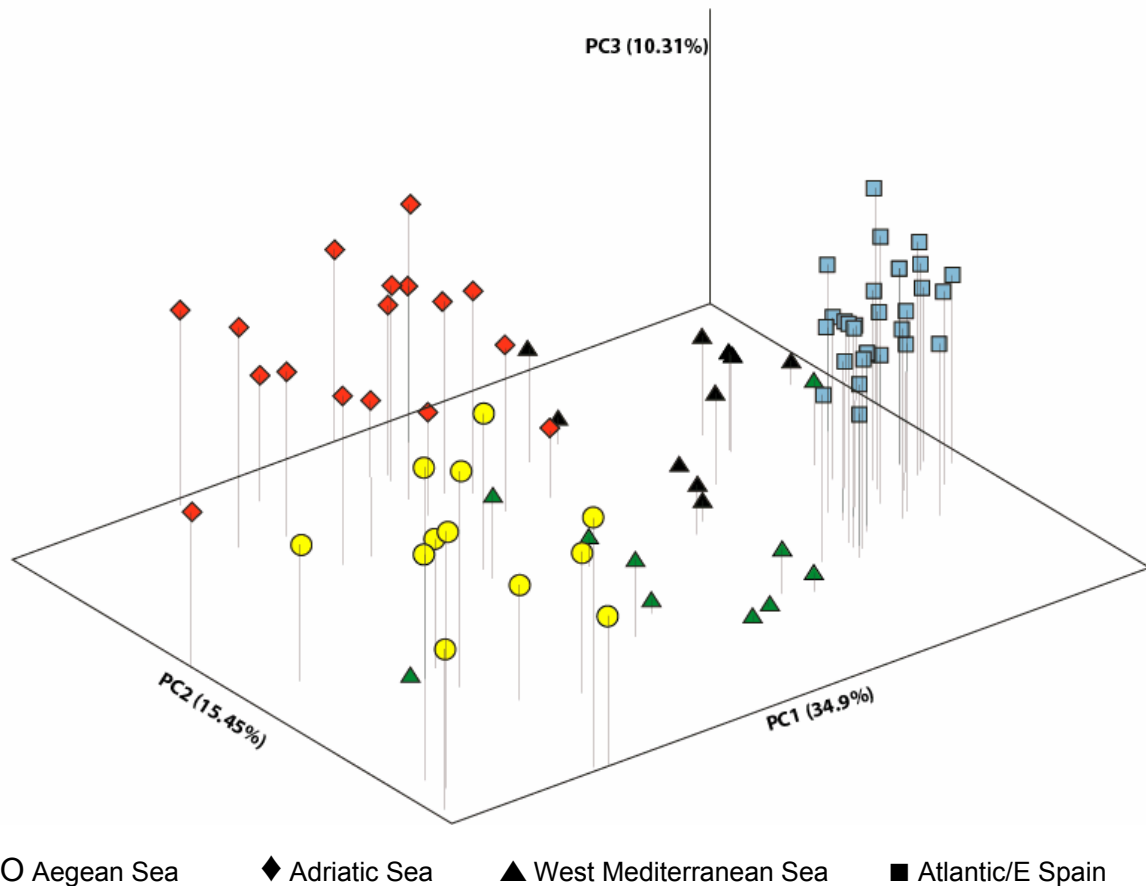
three individuals in the Aegean that clustered together with the Adriatic Sea material. Furthermore, it is also observed in the NJ tree that the majority of the accessions in the Atlantic/E Spain cluster exhibit shorter internal and terminal branches than other clusters in the tree (Figure 3.1). Another feature of the Atlantic/ E Spain cluster worth noticing is the absence of sub-structure in the genetic variation across this cluster's distributional range ( $\approx 5000$  km from northeast Spain to the English Channel).



**Figure 3.1 (a)** Unrooted neighbour-joining NJ phenogram based on Nei and Li's (1979) genetic distance of 89 polymorphic characters in 78 individuals of *Crithmum maritimum*. Symbols represent samples from the same locality (large dots in the map): S. England (■); W. Spain (●); W. Italy (▲); N.W. Turkey (★). The same tree with locality names is provided in appendix III. **(b)** Geographical distribution (thick coastline), analyzed accessions (dots) and geographical distribution of NJ clusters (colours) in *Crithmum maritimum*.

The 3-D plot of the principal coordinate analysis (PCO) of the squared Euclidean distances between all 78 polymorphic AFLP characters confirmed the same clustering pattern revealed by the NJ analysis (Figure 3.2). The 3-D plot shows clearly the three major groups, Aegean, Adriatic and Atlantic/E Spain, as well as placing the W Mediterranean accessions in an intermediate position. The first three coordinates accounted for 34.90%, 15.45% and 10.31% of the total variance. The Atlantic/E Spain group and the W Mediterranean accessions were separated from the remaining samples by the first coordinate (34.90%) and the Aegean group was better discriminated from the Adriatic by the second variate (15.45%).

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**Figure 3.2** Principal coordinate analysis PCO based on squared Euclidean distances from 78 individuals of *C. maritimum* and 89 polymorphic characters. Numbers represents the proportion of variation reflected within each axis.

Based on the clustering pattern revealed by the NJ and PCO analyses, partitioning of molecular variance among the four groups (AMOVA and  $F_{ST}$  values) was calculated and is presented in Table 3.2. The nonhierarchical AMOVA indicated a relatively high proportion of total AFLP variation among regions (32.79%). When comparing the groups pairwise with each other, the highest variation (50%) and  $F_{ST}$  value (0.50;  $P < 0.001$ ) among groups was obtained in the comparison between the Atlantic/E Spain and the Aegean. Comparisons involving only Mediterranean groups gave comparatively smaller values (18.01-38.50%) than comparing the Atlantic/E Spain group with any of the Mediterranean ones (25.82-48.03%; Table 3.2).

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**Table 3.2** Analysis of molecular variance (AMOVA) for AFLP phenotypes in *Crithmum maritimum* describes variation among different groups. The groups were identified based on NJ clustering and PCO analysis. df (A,W)= degrees of freedom of among and within groups comparisons.

Comparisons	df (A,W)	Among groups var. (%)	$F_{ST}$	$P$
<b>Non-hierarchical (all groups)</b>	(3,74)	32.79	0.32	<0.001
<b>Aegean vs. Adriatic</b>	(1,27)	24.30	0.24	<0.001
<b>Aegean vs. W Mediterranean (black)</b>	(1,20)	38.50	0.38	<0.001
<b>Aegean vs. W Mediterranean (green)</b>	(1,19)	28.74	0.28	<0.001
<b>Adriatic vs. W Mediterranean (black)</b>	(1,25)	22.06	0.22	<0.001
<b>Adriatic vs. W Mediterranean (green)</b>	(1,24)	27.40	0.27	<0.001
<b>Aegean + Adriatic vs. W Mediterranean (black and green)</b>	(1,44)	18.01	0.18	<0.001
<b>Atlantic/E Spain vs. all Mediterranean</b>	(1,74)	25.82	0.25	<0.001
<b>Atlantic/E Spain vs. Aegean</b>	(1,40)	50.52	0.50	<0.001
<b>Atlantic/E Spain vs. Adriatic</b>	(1,45)	40.60	0.40	<0.001
<b>Atlantic/E Spain vs. (black)</b>	(1,38)	27.42	0.27	<0.001
<b>Atlantic/E Spain vs. (green)</b>	(1,37)	40.99	0.40	<0.001
<b>Atlantic/E Spain vs. W Mediterranean (black and green)</b>	(1,47)	27.43	0.27	<0.001

A highly significant correlation between genetic and geographical distances, as shown by Mantel test, was obtained when all accessions along the entire sampling range were included ( $r_M = 0.537$ ;  $P = 0.001$ ). However, when correlation was tested separately for groups, only the W Mediterranean group (green) shows significant IBD ( $r_M = 0.457$ ;  $P = 0.049$ ), otherwise, the test resulted in nonsignificant correlations (Table 3.3).

**Table 3.3** Mantel test results of correlation between geographical (measured along the coastline) and genetic distances (Nei and Li, 1972) in *Crithmum maritimum* along the distributional range. \* $P = 0.05$  significance.

Group	( $r_M$ ) value	$P$
<b>All accessions included</b>	0.537	0.001
<b>Aegean Sea group</b>	0.278	0.128
<b>Adriatic Sea group</b>	0.200	0.090
<b>West Mediterranean (black + green)</b>	0.130	0.174
<b>West Mediterranean Sea (black)</b>	-0.047	0.432
<b>West Mediterranean Sea (green)</b>	0.457	0.049*
<b>Atlantic Ocean/ E Spain group</b>	0.074	0.206

The Shannon index  $S'$  was distinctly smaller in the Atlantic/E Spain area (0.118) than in the Mediterranean area (0.192).

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### 3.1.2. *Crithmum maritimum* ITS sequence:

The total length of *C. maritimum* ITS is 600 bp (ITS1 = 214, 5.8S = 163, ITS2 = 223 bp). A preliminary survey included four accessions from the Aegean Sea, the west Mediterranean and the Atlantic Ocean. All four accessions had identical ITS sequences. The complete ITS sequence (ITS1+5.8S+ITS2) of *C. maritimum* is presented in Appendix IV.

### 3.2. *Halimione portulacoides*

#### 3.2.1. AFLP analysis:

Eighty-six individuals from 71 populations were successfully evaluated and included in the AFLP analysis. Markers of three primer combinations (FAM, E39-M61; HEX, E45-M54; NED, E40-M57) in the range between 72-449 bp were successfully scored. The total number of characters in all primers was 295 (NED, 106; HEX, 105; FAM, 84). Of these 198 (67%) were polymorphic, 69 (23%) autapomorphic, and 28 (10%) were uniform across all accessions. Details about the AFLP markers in *H. portulacoides* are presented in Table 3.4.

**Table 3.4** Characteristics of AFLP markers derived from three primer combinations used on 86 individuals of *Halimione portulacoides*.

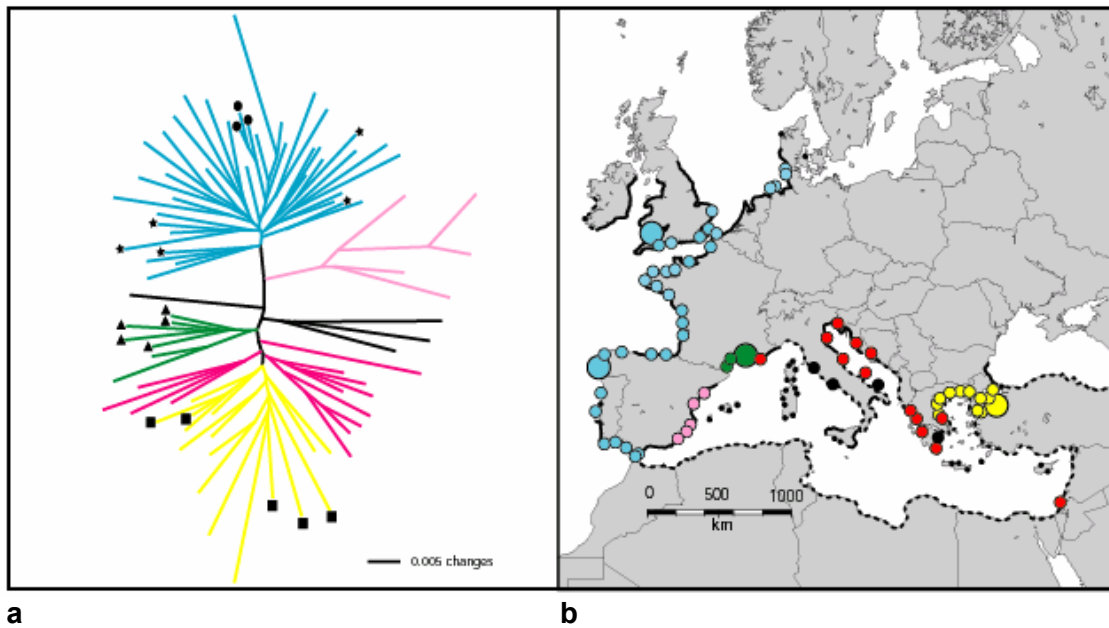
	Primer combination			Total	Average
	E40-M57	E45-M54	E39-M61		
No. of characters	106	105	84	295	98
No. of uniform characters	7	12	9	28	9
No. of unique characters	31	16	22	69	23
<b>No. polymorphic characters</b>	<b>68</b>	<b>77</b>	<b>53</b>	<b>198</b>	<b>66</b>
% of polymorphic characters	64	73	63	67	67
Scorable range in bases	72-428	76-449	75-417		

Mean genetic distance among individuals was  $0.051 \pm 0.0138$ . The NJ tree of the pairwise genetic distances between all individuals is shown in Figure 3.3. The clustering pattern in *H. portulacoides* reflects a clear spatial structure of the genetic variation along the entire sampling range from the Aegean to the North Sea, in addition to a clear Atlantic-Mediterranean subdivision. Five clusters or groups of genetically similar accessions can be recognized in the NJ tree: (i) samples from the Atlantic Ocean from the Strait of Gibraltar to Langeneß in the North Sea fall into one cluster (Atlantic). (ii) a cluster of five accessions from east Spain fall into an intermediate position between the Atlantic cluster and the remaining material from the Mediterranean (E Spain). (iii) seven individuals from south France (five from one population plus two from nearby populations) form one cluster (S France), which is also a neighbour cluster to three individuals (two from west and one from southeast Italy), and one individual from south Greece. (iv) samples from the Adriatic Sea, west Greece, one sample from east Greece and one sample from Israel fall into two

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genetically similar clusters (Adriatic). (v) samples from the north Aegean and Marmara Sea fall into one separate cluster (Aegean).

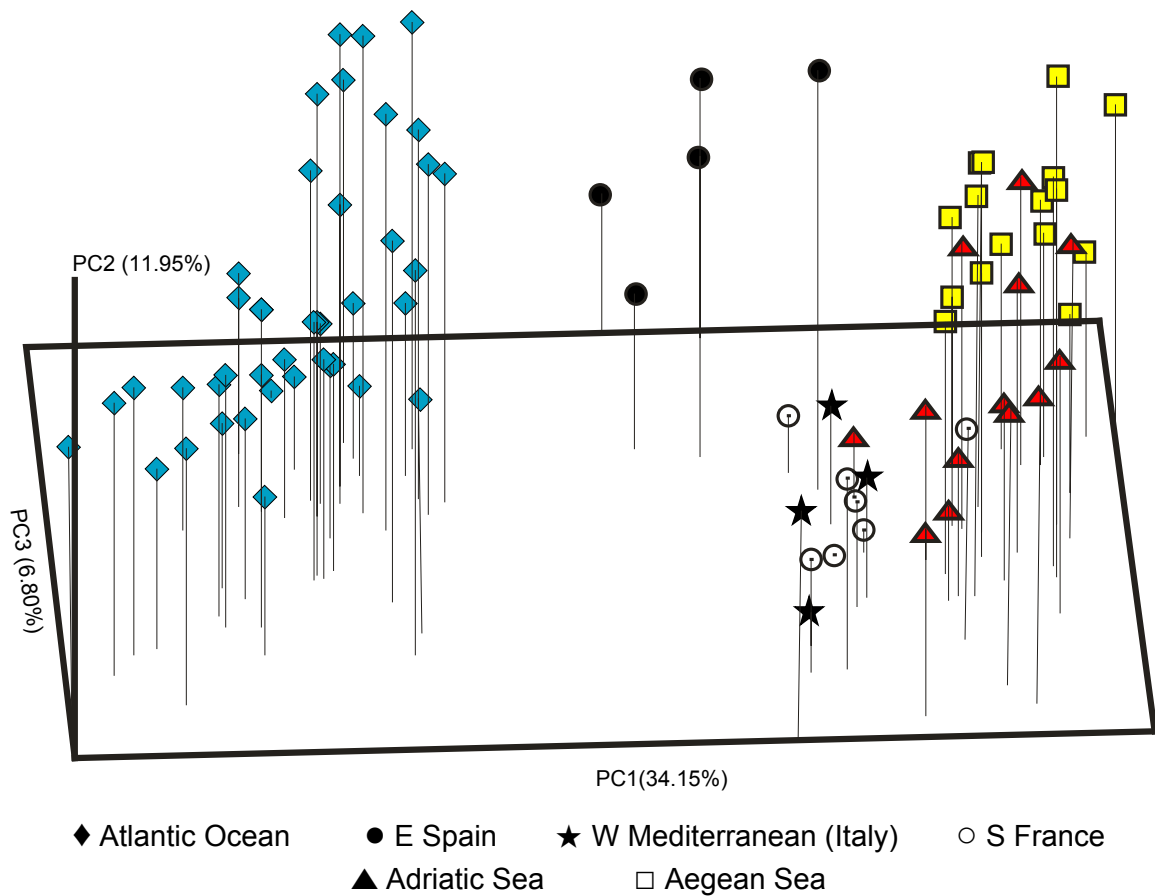
Population samples from two localities, one with three individuals from southwest England and one with five from south France, each form a distinct sub-cluster in the NJ tree. On the other hand, the population samples from the Aegean and Atlantic (west Spain) localities show no distinct clustering but are scattered within their respective geographical groups (see symbols in the tree; Figure 3.3). One sample from south France clustered with the Adriatic Sea individuals appears as an outlier.



**Figure 3.3** (a) Unrooted neighbour-joining NJ phenogram based on Nei and Li's (1979) genetic distance of 198 polymorphic AFLP characters in 86 individuals of *Halimione portulacoides*. Symbols represent samples from the same locality (large dots in the map): N. W. Turkey (■); S. W. England (●); S. France (▲); W. Spain (★). The same tree with locality names is provided in appendix III. (b) Geographical distribution (thick coastline), analyzed accessions (dots) and geographical distribution of NJ clusters (colours) in *H. portulacoides*.

The PCO analysis of the squared Euclidean distances based on the 198 AFLP polymorphic markers confirmed the clustering pattern as revealed by the NJ analysis (Figure 3.4). The first three principal coordinates accounted for 34.15%, 11.95% and 6.80% of the total variation. In the 3-D plot, a clear division between the Mediterranean and the Atlantic material can be observed. Moreover, the east Spain accessions show an intermediate position between the two previously mentioned groups. The Atlantic accessions are discriminated from other material by the first axis (34.15%). Also, it is possible to discriminate the east Mediterranean (i.e. Adriatic and Aegean) from the S France individuals by the first variate (Figure 3.4). No subgrouping can be observed in either the NJ or the PCO analysis within the Atlantic material in spite of the long distributional range (coastline  $\approx$  5000 km).

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**Figure 3.4** Three-dimensional principal coordinate analysis PCO of 198 AFLP markers in 86 individuals of *Halimione portulacoides*. Number on each axis represents the proportion of variation.

In accordance with the NJ and PCO analyses, accessions were divided into six groups to calculate the proportion of among group genetic variation and  $F_{ST}$  values (Table 3.5). The groups are (i) Aegean, (ii) Adriatic, (iii) Italy + S Greece (black in Fig. 3.3), (iv) S France, (v) E Spain and (vi) Atlantic. The among-group variation (nonhierarchical AMOVA) was 29.39% ( $P < 0.001$ ). Comparisons involving the Atlantic group with any of the Mediterranean groups always show a higher percentage of among group variation. On the other hand, when comparing the Mediterranean groups with each other (except E Spain), much lower variation among groups was obtained (Table 3.5). The amount of between group variation was lower when E Spain was compared with the Mediterranean (24.73%) than with the Atlantic group (29.62%).

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**Table 3.5** Analysis of molecular variance (AMOVA) for AFLP phenotypes in *Halimione portulacoides* describes variation among different groups. The groups were identified based on NJ clustering and PCO analysis. df (A,W)= degrees of freedom of among and within groups comparisons.

Comparisons	df (A,W)	Among groups variation (%)	$F_{ST}$	$P$
Non-hierarchical (all groups)	(4,81)	29.39	0.29	<0.001
Aegean vs. Adriatic	(1,26)	10.40	0.10	<0.001
Aegean vs. [Italy +S Greece] (black)	(1,18)	22.62	0.22	<0.001
Aegean vs. E Spain	(1,19)	31.93	0.32	<0.001
Aegean vs. Atlantic	(1,55)	36.17	0.36	<0.001
Adriatic vs. [Italy +S Greece] (black)	(1,14)	12.81	0.12	0.0026
Adriatic vs. E Spain	(1,15)	30.89	0.30	<0.001
Adriatic vs. Atlantic	(1,51)	33.56	0.33	<0.001
S France vs. Aegean	(1,21)	24.54	0.25	<0.001
S France vs. Adriatic	(1,17)	12.59	0.13	<0.001
S France vs. [Italy +S Greece] (black)	(1,9)	17.61	0.18	0.0039
S France vs. E Spain	(1,10)	34.92	0.35	0.0095
S France vs. Atlantic	(1,46)	31.31	0.31	<0.001
E Spain vs. [Italy +S Greece] (black)	(1,15)	30.86	0.31	<0.001
Atlantic vs. [Italy +S Greece] (black)	(1,51)	28.20	0.28	<0.001
E Spain vs. Atlantic	(1,44)	29.62	0.30	<0.001
E Spain vs. Mediterranean	(1,43)	24.73	0.25	<0.001

Correlation between geographical and genetic distances (Table 3.6) yielded significant correlation when all accessions were included ( $r_M = 0.578$ ;  $P = 0.001$ ), and within the Atlantic cluster ( $r_M = 0.149$ ;  $P = 0.008$ ). Otherwise, no significant IBD was obtained in any other group (Table 3.6).

**Table 3.6** Results of Mantel test of correlation between geographical (along the coastal line) and pairwise genetic distances of Nei and Li (1979) in *H. portulacoides*. \*\* $P = 0.005$  significant.

Group	( $r_M$ ) value	$P$
All groups included	0.5778	0.001**
Aegean	0.021	0.420
Adriatic	0.289	0.110
Italy +S Greece (black)	0.935	0.208
S France	0.378	0.170
E Spain	0.208	0.305
Atlantic	0.149	0.008**

The Atlantic region contained marginally lower genetic diversity ( $S_I = 0.117$ ) than the Mediterranean area ( $S_I = 0.125$ ).



## Results

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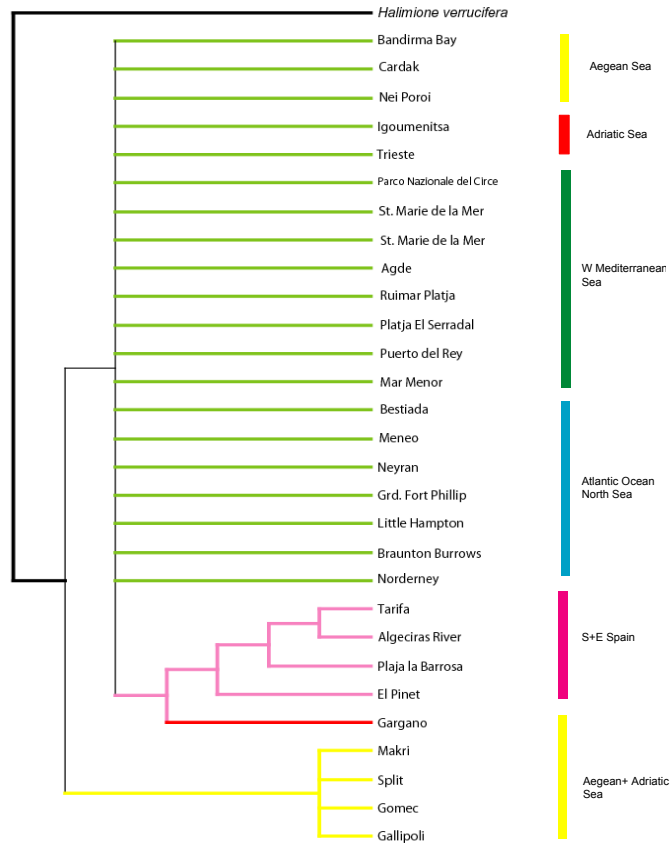
### 3.2.2. *Halimione portulacoides* ITS sequence:

Twenty-nine accessions of *Halimione portulacoides* from the Mediterranean and the Atlantic coasts, in addition to the outgroup taxa *H. verrucifera* and *H. pedunculata* were included in the analysis. A maximum parsimony MP analysis shows that *H. portulacoides* has a closer phylogenetic relationship to *H. verrucifera* than to *H. pedunculata*. The former differs from *H. portulacoides* in 12 substitutions compared to 35 substitutions from *H. pedunculata* (data not shown). Accordingly, *H. verrucifera* was used as an outgroup for further MP analysis. After alignment, the total length of the ITS region (18S+5.8S+26S) was 613 bp (ITS1 = 223 bp, 5.8S = 160 bp, and ITS2 = 230 bp). Within *H. portulacoides* ITS sequences, one indel (one bp insertion) event was seen in one individual from Igoumenitsa (west Greece). Table 3.7 lists the polymorphic nucleotides in all *H. portulacoides* accessions and the complete ITS sequences of *H. portulacoides* and *H. verrucifera* are provided in Appendix IV.

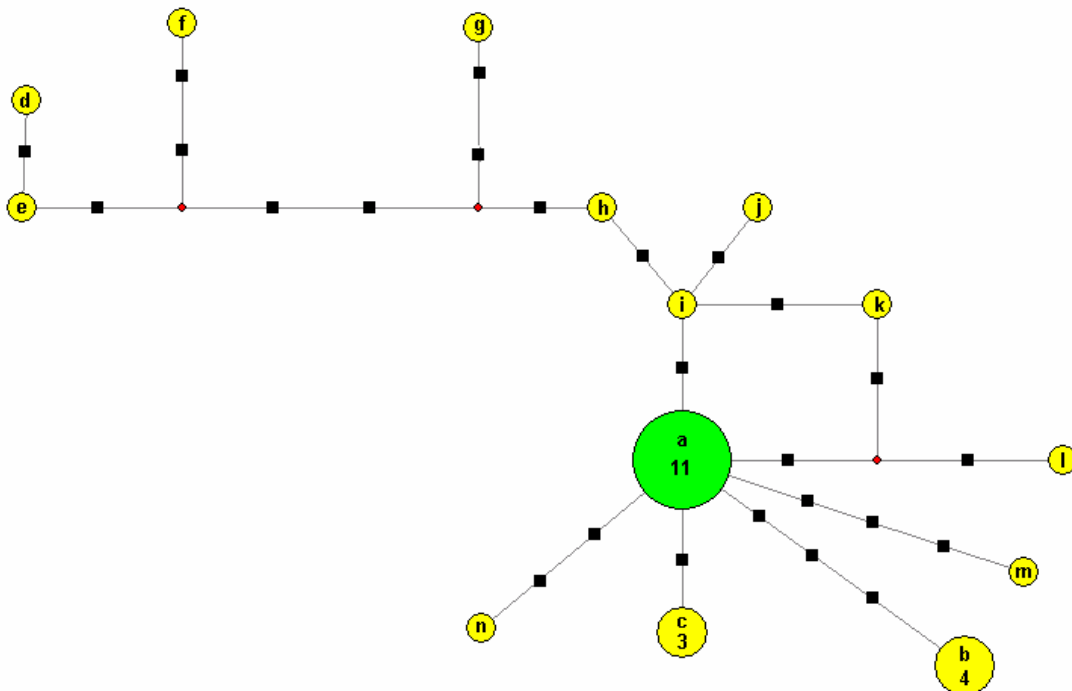
The unweighted MP analysis yielded 5895 maximally parsimonious trees of 36 steps and a consistency index (CI) of 0.889, a retention index (RI) of 0.871 and a homoplasy index (HI) of 0.111. A strict consensus tree was calculated and presented in Figure 3.5 shows that a common and widely-distributed ribotype appeared in accessions from the entire distributional range (Figure 3.7). Beside the major ribotype, accessions form another two sub-clades; one in the eastern Mediterranean (Aegean and Adriatic Sea) and the second mainly in west Mediterranean (south Spain). MP analysis of ITS data could not resolve phylogeographical relationships of *H. portulacoides* in as much detail as AFLPs.

The Median-Joining MJ network (Bandelt *et al.* 1999) is presented in Figure 3.6 and shows more details on mutational steps than the strict consensus tree. The major ribotype (symbol "a") consists of 11 sequences from the entire geographical range (5 Atlantic, 3 south France, 2 east Spain and 1 in the Aegean Sea). The east Mediterranean ribotype, "b", consists of four sequences (three Aegean; Gallipoli, Gömec and Makri, and one from the Adriatic Sea (Split)). Another ribotype, "c", involves three sequences: Puerto del Rey and Mar Menor from east Spain and one individual from west Portugal (Bestiada). Other ribotypes from the Mediterranean show additional character state changes from the common ribotype (see Figure 3.6 and Table 3.7). Three accessions from south Spain (Tarifa "e", Algeciras River "d" and Playa la Borrassa "f") have the most divergent ribotypes in the whole dataset. They differ from the common type "a" in 6 & 7 substitutions, respectively (Figure 3.6; Table 3.7).

## Results

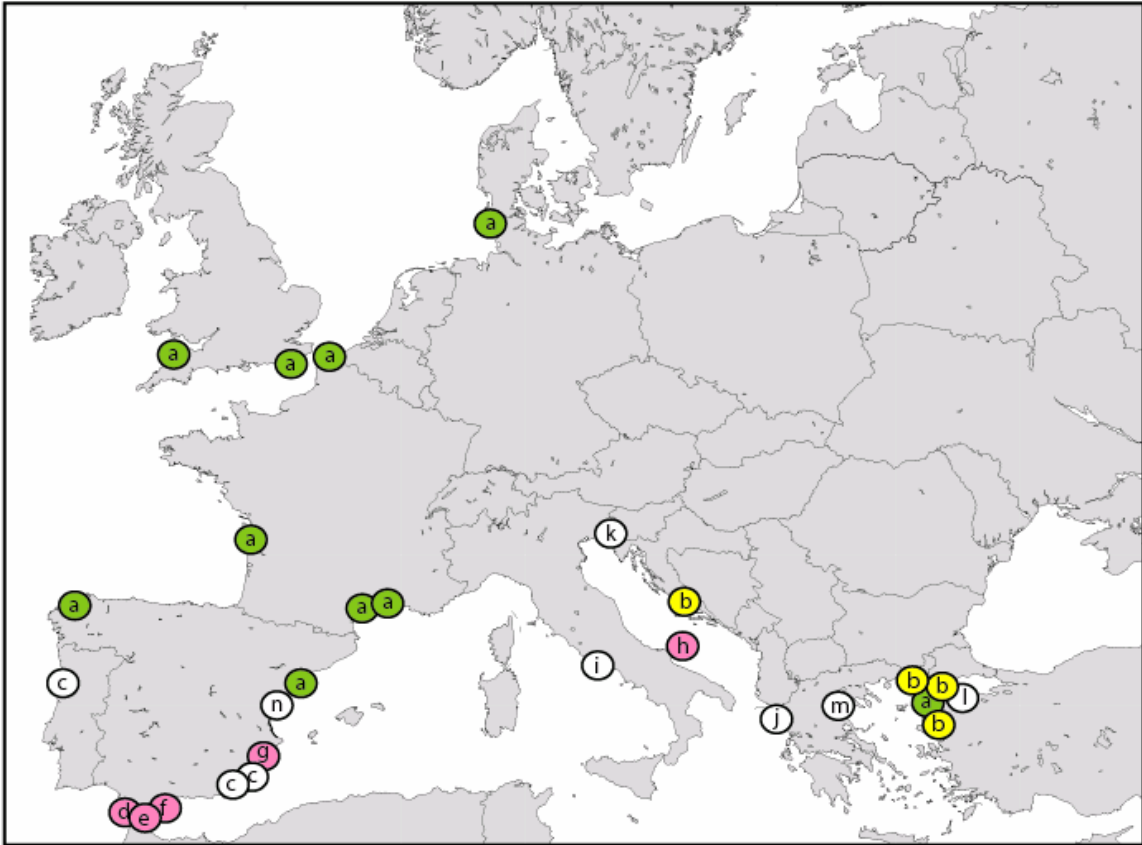


**Figure 3.5 (a)** Strict consensus tree of 29 accessions of *Halimione portulacoides* based on ITS sequence variation and rooted with *H. verrucifera*.



**Figure 3.6** Median-Joining MJ network of ITS sequence mutations depicting the variable nucleotide substitutions among 29 *H. portulacoides* ribotypes: the green circle represents the common ribotype (Aegean to North Sea; Table 3.7). The network was calculated assuming Epsilon = 0. Circle size is proportional to ribotype frequency (number of ribotypes is written in the circle or represents one sequence when absent). Squares between ribotypes indicate mutational steps. All letters refer to individuals in Table 3.7.

## Results



**Figure 3.7** Geographical distribution of *H. portulacoides* ITS ribotypes in the area of study. Letters correspond with table 3.7.

## Results

**Table 3.7** Variable nucleotide sites for ITS region in 29 *H. portulacoides* sequences from the Mediterranean and the Atlantic coasts. Sequence symbols: A, C, G, T = dATP, dCTP, dGTP, dTTP; Y=C/T; M=A/C; R=A/G; K=G/T; S=C/G. Other symbols: i = phylogenetically informative; a = autapomorphic; h = heterogeneous (variable but neither autapomorphic nor phylogenetically informative). MJ symbol = letters correspond with the MJ network.

MJ symbol	Locality	ITS1										ITS2													
		h	i	i	i	i	h	h	i	a	i	a	i	a	i	h	h	v	a	a	i	h	h	i	a
		21	58	73	77	78	118	131	178	182	200	399	40	40	405	409	41	42	51	51	52	54	55	58	60
<b>a</b>	Agde/ S. France	C	C	T	C	C	A	C	C	C	G	C	C	A	-	C	C	A	G	G	T	C	C	T	A
<b>a</b>	Little Hampton/ S. England	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<b>a</b>	St. Mar de la Mer/ S. France	.	.	.	.	.	.	.	.	.	.	.	M	-	.	.	.	.	.	.	.	.	.	.	.
<b>a</b>	Cardak/ Aegean Sea	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	R	.	.	.	.	.	Y	.	.
<b>a</b>	Neyran/ W. France	.	.	.	.	.	.	Y	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>a</b>	Braunton Burrows/ SW. England	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>a</b>	Grd. Fort Philipp/ W. France	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>a</b>	Ruimar Platja/ E. Spain	.	Y	.	.	.	M	.	.	.	K	.	.	-	.	S	.	.	.	.	.	Y	.	.	.
<b>a</b>	Norderney/ North Sea	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>a</b>	Meneo/ N. Spain	.	.	.	.	.	.	.	.	.	.	.	.	M	-	.	.	.	.	.	Y	.	.	Y	.
<b>a</b>	St. Mare del Mer (2)/ S. France	.	.	.	Y	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>b</b>	Gallipoli/ Aegean Sea	.	.	C	.	T	.	.	.	.	T	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>b</b>	Makri/ Aegean Sea	Y	.	Y	.	T	.	.	.	.	K	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>b</b>	Split/ Adriatic Sea	.	.	C	.	T	.	.	.	.	T	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>b</b>	Gomec/ Aegean Sea	.	.	C	.	T	.	.	.	.	T	.	.	-	.	.	.	.	.	.	.	.	.	.	.
<b>c</b>	Puerto del Rey/ E. Spain	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	C	.	.	.	.
<b>c</b>	Bestiada/ Portugal	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	C	.	.	.	.
<b>c</b>	Mar Menor/ E. Spain	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	C	.	.	.	.	.
<b>d</b>	Tarifa/ S Spain (Strait of Gibraltar)	.	T	.	.	.	C	.	G	.	T	.	.	C	-	.	.	.	.	C	.	.	.	C	.
<b>e</b>	Algeciras/ S Spain (Strait of Gibraltar)	.	T	.	.	.	C	.	G	.	T	.	.	C	-	.	.	.	.	C	.	.	.	C	.
<b>f</b>	Plaja la Borrassa/ E. Spain	.	Y	.	.	.	C	.	.	.	T	T	.	A	-	M	.	.	.	.	.	.	.	C	.
<b>g</b>	El Pinet/ E. Spain	.	Y	.	.	.	C	.	.	.	T	.	T	C	-	.	.	.	.	.	.	.	.	G	.
<b>h</b>	Gargano/ Adriatic Sea	.	.	.	.	.	C	.	.	.	.	.	.	C	-	.	.	R	.	.	.	.	.	.	.
<b>i</b>	Parco Nazioale del Circe/ W Italy	.	.	.	.	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	.	.	.	.	.
<b>j</b>	Igoumenitsa/ Ionian Sea	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	.	R	.	.	.	.	.	.	.
<b>k</b>	Riva Longa/ Adriatic Sea	.	.	.	.	.	.	.	.	.	.	.	.	C	-	.	.	G	.	.	.	.	.	.	.
<b>l</b>	Bandirma Bay/ Marmara Sea	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	G	.	.	.	.	.	T	.	.
<b>m</b>	Nei Poroi/ Aegean Sea	.	.	.	.	.	.	.	.	T	.	.	T	.	-	.	R	A	.	.	.	.	.	.	.
<b>n</b>	Plaja El Serradal/ S. Spain	.	.	.	.	.	.	.	.	.	.	.	.	-	.	G	.	.	.	.	T	.	.	.	.

## Results

### 3.3. *Salsola kali*

#### 3.3.1. AFLP analysis

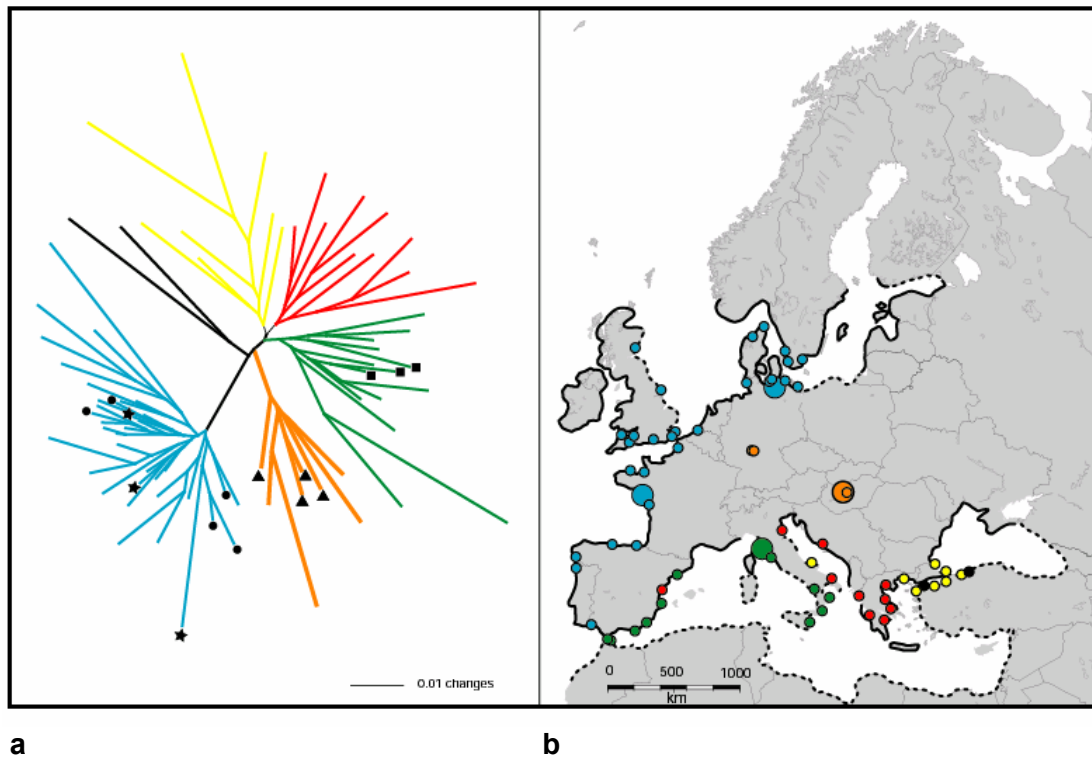
Seventy-nine individuals from 57 populations were included in the AFLP analysis of *Salsola kali*. Two primer combinations (FAM, E38-M55; HEX, E45-M57) produced 233 AFLP characters in the range between 90-412 bp, of which 177 (76%) were polymorphic, four (1.7%) uniform and 52 (22.3%) were autapomorphic. Characteristics of the AFLP profile of *S. kali* are presented in Table 3.8.

**Table 3.8** Characteristics of AFLP data derived from the two primer combinations used on 79 individuals of *Salsola kali*.

	Primer combination		Total	Average
	E38-M55	E45-M57		
No. of characters	79	154	233	117
No. of uniform characters	2	2	4	2
No. of unique characters	16	36	52	26
No. polymorphic characters	61	116	177	89
<b>% of polymorphic characters</b>	<b>77</b>	<b>75</b>	<b>76</b>	<b>76</b>
Scorable range in bases	90-380	99-412		

The mean genetic distance among individuals was  $0.0669 \pm 0.023$ . Similar to the previously investigated species (*H. portulacoides* and *C. maritimum*), the AFLP pattern in *Salsola kali* shows a clear spatial pattern of genetic variation. The unrooted NJ tree of genetic distance between all pairwise combinations of the polymorphic AFLP characters is shown in Figure 3.8. The phenogram clearly shows two main clusters reflecting a Mediterranean-Atlantic subdivision. Within the Atlantic cluster, accessions do not show sub-structure regarding their geographical origin, i.e., no sub-clustering of North Sea or Baltic Sea accessions can be observed in the NJ phenogram. On the other hand, the Mediterranean material shows a pronounced structure across the whole sampling range. In the east, accessions from the Black Sea and the northern Aegean fall into one cluster (except for one sample from the Black Sea and one from the Aegean, both coloured black, which fell outside of the Aegean cluster). The material from the Adriatic Sea and from both west and east Greece fall into separate cluster (plus one individual in east Spain). The third Mediterranean cluster contains all accessions from south Italy to south Spain (Strait of Gibraltar). Exceptionally, one sample from east Spain fall together with the Adriatic material. The inland material, two samples from Germany and four from Hungary, fall into a separate cluster that is more similar to the Mediterranean than to the Atlantic material (Figure 3.8). In all studied material, no separate clustering of individuals sampled from the same locality was observed. However, samples clustered within the major cluster of the recognized geographical region.

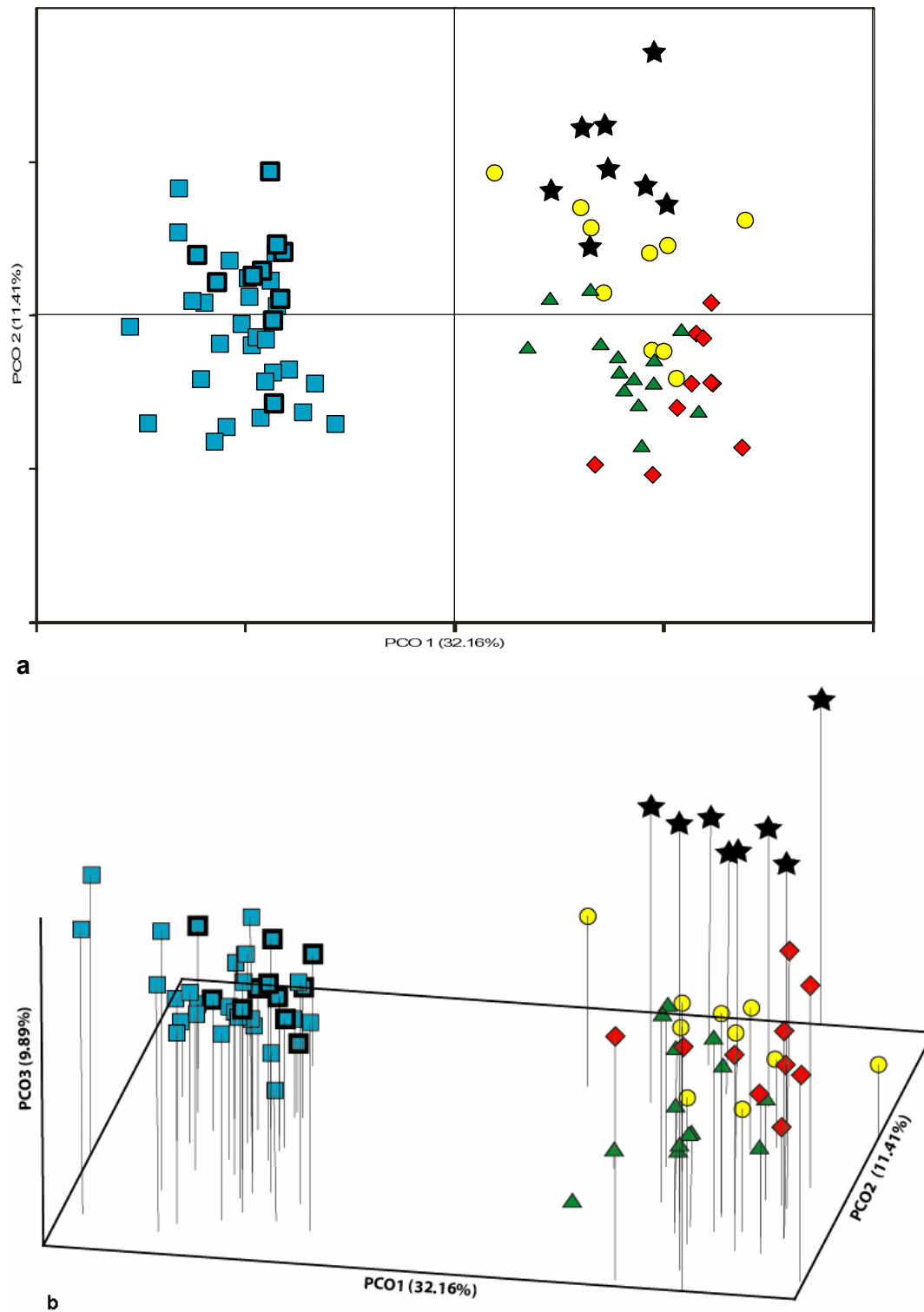
## Results



**Figure 3.8 (a)** Unrooted neighbour-joining NJ phenogram based on Nei and Li's (1979) genetic distance of 233 AFLP characters from 78 individuals of *Salsola kali*. Symbols represent samples from the same locality (large dots in the map): W. Italy (■); N. Germany (●); Hungary (▲); W. France (★). The same tree with locality names is provided in appendix III. **(b)** Geographical distribution (thick coastline), analyzed accessions (dots) and geographical distribution of NJ clusters (colours) in *S. kali*.

The 2-D and 3-D PCO plots were generated using the squared Euclidean distances of the 177 polymorphic AFLP markers. The first three variates accounted for 32.16%, 11.40% and 9.89% of the total variation. Both 2-D and 3-D PCO plots (Figure 3.9a+b) clearly confirm the pattern revealed by NJ analysis. In both, the Mediterranean-Atlantic subdivision can be clearly visualized and samples are discriminated by the first variate (32.16%). Moreover, the subgrouping of the Mediterranean accessions is better visualized by the 2-D plot where samples are discriminated by the second variate (11.41%). In the case of inland accessions, they can be better visualized in the 3-D plot by the third variate (9.89%). Similar to the NJ phenogram, the material from the Atlantic Ocean/North Sea/ Baltic Sea shows no sub-structure in the PCO as well.

## Results



□ Atlantic Ocean/North Sea/Baltic Sea ★ Inland (Germany & Hungary)

▲ West Mediterranean Sea ◆ Adriatic Sea + E Greece ○ Black/Aegean Sea

**Figure 3.9** (a) Two-dimensional and (b) Three-dimensional PCO plots of 177 polymorphic AFLP markers in 79 accessions of *S. kali* based on their squared Euclidean distances. Numbers represent the proportion of variation accounted by each axis. Bold squares in the Atlantic group represent the Baltic Sea individuals.

## Results

Based on the clustering pattern revealed by the NJ and PCO analyses, analysis of molecular variance (AMOVA) was performed for the recognized groups (Table 3.9). The groups were: (i) Black/Aegean Sea, (ii) Adriatic Sea, (iii) west Mediterranean Sea, (iv) Atlantic Ocean/North Sea/Baltic Sea and (v) inland material from Hungary and Germany. A nonhierarchical AMOVA involving all material resulted in 33.39% of genetic variation to occur among the groups ( $F_{ST} = 0.33$ ;  $P < 0.001$ ). Excluding the inland accessions, the highest among group genetic variation was obtained in the comparison between the Atlantic versus Black/Aegean Sea groups (39.56%;  $F_{ST} = 0.39$ ;  $P < 0.001$ ). The lowest value was obtained in a comparison between the Adriatic Sea and Black/Aegean Sea groups (8.39%;  $F_{ST} = 0.08$ ;  $P < 0.001$ ). When comparing the Mediterranean groups with each other, low percentages of genetic variation was partitioned among groups. On the other hand, when comparisons include Mediterranean and Atlantic groups, higher percentages of between group variation was obtained (Table 3.9). The group of inland accessions shows highest variation among groups when compared with the Atlantic group (44.52%;  $F_{ST} = 0.44$ ;  $P < 0.001$ ), and the lowest when compared with the Adriatic Sea group (22.84%;  $F_{ST} = 0.22$ ;  $P < 0.001$ ).

**Table 3.9** Partition of molecular variance (AMOVA), and  $F_{ST}$ -values calculated from different comparisons of *S kali* groups. df (A,W)= degrees of freedom of among and within group comparisons.

Comparison	df (A,W)	Among groups var. (%)	$F_{ST}$	$P$
<b>Non-hierarchical (all groups)</b>	(4,74)	33.39	0.33	<0.001
<b>Black/Aegean Sea vs. Atlantic/North Sea/Baltic Sea</b>	(1,46)	39.56	0.39	<0.001
<b>Adriatic Sea vs. Atlantic/North Sea/Baltic Sea</b>	(1,46)	36.37	0.36	<0.001
<b>W Mediterranean Sea vs. Atlantic/North Sea/Baltic Sea</b>	(1,50)	38.57	0.38	<0.001
<b>Inland vs. Atlantic/North Sea/Baltic Sea</b>	(1,44)	44.52	0.44	<0.001
<b>Adriatic Sea vs. W Mediterranean Sea</b>	(1,23)	14.90	0.14	<0.001
<b>Black/Aegean Sea vs. W Mediterranean Sea</b>	(1,23)	17.82	0.178	<0.001
<b>Inland vs. W Mediterranean Sea</b>	(1,21)	33.72	0.337	<0.001
<b>Black/Aegean Sea vs. Atlantic/North Sea/Baltic Sea</b>	(1,19)	8.39	0.08	<0.001
<b>Adriatic Sea vs. Inland</b>	(1,17)	22.84	0.228	<0.001
<b>Black/Aegean Sea vs. Inland</b>	(1,17)	25.7	0.25	<0.001



## Results

Correlation between genetic and geographical distances (Mantel test) is presented in Table 3.10. A highly significant correlation ( $r_M = 0.619$ ;  $P = 0.0001$ ) was obtained when all accessions from the entire range (except inland material) were included in the analysis. Low but significant correlation ( $r_M = 0.205$ ;  $P = 0.010$ ) was calculated for Atlantic/N Sea/Baltic Sea accessions in spite of their extended distributional range ( $\approx 6000$  km of coastline), and no significant IBD was found in any of the other groups identified.

**Table 3.10** Mantel test results of correlation between geographical (coastal line) and genetic distance in *S. kali*. \* $P =$  significant at 0.05.

Groups	( $r_M$ ) value	$P$
All populations (inland included)	0.484	0.0010
All populations (inland excluded)	0.619	0.0001
Black/Aegean Sea	-0.272	0.170
Adriatic Sea	-0.125	0.209
W Mediterranean Sea	0.105	0.185
Atlantic Ocean	0.205	0.010*

In the Atlantic area,  $SI$  was distinctly lower (0.102) than in the Mediterranean area (0.148).

### 3.3.2 *Salsola kali* ITS sequence:

Accessions of *Salsola kali* from the Black Sea to the Baltic Sea coasts, in addition to inland localities from Spain, Germany and Hungary were included in this survey. In total, 83 sequences and cloned ITS copies (Table 3.11) were successfully amplified and sequenced.

The four accessions from Iran have ITS sequences identical with *Salsola tragus*. A preliminary MP analysis had shown that *S. kali* has a closer phylogenetic relationship to *S. tragus* than to any other taxon tested as a candidate outgroup (data not shown). Accordingly, *S. tragus* is used as an outgroup for rooting the MP trees. After alignment, the length of the ITS region (18S+5.8S+26S) ranged from 601-611 bp (ITS1 = 232-242 bp, 5.8S = 161 bp, and ITS2 = 208 bp). Table 3.11 shows the polymorphic positions in all sequenced accessions including the outgroup taxon. Excluding the outgroup, 13 nucleotide substitutions in ITS1 (4 autapomorphic, 7 synapomorphic and 2 additive), five in 5.8S (4 autapomorphic and 1 synapomorphic) and 11 in ITS2 region (4 autapomorphic, 4 synapomorphic and 3 additive) were observed. In total, 12 phylogenetically informative substitutions among *Salsola kali* accessions and cloned sequences were found (Table 3.11). The complete ITS sequences of *S. kali* and *S. tragus* are presented in Appendix IV.

## Results

**Table 3.11** Variable nucleotide sites in 83 *Salsola kali* ITS sequences from different regions and the outgroup *S. tragus*. Sequence symbols: A, C, G, T = dATP, dCTP, dGTP, dTTP; Y=C/T; M=A/C; R=A/G; K=G/T; S=C/G. Other symbols: i = phylogenetically informative; a = autapomorphic; h = heterogeneous/additive (variable but neither autapomorphic nor phylogenetically informative); (Ω) = indel; (=) = gab; Bold numbers = cloned ITS sequences; Numbers in brackets = different samples from the same locality. ITS Types: EM, east Mediterranean; WM, west Mediterranean; AM, Atlantic mixed and B, Baltic Sea.

Taxon origin	Region in ITS	ITS1														5.8S			ITS2																			
		1	5	6	6	9	0	0	1	4	5	8	9	1	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	5	5	5	5	5	5	6		
<i>S. tragus</i>		C	G	C	G	G	A	C	T	C	A	C	G	A	A	C	G	T	T	C	C	C	C	C	C	C	C	C	G	C	T	C	C	G	C	T	C	C
EM_Eregli_Black Sea		C	C	C	T	G	A	C	T	C	A	C	G	A	A	T	G	T	T	C	C	C	C	C	C	C	C	T	C	C	C	G	C	T	T	C	C	
EM_Karasu_Black Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EM_Erasmio_Aegean Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Ignaeda_Black Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Nie Anchialos_Aegean Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Yaluva_Aegean Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Githio_S Greece		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Lehena_1+3+5+6+7_W Greece		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Igoumenitsa_W Greece		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Split_Adriatic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Albegna_W Italy		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Gonsenheim_Germany		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Budenheim_Germany		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Soroksar_Hungary		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Tahi_Hungary		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Lleida_inland-Spain		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Huescar_inland-Spain		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Sale_5_Morocco		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Sale_6_Morocco		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Sale_7_Morocco		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
EM_Sale_3_Morocco		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_Lehena_2_W Greece		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_M_Lesina_Adriatic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_Villamare_S Italy		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_Canet_S France		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_Pi_Olivia_(1+2)_E Spain		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_Pi_Olivia_4+5+7+8+10+11_E Spain		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_Pi_Olivia_6_E Spain		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_Pi_Olivia_9_E Spain		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
WM_Sale_4_Morocco		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
AM_St_Benoit des Ondes_3		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
AM_St_Benoit des Ondes_9		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
AM_Lyngsa_3+4+7+11_Kattegat Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
AM_Lyngsa_10_Kattegat Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
AM_Zinnowitz_5_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
AM_Zinnowitz_1_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
AM_Zinnowitz_4_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_St_Benoit des Ondes_12+5+6+7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_St_Benoit des Ondes_10		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Nyehusen_3+4+5+8+9+10+12		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Nyehusen_(1+2+3)_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Nyehusen_7_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Logstor_Skagerak Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Lyngsa_5+6+12_Kattegat Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Lyngsa_9_Kattegat Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_St_Peter Ording_(1+2)_North Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Boinsdorfer_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Heiligen Hafen_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Prerow_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Zinnowitz_clone-2+6+7+8+9_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
B_Zinnowitz_clone-3_Baltic Sea		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

## Results

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The ITS sequences of *S. kali* from the European, northwest African coasts and inland localities contain polymorphic and heterogeneous (additive) character states in many positions, however, they can be classified into four major types:

**1- Eastern Mediterranean type (EM):** ITS of this type is 611 bp in length and is mainly found in accessions from the east Mediterranean Sea (Black/Aegean Sea and the Adriatic Sea). This type was also found in the accessions from Spanish, Hungarian and German inland material (Table 3.11). This type shows the same ITS length as the outgroup taxon and was given the yellow color in the map and the MP cladogram.

**2- Western Mediterranean type (WM):** This type appears in accessions from the central and west Mediterranean coasts (southwest Italy, south France, east and southeast Spain). When aligned with the EM type, it has an indel in ITS1 region at position 189 (missing C), thus, its length = 610 bp. Besides the indel, it differs from the EM type in at least two synapomorphic positions (Table 3.11). This ribotype was represented using red color in the map and the MP cladogram

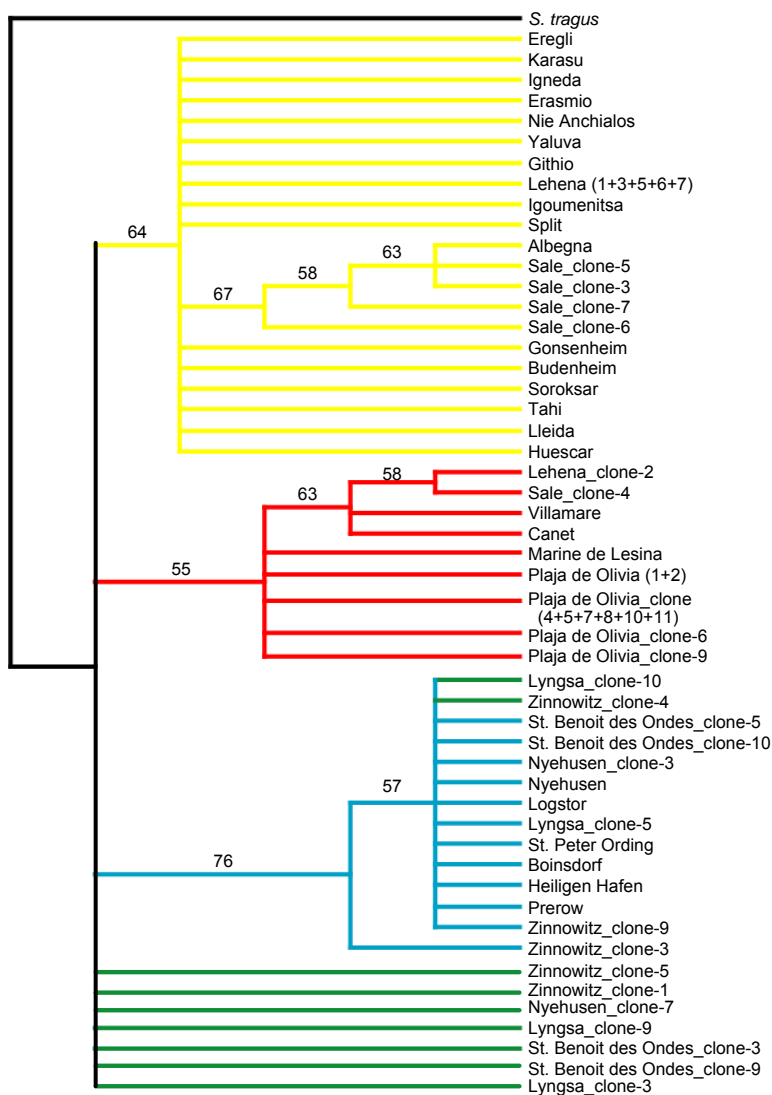
- Accessions in two region in the Mediterranean, particularly south Greece and south Spain / north Morocco contain both ITS types (EM and WM). The amplified sequence appears heterogeneous (double signal in the electropherogram) from the position of the previously mentioned indel (in WM) to the end of the ITS region. Cloning this heterogeneous ITS type produced both the WM and the EM ribotypes (see Table 3.11). Accessions which have this ribotype were colored red-yellow in the map.

**3- Baltic type (B):** the ITS length of this type is 10 bases shorter than the EM type (deletion from bases 107-117 in ITS1). Besides the indel it differs from the EM type by six substitutions and from WM type by three substitutions (see Table 3.11 for details). The B type is found in four localities in the Baltic Sea, and also found in Sankt Peter Ording (North Sea) and Løgstør (Skagerak Sea/ north Denmark). A blue color denotes this ribotype in the MP cladogram (Figure 3.10) and in the map (Figure 3.11).

**4- The Atlantic accessions from south Portugal to the Baltic Sea, including south and east Britain contain an additional ITS copy beside the B ribotype (Mixed Atlantic "AM").** The sequence appears heterogeneous from the indel mentioned in B type till the end. Cloned sequences show both the B type and another type that has the same ITS length as the outgroup. It differs from the EM type by eight, from WM type by six and from the B type by five nucleotide substitutions. This ITS type was given the green-blue colour in Figure 3.11 and green branches in the MP tree in Figure 3.10.

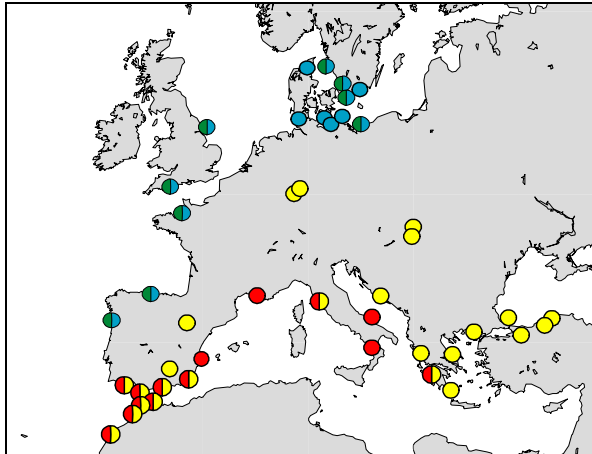
## Results

The unweighted MP analysis of the ITS data of 52 ribotypes/35 characters resulted in 34 most parsimonious cladograms of 43 steps in length (CI = 0.721, RI = 0.881, HI = 0.279). One of the shortest trees (Figure 3.10) with 100 bootstrap replicates divides the ribotypes into three main clades and seven non-resolved accessions. The clade which contains all of the east Mediterranean and inland material shows a bootstrap value of 64% and the clade containing the Baltic ribotypes shows the highest bootstrap value in the cladogram (76%). The third major clade contains the west Mediterranean material from south Italy to east Spain plus cloned sequences from Morocco and Greece with low (55%) bootstrap value.



**Figure 3.10** Rooted bootstrap tree (100 replicates) for *Salsola kali* ITS sequences constructed by parsimony method. The numbers at the internal branches of the tree represent the bootstrap values for each clade. Sequences used for this analysis are listed in Table 3.11 and illustrated in Figure 3.11.

## Results



**Figure 3.11** Geographical distribution of the four *Salsola kali* ITS ribotypes in the area of study

### 3.4. *Calystegia soldanella*

#### 3.4.1. AFLP analysis of the entire range sampling:

Seventy-three accessions from 53 localities sampled along the entire distributional range were evaluated using two AFLP primer pairs (FAM, E39-M61; HEX, E33-M57). Together, the two primers yielded 98 AFLP characters within the scorable range 70-416 bp (FAM, 59; HEX, 39). Sixty-eight of the characters (69%) were polymorphic, 13 (13.2 %) were monomorphic and 17 (17.3%) were autapomorphic (appeared only in one individual). Table 3.12 presents the main characteristics of AFLP phenotypes in *C. soldanella* in the entire-range sampling approach.

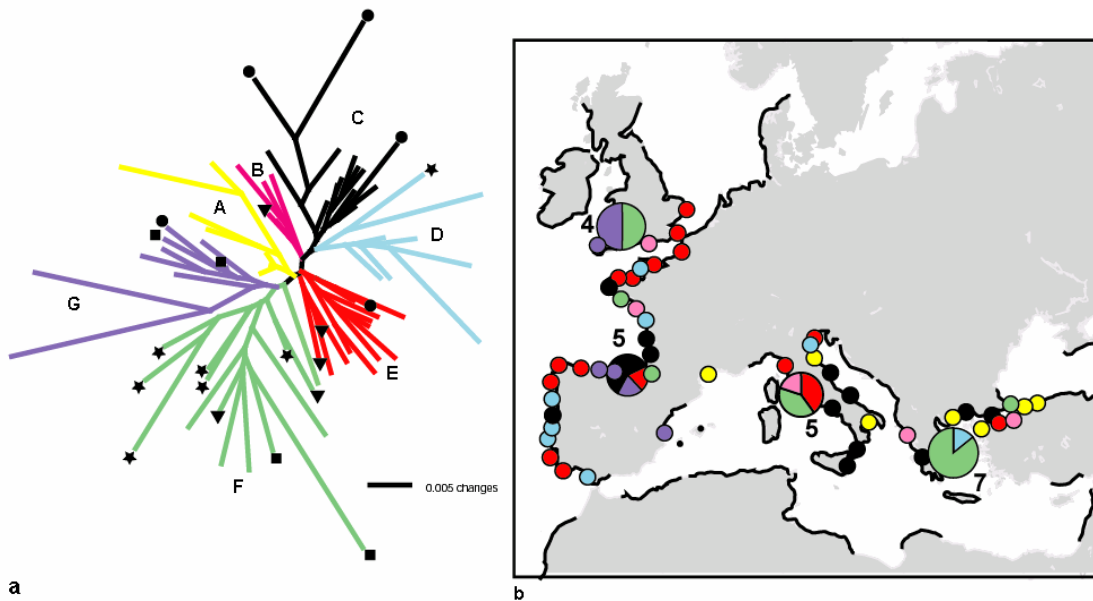
**Table 3.12** Characteristics of AFLP markers derived from the two primer combinations used in the *C. soldanella* entire range sampling approach.

	Primer combination		Total	Average
	E39-M61	E33-M57		
No. of characters	59	39	98	49
No. of uniform characters	8	5	13	7
No. of unique characters	10	7	17	9
<b>No. polymorphic characters</b>	<b>41</b>	<b>27</b>	<b>68</b>	<b>34</b>
% of polymorphic characters	69	69	<b>69</b>	69
Scorable range in bases	70-304	72-416		

There were no samples of 100% genetic similarity found in the studied material. Mean genetic distance among individuals was  $0.0309 \pm 0.011$ . The most similar accessions in the pairwise genetic-distance matrix (GD = 0.00224; geographic distance  $\approx$  100km) were samples from Marina Romea and Marina di Pisticci (both from the Adriatic Sea). The next most similar were samples from Marina di Pisticci/ Adriatic Sea and from Las Castellas/ south France (GD = 0.0023; geographic distance  $\approx$  3500 km along the coast). The most genetically different samples were from Foz Arelho/ west Portugal and Braunton Burrows / south England (GD = 0.0798; geographic distance  $\approx$  3500km).

## Results

Different from the other three investigated species (*Halimione portulacoides*, *Crithmum maritimum* and *Salsola kali*), the unrooted NJ phenogram (Figure 3.12) shows a pattern that does not reflect any spatial structure of genetic variation along the sampling range, and the majority of accessions do not cluster according to their geographical origin. Even individuals sampled from the same populations from west Italy, north Spain, and southwest England do not fall into a separate cluster. However, only the individuals from an Aegean Sea population fall into one cluster (Figure 3.12a, cluster "F"). Essentially seven clusters (A-G) have been recognized for further analysis.



**Figure 3.12** (a) Unrooted NJ phenogram based on Nei and Li's (1978) genetic distance of 69 AFLP polymorphic markers in 73 *C. soldanella* accessions. (b) Distribution of *C. soldanella* distribution (bold coastline), sampled localities (circles). Colours correspond with clustering pattern in the NJ analysis. Large circles and numbers stands for localities where more than one individual are included in the analysis; S. W. England (■); N. Spain (●); W. Italy (▲); Greece (★). The same tree with locality names is provided in appendix III.

The total amount of variation among clusters was 25.64% ( $P < 0.001$ ), and the strongest differentiation was between clusters "C" and "G" (35.34%;  $P < 0.01$ ). The variation among groups ranges between 11.95% - 35.34% (Table 3.13).

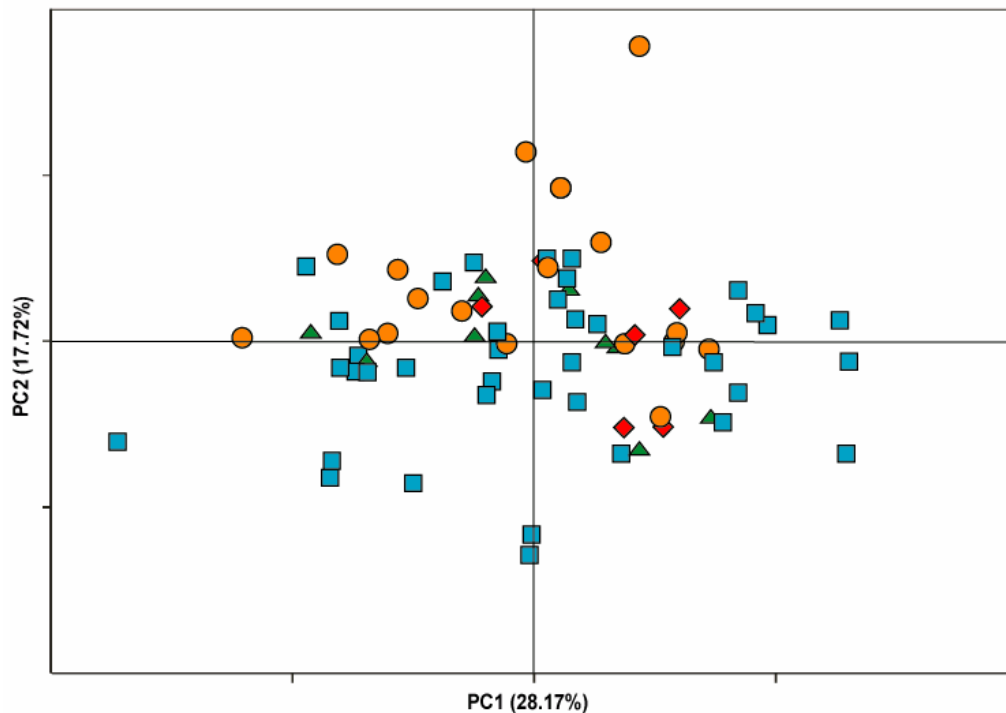
Correlation between geographical and genetic distances (Mantel test) resulted in low but significant IBD along the sampled range ( $r_M = 0.116$ ;  $P = 0.0391$ ).

## Results

**Table 3.13** Pairwise  $F_{ST}$ -values of the identified groups (A-G in NJ tree) over all loci in the AFLP data. The statistical significance of the observed values of  $F_{ST}$  was tested by comparison with 1023 permutations. \*  $P < 0.05$  level, \*\*  $P < 0.01$  level.

	A	C	D	B	G	F	E
A	0.0						
C	0.34319**	0.0					
D	0.27946**	0.19317**	0.0				
B	0.27526*	0.19735**	0.21030**	0.0			
G	0.26675**	0.35346**	0.34900**	0.32304**	0.0		
F	0.25121**	0.30484**	0.33411**	0.28508**	0.11946**	0.0	
E	0.19196**	0.23649**	0.26389**	0.20047**	0.16934**	0.20325**	0.0

The two-dimensional PCO plot confirms the overall pattern revealed by the NJ analysis (Figure 3.13). The first two PCs accounted for 28.17% and 17.72% of the total variation. Samples in the plot were given symbols according to their geographical origin (Atlantic, west Mediterranean, Adriatic and Aegean Sea), but no grouping could be observed.



■ Atlantic Ocean ▲ West Mediterranean Sea ◆ Adriatic Sea ● Black/Aegean Sea

**Figure 3.13** Two-dimensional PCO plot of 65 polymorphic AFLP markers in 74 individuals of *C. soldanella* sampled from the European coasts.

### 3.4.2. AFLP analysis of the population level approach:

The second approach in the *C. soldanella* phylogeography is a population level sampling, in which 61 individuals from seven populations were included (Figure 2.1). Three primer combinations were used and successfully evaluated in the AFLP procedure (FAM, E39-M61; HEX, E45-M57; NED, E37-M54). Within the range of 70-455 bp, 154 characters were successfully scored, of which 106 (68%) were polymorphic, eleven (7%)

## Results

autapomorphic, and thirty-seven (24%) were uniform across all accessions. The average number of scorable characters/primer was 51. Table 3.14 summarizes AFLP characteristics in the *C. soldanella* population level sampling.

**Table 3.14** Characteristics of AFLP markers derived from three primer combinations used in the population level approach of *C. soldanella*.

	Primer combination			Total	Average
	E39-M61	E45-M57	E37-M54		
No. of characters	59	50	45	154	51
No. of uniform characters	15	11	11	37	12
No. of unique characters	3	4	4	11	4
<b>No. polymorphic characters</b>	<b>41</b>	<b>35</b>	<b>30</b>	<b>106</b>	<b>35</b>
% of polymorphic characters	69	70	67	69	69
Scorable range in bases	70-455	70-325	70-325		

The mean genetic distance among individuals was  $0.0294 \pm 0.0102$ . No identical genotypes were found in the pairwise similarity coefficient matrix within the studied material. The most similar genotypes were two samples from Porto-Garibaldi population in the Adriatic Sea with the GD = 0.00533, and the most different genotypes were one from the west Italy population and one from Cavallino population/Adriatic Sea with the GD = 0.0825.

The overall pattern in the NJ tree (Figure 3.15) does not reflect a clear spatial structure of genetic variation. Of the 61 included accessions, three from Oreio/Aegean Sea and 15 Adriatic Sea samples fall into a separate cluster each. The remaining samples from next locality and from west Italy and the English Channel populations are scattered in the tree without geographical pattern. Accordingly, the same overall pattern as in the entire range sampling is revealed by the population level approach.

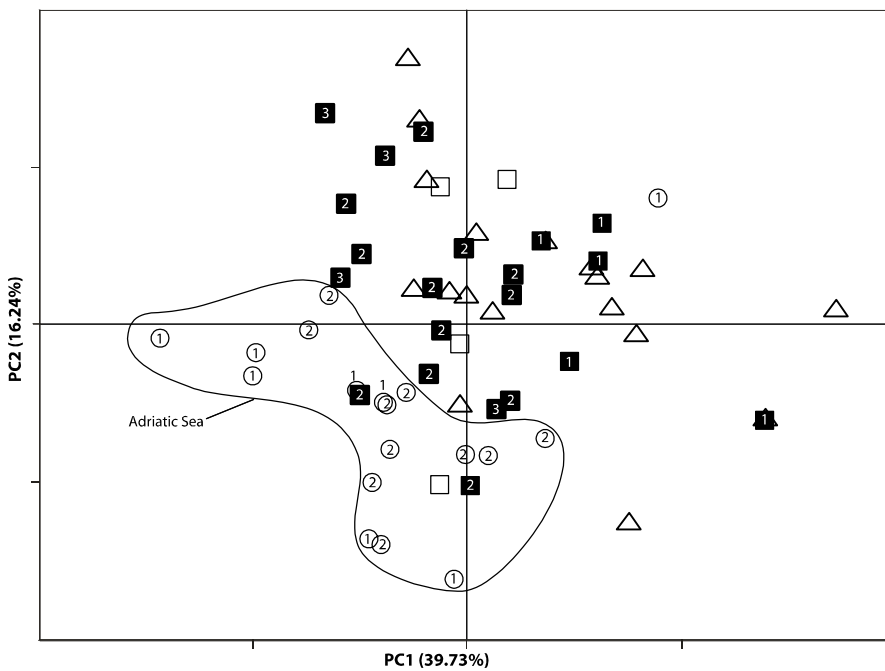
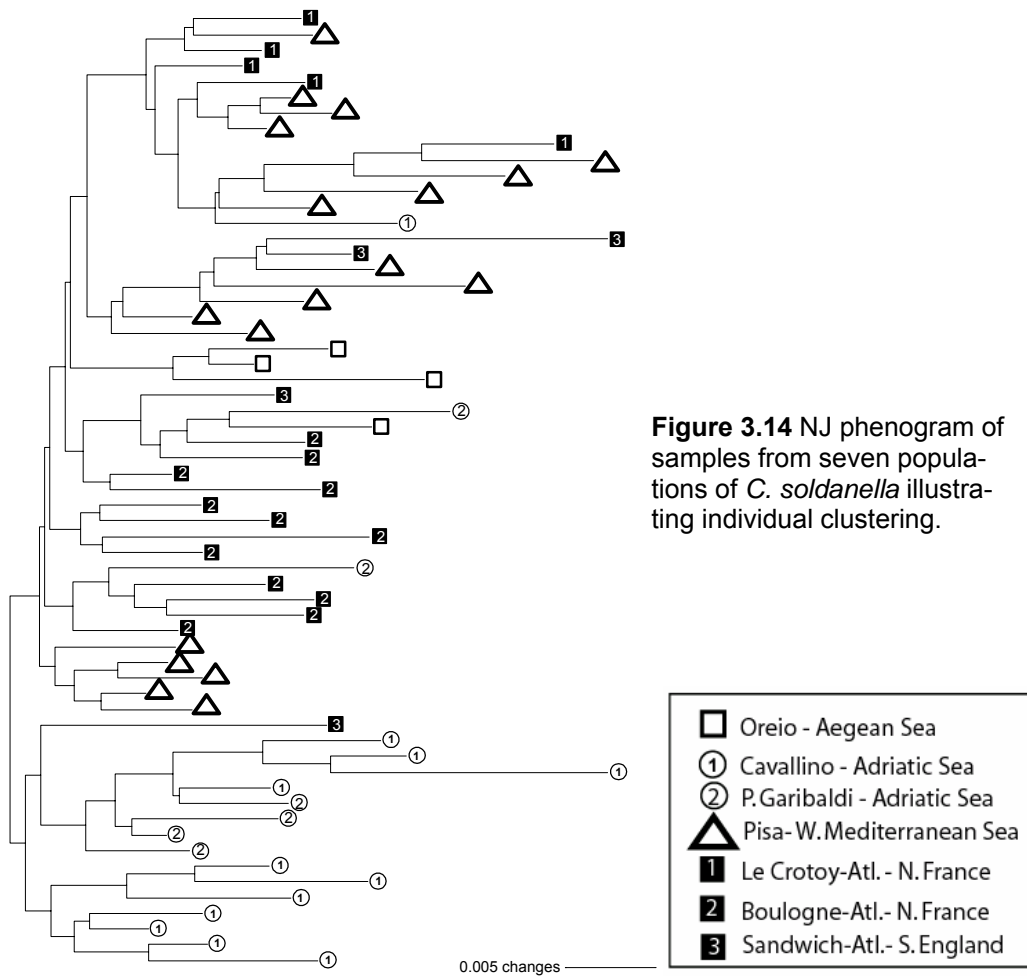
The 2-D PCO of the squared Euclidean distances between all 106 polymorphic AFLP characters confirms the same picture as revealed by the NJ analysis (Figure 3.16). The 2-D plot (39.73% and 16.24% of the total variation for the first two axes) clearly shows the overlapping of the Mediterranean and the Atlantic populations. On the other hand, it also shows the discrimination of the Adriatic Sea accessions, mainly by the second variate (16.24%).

A single Mantel test involving all populations yielded a statistically nonsignificant correlation between genetic and geographical distance ( $r_M = 0.1083$ ;  $P = 0.3301$ ).

Genetic diversity *S<sub>I</sub>* in the population Boulogne/Atlantic was lower (0.117) than the three Mediterranean populations (Pisa, 0.140; Cavallino, 0.147; Porto-Garibaldi, 0.128).



## Results



## Results

Partitioning of the molecular variance (percentage of genetic variation among and within populations), and  $F_{ST}$ -values are presented in table 3.15. The calculations were set up at three different levels: (i) AMOVA was calculated for all seven populations in a nonhierarchical manner. The percentage of variation among populations in this approach was 18.02% ( $F_{ST} = 0.18$ ;  $P < 0.001$ ). (ii) AFLP characters were combined according to region-population-basis in order to form three regional groups. Thus, the populations from Le Crotoy, Sandwich and Boulogne were combined to one population from the Atlantic, Porto Garibaldi and Cavallino was treated as another population from the Adriatic Sea and the third group contained the population from west Italy. The Greece population (four individuals) was excluded from this analysis because of low sampling size. In this case, the percentage of among population variation decreased to 14.20% ( $F_{ST} = 0.14$ ;  $P < 0.001$ ). (iii) The seven populations were divided into three groups according to their regional distribution (English Channel, west Mediterranean and Adriatic Sea) in a hierarchical AMOVA. In this case, the percentage of (among populations within groups' variation) was 10.08% with  $F_{ST}$  value = 0.182 ( $P < 0.001$ ).

**Table 3.15** Analysis of molecular variance (AMOVA) of AFLP phenotypes in *C. soldanella* calculated between and within all populations in different hierarchical settings .

	Among groups var. (%)	Among populations var. (%)	Within populations var. (%)	$F_{ST}$
<b>Nonhierarchical AMOVA (7 populations)</b>	-	18.02	81.98	0.18019 $P < 0.001$
<b>Three regions</b>	-	14.20	85.80	0.14 $P < 0.001$
<b>Three groups /regional (7 populations)</b>	8.18	10.08	81.74	0.182 $P < 0.001$

### 3.4.2. *Calystegia soldanella* ITS sequence:

The total length of the ITS region in *C. soldanella* was 599 bp (ITS1 = 223, 5.8S = 150, ITS2 = 226 bp). A survey including two accessions from the Aegean Sea and the Atlantic Ocean coasts gave 100% identical ITS sequences. Because of the lack of variation, no further ITS sequencing was carried out. The complete ITS sequence (ITS1+5.8S+ITS2) of *C. soldanella* is presented in Appendix IV.

### 4. DISCUSSION

At the range-wide geographical scale, the AFLP (NJ and PCO analyses) and ITS sequence data show pronounced genetic subdivisions and spatial structure of genetic variation in three of the studied species (*Salsola kali*, *Halimione portulacoides* and *Crithmum maritimum*). These subdivisions were also confirmed by the relatively large amount of variation apportioned among identified groups in the AMOVA analysis. Based on these analyses, a number of similarities and dissimilarities in the species' phylogeographic pattern can be recognized and summarized as follows:

- in the three species, a separate Black Sea/Aegean Sea cluster was revealed. However, in *S. kali*, only the samples from the northern part of the Aegean belong to this cluster.
- all species have an Adriatic Sea cluster. This cluster also contains some individuals from the western and eastern coast of Greece (as in *S. kali* and *C. maritimum*) and the southern coast of Italy (*H. portulacoides* and *S. kali*).
- Furthermore, in both *H. portulacoides* and *S. kali*, a separate ITS sequence clade in the material from the entire eastern part of the Mediterranean (Black/Aegean and Adriatic Sea) was revealed.
- in the west Mediterranean from south Italy to south France, *S. kali* shows a well defined AFLP cluster in addition to a separate clade of ITS sequences. *Halimione portulacoides* material from the west Mediterranean is divided into a separate cluster in the western part (E Spain), or falls into small neighbouring clusters (from south France to south Italy) which in general are more similar to the east Mediterranean groups (Adriatic and Aegean). In south Spain, the ITS sequences of *H. portulacoides* form a separate clade and characterized by higher number of nucleotide substitutions compared with other regions. The material of *C. maritimum* from eastern Spain does not fall into one separate cluster but it groups together with the Atlantic. The rest of the material in the eastern part of west Mediterranean appears in an intermediate positions between the three groups (Atlantic/E Spain, Adriatic Sea and Black/Aegean Sea). This group of genetically similar individuals is distributed also from south Italy to south France.
- the material from the Atlantic coast fall into one separate cluster from the Strait of Gibraltar to the North Sea (*H. portulacoides*) and to the Baltic Sea (*S. kali*). A well supported clade of ITS sequences in *S. kali* was also uncovered in the Atlantic accessions. However, despite the large spatial scale (coastline >5000 km), these accessions show hardly any regional structure across the whole range, i.e., no sub-

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clustering was observed in the English Channel, the North Sea or the Baltic Sea material even when the Atlantic accessions were analyzed separately (data not shown). In the fourth species, *C. soldanella*, unexpectedly an absence of geographical structure of the AFLPs was revealed in the entire range and the population level sampling approaches. This pattern will be discussed in a separate part of this discussion.

The species included in this study show nearly similar mean genetic distances which suggests roughly similar absolute time of genetic diversification. Accordingly, they are assumed to have experienced the same history which is a necessarily prerequisite for the purpose of comparison.

To interpret the revealed pattern, three pieces of information are needed: (i) since it is assumed that temperature (or other factors related to temperature) is one of the main limiting factors to the northern distributional limits of coastal species (e.g. Chapman, 1960; Scott, 1963), knowledge about historical temperature distribution during the Quaternary or particularly during the last glacial maximum (LGM  $\approx$  21 – 18000 y B.P) in addition to knowledge about the geographic history of the European coasts are needed. (ii) information about contemporary abiotic factors: the plants are benefiting from sea currents as a dispersal agent, thus, information about positions of sea currents and their directions is also required. (iii) also there is a need for enough information about the biology and ecology of each species such as dispersal mechanisms, breeding system, life history, habitat etc.

### **AFLP clustering pattern in the Black, Aegean and the Adriatic Sea region:**

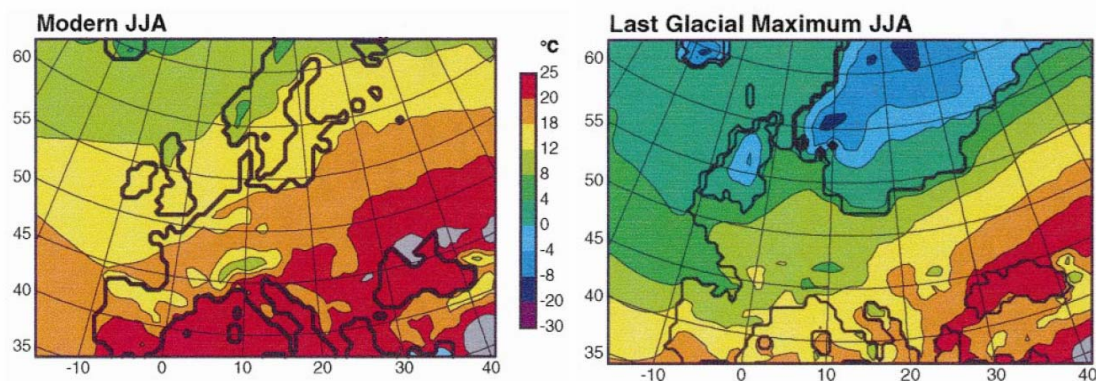
At the regional scale from the Black Sea to Marmara Sea and north and west coasts of the Aegean Sea, the different analyses (NJ, PCO and AMOVA) indicate a separate cluster of individuals from the mentioned region in all three species. In that particular region, disconnection between the Black and the Aegean Seas due to the decrease in sea level during the LGM took place (Perissoratis and Conispoliatis, 2003). Summer temperature of June-July-August (JJA) ranged between 18 - 25°C at that time (Figure 4.1). Today's northern distribution of the three species in summer correlates with the isotherms of 14°C for *S. kali* and 16°C for both *H. portulacoides* and *C. maritimum*. Accordingly, the past distribution of the three species was not interrupted due to temperature drop during the LGM, and can explain the persistence of each species' populations in the Aegean and the Black Sea area during the LGM. On the other hand, our insufficient sampling from the Black Sea in *S. kali* and *C. maritimum* can not distinguish a separate Black Sea cluster,

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thus no further conclusions regarding the subdivision between the Black and Aegean Seas material can be drawn.

The same conclusion can be reached in the Adriatic Sea region. The LGM isotherms in summer were around 18-20°C (Figure 4.1) which also implies that populations of the three species were able to survive the LGM at the former Adriatic Sea coasts without interruption which is a necessary condition for genetic divergence to evolve.

In summary, the pattern in the east Mediterranean coasts can be explained by referring to the historical temperatures in relation to the species' modern distribution temperatures.

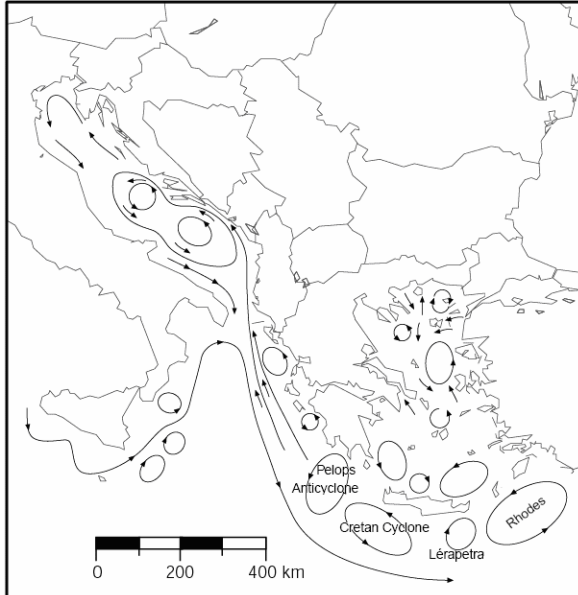


**Figure 4.1** Modern and simulated LGM temperature (ca. 21,000 yr B.P.) in Europe for summer (June, July, August [JJA]). Isotherm scale in  $\pm^{\circ}\text{C}$ . Coastline is marked with heavy black line. After van Andel (2003).

The subdivision between the Adriatic and Aegean/Black Sea material in the three species can have a contemporary abiotic explanation, particularly by major sea surface- currents which act as primary dispersal agent for the three species. As illustrated in figure 4.2, there are permanent cyclonic and anticyclonic gyres (four main water cyclones; Rhodes, Lerapetra, Cretan cyclone and Pelops anticyclone) bordering the Aegean Sea at its southern edges. Due to these gyres, no major surface flow is connecting the Aegean basin directly with other neighbouring ones like the Adriatic Sea or other parts of the Mediterranean. Also the Adriatic Sea is characterized by having two large circular current systems at its southern and middle parts and other anticyclonic gyres to the south of Italy separating it from other water masses in the Mediterranean (Artegiani *et al.* 1997a,b; Ovchinnikov, 1966; Lykousis *et al.* 2002). These current features create an isolated population systems in these regions with minor gene flow into or out of each basin and forcing genetic divergence to evolve. Our sampling strategy prevents drawing any detailed conclusions regarding possible gene flow between populations of these two distinct regions. However, in the ITS data of *S. kali*, one individual from west Greece (Lehena) show an additive ITS sequence that includes both east and west Mediterranean ribotypes.

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Accordingly, this can be an evidence for hybridization which is taking place in border zones of the described regions but this still speculative without detailed sampling.



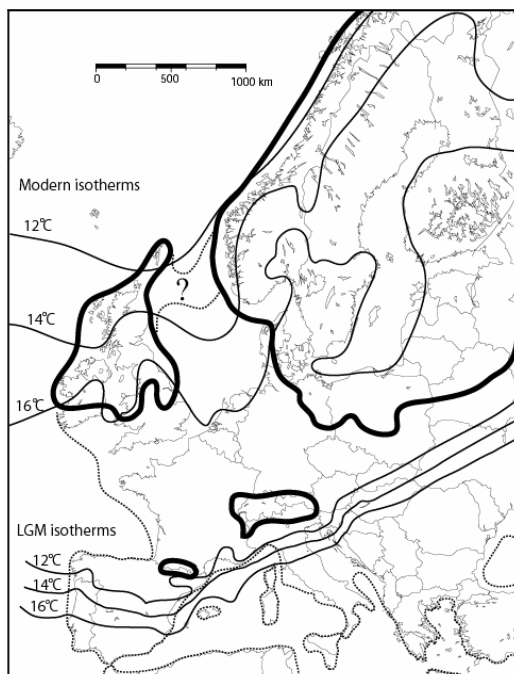
**Figure 4.2** Major surface currents in the Aegean, Ionian and the Adriatic Seas. Redrawn from Ovchinnikov (1966), Rey (1983), Malanotti-Rizzoli *et al.* (1997), Millot (1999) and Lykousis *et al.* (2002).

In summary, within the region of Black/Aegean and Adriatic Seas, both the historical climate and contemporary abiotic factors (sea currents) offer an explanation for the existence and persistence of genetic divergence and subdivisions in the three species. Genetic breaks were also reported in that area in many marine studies; molecular variation in a lagoon cockle (*Cerastoderma glaucum*) was examined across the species range along the European coasts and showed a major phylogeographic break in the mtDNA that separates a group of Aegean Sea haplotypes from those to the west of the Peloponnesian Peninsula in the Mediterranean (Nikula and Väinölä, 2003). This genetic break was attributed to a long-term isolation of populations in parts of the eastern Mediterranean and Black Sea basins through the Pleistocene. In a study by Borsa *et al.* (1997), a distinct Aegean lineage was reported for flounders (*Platichthys* species) using allozyme evidence. Olsen *et al.* (2004) reported a distinct genetic lineage in the Black Sea in comparison to the Mediterranean Sea in the sea grass *Zostera marina*. Interestingly, the Aegean/Black Sea area has been recognized as a separate biogeographic region in many publications (e.g. Adamovic, 1909; Tahktajan, 1986; Meusel and Jäger, 1992).

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### AFLP clustering pattern in the west Mediterranean basin

The climatic features of west Europe during the LGM differs from its eastern part. The west Mediterranean basin was characterized by lower temperatures than the east (Figure 4.1), and in general isotherms were pushed more to the south in the western regions (e.g., the 14°C JJA isotherm was roughly parallel to the northern coasts of the west Mediterranean during the LGM, but nowadays it crosses Scotland and reaches north Scandinavia (Figure 4.3)). Another difference between the east and west Mediterranean geography is the slight shift of the coastline during the LGM compared to the dramatic shifts in the east like the Adriatic Sea or the Black/Aegean Seas. An exception to this is the south coast of France coast (Gulf of Leon) where the coastline was displaced around 50 - 60 km southwards (Figure 4.3).



**Figure 4.3** Modern (northern Europe) and reconstructed (southern Europe) last glacial maximum isotherms. The 12°C, 14°C and 16°C mean July isotherms (modern) or June-July-August (LGM) are shown. The position of the 14°C and 16°C LGM isotherms were interpolated from the position of the 12°C and 18°C isotherms. Modern isotherms were redrawn from Hann (1982), and LGM isotherms from van Andel (2002). The LGM coastline is shown as a dotted line (Lang 1994).

Regarding the sea surface-currents relevant to our study, the main features in the west Mediterranean are a northwestwards current parallel to the Italian coasts and southwestwards current parallel to south France and east Spanish coasts (Millot 1999).

The results obtained in the west Mediterranean coasts vary between the three species but can be explained by the previously described climatic and geographical features. Based on the assumption of summer temperature being one of the major factors that limits the coastal species' distribution, only populations of *S. kali* were able to inhabit the entire west Mediterranean coasts without interruption by the temperature drop during the LGM. A separate AFLP cluster and ITS clade in the west Mediterranean provide evidence for

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undisturbed and continuous inhabitation of *S. kali* populations in the west Mediterranean coasts. An exception are the populations at the most southern coasts of Spain and north Morocco. In that region, the ITS sequences show the additive pattern of both east and west Mediterranean types. Interestingly, we also found the eastern Mediterranean ITS-type in two inland Spanish localities (Huescar and Lleida) which could explain the reason behind finding that type in south Spain and north Morocco if hybridization is taking place between inland and coastal populations. However, our small sampling in the AFLP and ITS analyses in that region is not enough to detect this possible hybridization or draw further conclusions. Interestingly, in *S. kali*, a congruency in the ITS and AFLP data within the Mediterranean was observed; the separate west Mediterranean group was revealed in both markers and the eastern division as well.

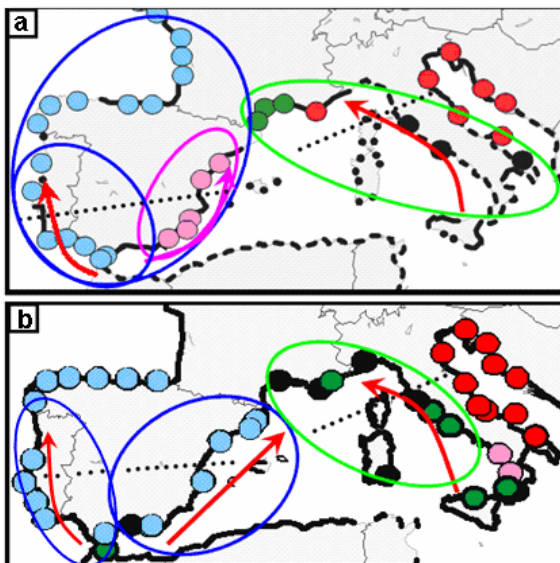
On the other hand, the other two species (*H. portulacoides* and *C. maritimum*), which are more cold sensitive, are not likely to have survived the LGM at the northern coasts of the west Mediterranean (southern France and parts of northwest Italy and northeast Spain). We assume that cold-sensitive species retreated towards the southern coasts of Italy and Spain and this assumption is highly supported by the pattern we revealed. Since both species are limited by the 16°C July isotherm, this temperature isotherm was displaced in south Spain and south Italy during the LGM. Consequently, a distribution gap particularly in south France arose. The subdivision of the material in the west Mediterranean suggests occurrence of two putative glacial refugia, one in the east (west Italy) and the second in the west (east Spain). Looking at *H. portulacoides* AFLP-NJ and PCO patterns, the samples from south France show more similarity to those in west Italy and to east Mediterranean clusters (Aegean and Adriatic). This suggests that the material originated from eastern populations which survived the LGM in the eastern Mediterranean and/or south Italy (Figure 4.4a). Accessions in the westernmost part of the Mediterranean (E Spain) which have their own separate AFLP cluster and a high number of ITS substitutions may have survived isolated in that area during the Quaternary.

In *C. maritimum*, the recolonization scenario of the northeastern part in the west Mediterranean is somehow similar to *H. portulacoides*. In spite of the shallow structure in our data from south France and north Italy, samples there show more similarity to those from south Italy suggesting that these populations may have an eastern origins. Figure 4.4b illustrates hypothetical scenarios of recolonization in the west Mediterranean coasts. On the other hand and different from *H. portulacoides*, no east-west Spain subdivision (or further Atlantic-Mediterranean) could be detected, instead, the material from east Spain falls within the whole Atlantic group (Figure 4.4b). We speculate this pattern to be (i) a



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recent range-expansion during the Holocene from southern Spanish coasts to northwards in both directions. This can also be concluded from the nonsignificant IBD and the pronounced difference in  $S_i$  between both materials (0.118, Atlantic; 0.192, Mediterranean) and considering that new populations are usually characterized by lower genetic diversity (Hewitt, 1996; Widmer and Lexer, 2001). (ii) a continuous gene flow and ongoing equilibrium between east and west Spain populations that took place during the LGM and Holocene which consequently resulted in no genetic break to evolve. Quite similar results were obtained by Zardoya *et al.* (2004). They found extensive gene flow between Mediterranean Sea and Atlantic Ocean populations of chub mackerel which are organized into a larger panmictic unit. In contrast, Mediterranean Sea populations of mackerel show some degree of genetic differentiation and are structured along an east–west axis. The analyzed eastern Mediterranean Sea mackerel populations (Greece, Italy) are clearly separated from that of the western Mediterranean Sea (Barcelona), which forms a panmictic unit with eastern Atlantic Ocean populations. No genetic break between Atlantic and Mediterranean scale studies was also revealed in the European Anchovy (*Engraulis encrasicolus*) by Magoulas *et al.* (1996) and in five marine fish of the family Sparidae (seabreams) Bargelloni *et al.* (2003).



**Figure 4.** Suggested post-glacial recolonization routes (colored arrows) in the west Mediterranean region based on AFLP-NJ and PCO clustering patterns in (a) *Halimione portulacoides* and (b) *Crithmum maritimum*. Dotted line represents approximate 16°C isotherm during the LGM. After van Andel (2003).

In summary, genetic break between east vs. west Mediterranean was found in the three species and also in other two coastal plant species (*Cakile maritima* and *Eryngium maritimum*, Westberg and Kadereit unpubl. data). Mediterranean vs. Atlantic subdivision was also obtained in two of our species (*S. kali*, *H. portulacoides*) in addition to *E. maritimum* and *C. maritima* (Westberg and Kadereit unpubl. data).

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The same results are also found in conspecific populations of many marine organisms. For example, in a primary survey using allozyme markers, Mittiangeli *et al.* (2003) revealed a highly significant differentiation between a poor cod population from the Aegean and two other populations in the west Mediterranean. In other study by Bahri-Sfar *et al.* (2000) a well separated cluster and significant divergence was found between eastern and west Mediterranean populations of a sea bass. Another well supported lineage (89% bootstrap) of sand goby using mtDNA in the Adriatic Sea was obtained in an European wide-scale study by Stefanni and Thorley (2003), and in the same study a Mediterranean-Atlantic subdivision was also revealed.

### **The genetic break at the Strait of Gibraltar and the AFLP clustering pattern in the Atlantic Ocean, the North Sea and the Baltic Sea:**

The last group in our study includes the Atlantic Ocean/North Sea/Baltic Sea accessions starting near the Strait of Gibraltar to northwards (except in *C. maritimum*). A pronounced gap in the NJ, PCO and high  $F_{ST}$  values is separating the Atlantic from the Mediterranean material. This gap is most pronounced in *S. kali* ( $F_{ST} = 0.39$ ;  $P < 0.001$ ) and to a lesser extent in *H. portulacoides* ( $F_{ST} = 0.30$ ;  $P < 0.001$ ). However, none of the species' material shows any clear sub-structuring within the Atlantic sampling range ( $\approx 4000 - 5000$  km of coastline). These results are concordant with the findings of Clausen *et al.* (2000) and a population sampling approach of *Eryngium maritimum* (Westberg and Kadereit, unpubl. data). Our results and results in similar studies confirm the existence of a strong barrier to gene flow between southeast and southwest Spain. Both *H. portulacoides* and *S. kali* could have occupied the southern coasts of Spain during the LGM which make the appearance of such a genetic gap difficult to understand. In marine species (e.g. two species of *Chthamalus*, Pannacciulli *et al.* 1997; *Zostera marina*, Olsen *et al.* 2004; a sea star, *Coscinasterias*, Waters and Roy 2003; a cuttlefish, *Sepia officinales*, Perez-Losada *et al.* 1999), an east-west Gibraltar subdivision was observed and attributed to one or both of the following reasons: (i) a historical event represented by a reduction in the width and depth of the Gibraltar Strait during the glacial period (Nilsson, 1982) and due to the decrease in sea level which might have decreased the magnitude of exchanged material at both sides of Gibraltar Strait. (ii) present-day hydrographic barrier; the massive flow of water from the Atlantic Ocean towards the east (14 - 70 cm/s, Salas *et al.* 2001) generates two permanent anticyclonic gyres in what is called Almeria-Oran front (Arnone *et al.* 1990; Figure 4.5). The Almeria-Oran front may be implicated in the observed



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The inland material in *S. kali* is characterized by a separate AFLP cluster that is closer to the east Mediterranean material and identical ITS sequences with east Mediterranean accessions. Possibly, the inland material was originated recently from coastal populations. The Inland form of *S. kali* has been defined as subsp. *ruthenica* (Iljin) Soó, however, after these results, this division is debated especially because different *S. kali* subsp. are characterized by high variation in phenotypic characters at different developmental stages and under different environmental conditions (Rilke and Reimann, 1996).

### Scale of dispersal

Some exceptions in our data set permit to draw conclusions about the scale of long distance dispersal. It is justified to assume that long distance dispersal is taking place within the major phylogenetic group, i.e., in general, individuals included from the same population did not form one cluster (an exception are five samples from the south France population of *H. portulacoides*). Accordingly, the dispersal in general is likely taking place within the major phylogeographic area (Black/Aegeann Sea, Ardiatic Sea, west Mediterranean area and Atlantic Ocean coasts). By excluding artifacts in the AFLP data collection, occasional dispersal over a large distance, i.e., out of the cluster area found, is possible. An example are two neighbour Atlantic samples in *C. maritimum* which fall into the Mediterranean cluster in also a neighbouring position.

### *Calystegia soldanella* AFLP pattern

The AFLP analyses of *C. soldanella* suffered from a technical problem resulting in the use of different primer combinations. Consequently, the gels could not be compared between the two sampling levels, and because of this, different numbers of characters were obtained in each sampling strategy (entire range: 98; populations: 154 characters). However, the percentage of polymorphic AFLP characters was 69% in both sampling approaches which is not distinctly different from two of the studied species (*S. kali*: 76%; *H. portulacoides*: 67%). Also, the mean genetic distances were similar at both the entire range and the population level sampling ( $0.0309 \pm 0.011$  and  $0.0294 \pm 0.0102$ , respectively). These values are marginal lower when compared with other species (see results), but closest to the value obtained in *Eryngium maritimum* (0.0396; Westberg and Kadereit unpubl. data). Thus, it is assumed that the history of *C. soldanella* is of a roughly the same absolute age as that of the other species studied. Moreover, the total amount of genetic variation among seven arbitrary clusters identified in the entire sampling approach was 25.64% ( $F_{ST} = 0.26$ ,  $P < 0.001$ ). This value is also comparable with the other coastal

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species in this study (*H. portulacoides*; 29.3%; *S. kali*, 33.39%; *C. maritimum*, 32.79%), and to other studied species, e.g., *Cakile maritima*; 25.8% (Westberg and Kadereit unpubl. data).

Despite the fact that the three species described above inhabit different ecosystems, they share having a structured pattern of genetic variation with noticeable similarities in many regions. Compared to this, the absence of phylogeographic structure in *C. soldanella* at both the entire range and population level is exceptional. This can easily be seen from the NJ clustering and PCO analysis, in addition to the low significant IBD of the entire range sampling and the nonsignificant IBD at the population level. Accordingly, since all four species are codistributed on a broad scale and were influenced by the same climate and the same abiotic contemporary factors, this makes the absence of spatial structure in genetic data difficult to understand. Here, we will try to explain this unexpected pattern by referring to *C. soldanella* biotic properties which discriminate it from the other species studied.

As concluded from all analyses, there is a strong indication that gene flow is taking place between distant regions. *Calystegia soldanella* is a coastal species that benefits from sea water in its dispersal, and the seeds are able to float for a long time with maintaining viability. To the best of our knowledge, there is no comprehensive study that has investigated the seed buoyancy, viability and germinability of *C. soldanella* after long periods of floating and immersion in sea water. Ridley (1930) documented that 30% of *C. soldanella* seeds float unharmed for 1½ years and 40 - 50% for six months, and in a small scale experiment we recorded 90% seed germination after nine months of immersion in sea water. Accordingly, long buoyancy and exchange of seed material between distant populations can explain the high genetic similarities between individuals which were sampled from widely distant localities. Kim and Chung (1995) found absence of geographic structure between 13 populations of *C. soldanella* from the Korean Peninsula. They assumed that dispersal by seeds via sea water contributes to the relatively low level of genetic divergence between populations. A similar conclusion was reached by Franks *et al.* (2004) in the dune species *Uniola paniculata*. The exceptional clustering of the Adriatic individuals at the population level is likely because the northern coast of the basin is more isolated, thus less gene flow is taking place into or out of it.

Another important factor may be contributing to the lack of geographical structure of the genetic variation is the longevity of the growth type. *Calystegia soldanella* has rhizomes which can grow more than 70 cm under sand cover (Figure 2.7). This provides protection

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to the growing parts which will give rise to new shoots during the growing season and in the overwintering time. This suggests that the individuals (the genet) may be able to live for long period of time and resulting in populations containing genetic material from different and distant origins. It is documented in the literature that some vegetatively propagated species may have the ability to reach very high ages. An example is the huckleberry *Gaylussacia brachycerium* and quaking aspen *Populus muloides* which can live for at least 13,000 and 10,000 years respectively (Cook, 1983).

In summary, seeds long floating time and possibly the high longevity of genets increase the likelihood of long distance dispersal of *C. soldanella* preventing any spatial structure of genetic variation to evolve.

### 5. CONCLUSIONS

Under the broad title “coastal phylogeography”, the patterns we revealed at the scale analyzed and according to our sampling strategy are believed to have resulted from both biotic and abiotic factors. The three species occur in a linear ecosystem, however, along the large distributional range, the species encounter an array of different historical-climatic and contemporary factors which influence the overall genetic pattern.

Based on their northern distribution limits and the modern temperature isotherms and considering the historical isotherms, phylogeographic patterns in the Black/Aegean and Adriatic Sea areas are likely result of undisturbed historical distribution and not to have been influenced by temperature changes during the LGM. Furthermore, differences in genetic pattern in the west Mediterranean coasts are mainly due to differences in each species' summer temperature isotherm during the LGM; the cold-sensitive species (*H. portulacoides* and *C. maritimum*) had to retreat towards southern coasts which is recognized in the subdivision of the material in the west Mediterranean into eastern and western groups. This is not the case in the more cold-tolerant species *S. kali* which shows a uniform group because of undisturbed distribution.

Seed dispersal by sea water and the present-day surface currents explain the genetic breaks and the persistence of the genetic patterns. This generalization agrees with the distinct Black/Aegean Sea group, the distinct Adriatic Sea group and the Gibraltar gap as well. An exception to this is *C. maritimum* along the Spanish coasts.

It is quite surprising to have found great similarity in the genetic patterns between a wide range of marine organisms and coastal plants. This similarity probably implies that dispersal in these plants is mainly by sea currents. This leads also to the conclusion that dispersal of seeds and fruits by sea-water is of overriding importance in shaping the genetic structure of geographical ranges.

Exceptionally, the pattern in the fourth species *C. soldanella* reflects no geographical structure of the genetic variation either at the entire range sampling or population level approach. This is attributed to the remarkable seed dispersal ability and possibly plant longevity and clonal growth. Long floating time of *C. soldanella* seeds in sea water make gene flow across long distances possible and overcome the role of sea current on regional scale dispersal. Plant longevity and clonal growth preserve the mixed pattern of genetic variation build by long distance dispersal over long time periods.

### 6. Summary

The intraspecific phylogeography of four European coastal plants, *Crithmum maritimum*, *Halimione portulacoides*, *Salsola kali* and *Calystegia soldanella*, was inferred from AFLP and ITS data. Only in *C. maritimum*, *H. portulacoides* and *S. kali*, a spatial genetic structure was revealed. The phylogeographic similarities and dissimilarities of these species include: (1) All three have distinct Black/Aegean and Adriatic Sea clusters. (2) *Salsola kali* and *H. portulacoides* show a distinct Atlantic/North Sea/Baltic Sea cluster, while Atlantic and eastern Spanish material of *C. maritimum* clustered together. (3) In the west Mediterranean, only *S. kali* forms a single cluster, while both *H. portulacoides* and *C. maritimum* display a phylogeographic break in the vicinity of the southern French coast. For *S. kali*, AFLP and ITS data concur in identifying separate Atlantic, east and west Mediterranean clades. All these patterns are postulated to result from both temperature changes during the last glacial and contemporary sea currents. No geographic AFLP structure was revealed in *C. soldanella*, both at the range-wide and population level. This was attributed to the remarkable seed dispersal ability of this species and possibly its longevity and clonal growth, preserving a random pattern of genetic variation generated by long-distance seed dispersal over long time periods.

### 7. Zusammenfassung

Die intraspezifische Phylogeographie der vier Küstenpflanzen *Crithmum maritimum*, *Halimione portulacoides*, *Salsola kali* und *Calystegia soldanella* wurde mit Hilfe von AFLPs und ITS-Sequenzen untersucht. Nur bei *C. maritimum*, *H. portulacoides* und *S. kali* wurde eine geographische Struktur der genetischen Variation beobachtet. Folgende phylogeographischen Ähnlichkeiten bzw. Unterschiede wurden festgestellt: (1) alle drei Arten hatten ein distinktes Cluster im Schwarzen/Ägäischen und im Adriatischen Meer. (2) Während *S. kali*- und *H. portulacoides*-Proben ein distinktes Cluster im Atlantischen Ozean/Nordsee/Ostsee bildeten, fielen Proben von *C. maritimum* aus dem Atlantik mit ostspanischem Material zusammen. (3) Im westlichen Mittelmeer bildete nur *S. kali* ein einzelnes Cluster, während *H. portulacoides* und *C. maritimum* im Bereich der südfranzösischen Küste einen phylogeographischen Bruch zeigten. Bei *S. kali* identifizierten AFLP und ITS eine separate atlantische, eine ost- und eine westmediterrane Gruppierung. Diese Muster könnten sowohl aus Temperaturveränderung während der letzten Eiszeit, als auch aus gegenwärtigen Meeresströmungen resultieren. Bei *C. soldanella* wurde weder auf dem Populationsniveau noch im Gesamtverbreitungsgebiet eine geographische Struktur mit AFLP gefunden. Das wurde der außergewöhnlichen Samenausbreitungsfähigkeit, in Kombination mit einer möglichen Langlebigkeit und dem klonalen Wachstum der Art zugerechnet. Diese Faktoren erhalten ein Zufallsmuster der genetischen Variabilität über lange Zeiträume, die durch Samenausbreitung über lange Distanzen hervorgerufen wurde.



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## Appendix I

**Table 1.** List of localities, origin, geographical coordinates and collectors of the plant material in the four species studied. N.A. = not available.

Species	Country	Locality name	Geographical coordinates		Collector / vendor
			North	East-West	
<i>Crithmum maritimum</i>	Turkey	Eregli - Black Sea	41°19'	31°26'	Rami Arafah & Erik Westberg
	=	Bandirma Bay – Marmara Sea	40°23'	27°32'	Rami Arafah & Erik Westberg
	=	Esenköy – Marmara Sea	40°37'	28°57'	Rami Arafah & Erik Westberg
	=	Gaziköy – Marmara Sea	40°43'	27°18'	Rami Arafah & Erik Westberg
	=	Kucukkuya – Aegean Sea	39°31'	26°26'	Rami Arafah & Erik Westberg
	=	Sevketiye – Marmara Sea	40°24'	26°40'	Rami Arafah & Erik Westberg
	Greece	Agria – Aegean Sea	39°21'	22°59'	Rami Arafah & Erik Westberg
	=	Anthidona – Aegean Sea	38°30'	23°26'	Rami Arafah & Erik Westberg
	=	Igoumenitsa – Ionian Sea	39°31'	20°11'	Rami Arafah & Erik Westberg
	=	Makri – Aegean Sea	38°00'	23°05'	Rami Arafah & Erik Westberg
	=	Olimbiada – Aegean Sea	40°35'	23°50'	Rami Arafah & Erik Westberg
	=	Palea Epidavros – Aegean Sea	37°38'	23°10'	Rami Arafah & Erik Westberg
	=	Aktio / Prevesa – Aegean Sea	38°56'	20°45'	Rami Arafah & Erik Westberg
	=	Githio – South Aegean	36°46'	22°34'	Rami Arafah & Erik Westberg
	Italy	Egnazia - Adriatic Sea	40°54'	17°22'	Rami Arafah & Erik Westberg
	=	Gargano - Adriatic Sea	N.A	N.A	W. Licht – Uni. Mainz
	=	Laguna di Vinicia (Cavallino)- Adriatic Sea	45°28'	12°34'	J. W. Kadereit- Uni. -Mainz
	=	Marina di Montenero - Adreatic Sea	42°04'	14°48'	Rami Arafah & Erik Westberg
	=	Marina di Savito - Adriatic Sea	42°18'	12°51'	Rami Arafah & Erik Westberg
	=	Porto Civitiana - Adriatic Sea	43°18'	13°44'	Rami Arafah & Erik Westberg
	=	Trieste - Adriatic Sea	54°36'	13°47'	Rami Arafah & Erik Westberg
	=	Longobardi – Adriatic Sea	39°12'	16°04'	Rami Arafah & Erik Westberg
	=	Vilasimum - Sardinia	39°08'	9°03'	Rami Arafah & Erik Westberg
	=	Villamare – W. Mediterranean Sea	40°05'	15°31'	Rami Arafah & Erik Westberg
	=	Rocella – W. Mediterranean Sea	38°20'	16°24'	Rami Arafah & Erik Westberg
	=	Moneglia – W. Mediterranean Sea	44°14'	9°29'	Rami Arafah & Erik Westberg
	=	Parco Nazionale del Circe – W. Italy	41°22'	12°57'	Rami Arafah & Erik Westberg
	=	Bergeggio – W. Mediterranean Sea	44°14'	8°27'	Rami Arafah & Erik Westberg
	=	Albinia – W. Mediterranean Sea	42°30'	11°11'	Rami Arafah & Erik Westberg
	=	Pyros (Pyrgi) – W. Mediterranean Sea	42°02'	11°57'	Rami Arafah & Erik Westberg
	=	San Ferdinando - W. Mediterranean Sea	38°30'	15°45'	Rami Arafah & Erik Westberg
	=	Santa Polina - Sicily	38°01'	14°10'	Rami Arafah & Erik Westberg
	Croatia	Senj- Adriatic Sea	45°00'	14°54'	Erik Westberg
=	Split- Adriatic Sea	43°30'	16°27'	Erik Westberg	

## Appendix I

	=	Zadar- Adriatic Sea	44°06'	15°15'	Erik Westberg
	France	Gines – W. Mediterranean Sea	43°02'	6°06'	Rami Arafah & Erik Westberg
	=	St. Aygulf – W. Mediterranean Sea	43°24'	6°44'	Rami Arafah & Erik Westberg
	=	Pen-Ar-Ran- Atlantic Ocean	47°22'	-2°33'	Rami Arafah & Erik Westberg
	=	Port Loius - Atlantic Ocean	47°49'	-3°17'	Rami Arafah & Erik Westberg
	=	Beg Legiur – Atlantic Ocean	48°44'	-3°32'	Rami Arafah & Erik Westberg
	=	Natinal Parc de la Joie – Atlantic Ocean	48°40'	-3°39'	Rami Arafah & Erik Westberg
	=	Le Collet – Atlantic Ocean	47°02'	-2°02'	Rami Arafah & Erik Westberg
	Portugal	Armacao de Pera – Atlantic Ocean	37°06'	-8°21'	Rami Arafah & Erik Westberg
	=	Plaja de Vicera – Atlantic Ocean	39°52'	-8°59'	Rami Arafah & Erik Westberg
	=	Porto Covo Atlantic Ocean	37°52'	-8°47'	Rami Arafah & Erik Westberg
	=	Largo de Praja da Arguda – Atlantic Ocean	38°51'	-9°28'	Rami Arafah & Erik Westberg
	Spain	Las Alberquilles – W. Mediterranean Sea	36°44'	-3°48'	Rami Arafah & Erik Westberg
	=	Cueta- S. Spain	35°54'	-5°17'	Rami Arafah & Erik Westberg
	=	Punto de Tordera – Mediterranean	41°39'	2°46'	Rami Arafah & Erik Westberg
	=	Rinlo – Atlantic Ocean	43°33'	-7°05'	Rami Arafah & Erik Westberg
	=	Ruimar Platja – Mediterranean	40°43'	0°50'	Rami Arafah & Erik Westberg
	=	Plaja del Madraba- Mediterranean	38°51'	0°01'	Rami Arafah & Erik Westberg
	=	Sant Pol del Mar – Mediterranean	41°36'	2°37'	Rami Arafah & Erik Westberg
	=	Plaja de Layda- Mediterranean	43°24'	-2°40'	Rami Arafah & Erik Westberg
	=	Ailla d'Arrousa – Atlantic Ocean	42°32'	-8°52'	Rami Arafah & Erik Westberg
	=	Plaja de la Barrosa – Mediterranean	36°21'	-6°10'	Rami Arafah & Erik Westberg
	=	Paredes – Atlantic Ocean	43°24'	-8°12'	Rami Arafah & Erik Westberg
	=	Plaja de San Miquel – Mediterranean	36°43'	-2°52'	Rami Arafah & Erik Westberg
	=	Plaja Espasa – Atlantic Ocean	43°29'	-5°13'	Rami Arafah & Erik Westberg
	=	Linecres- Atlantic Ocean	43°26'	-3°58'	Rami Arafah & Erik Westberg
	England	Sandwich	51°17'	1°23'	Rami Arafah & Erik Westberg
	=	Lullworth Cove	50°37'	-2°14'	Rami Arafah & Erik Westberg
	=	Little Hampton	50°47'	-0°34'	Rami Arafah & Erik Westberg
	=	Sandy Bay	51°22'	-2°57'	Rami Arafah & Erik Westberg



## Appendix I

Species	Country	Locality name	Geographical coordinates		Collector / vendor
			North/	East- West	
<i>Salsola kali</i>	Turkey	Ignaeda – Black Sea	41°53'	28°03'	Rami Arafeh & Erik Westberg
	=	Sahilköy – Black Sea	41°12'	29°23'	Rami Arafeh & Erik Westberg
	=	Karasu – Black Sea	41°08'	30°40'	Rami Arafeh & Erik Westberg
	=	Eregli - Black Sea	41°19'	31°26'	Rami Arafeh & Erik Westberg
	=	Yaluva – Marmara Sea	40°39'	29°12'	Rami Arafeh & Erik Westberg
	=	Bandirma Bay – Marmara Sea	40°23'	27°23'	Rami Arafeh & Erik Westberg
	=	Sevketeye – Marmara Sea	40°24'	26°40'	Rami Arafeh & Erik Westberg
	=	Kumkale – Marmara Sea	40°01'	26°11'	Rami Arafeh & Erik Westberg
	Greece	Erasmio Beach – Aegean Sea	40°52'	24°50'	Rami Arafeh & Erik Westberg
	=	Potamos – Aegean Sea	40°23'	22°55'	Rami Arafeh & Erik Westberg
	=	Nei Anchialos – Aegean Sea	39°15'	22°49'	Rami Arafeh & Erik Westberg
	=	Anthidona – Aegean Sea	38°30'	23°26'	Rami Arafeh & Erik Westberg
	=	Nafplio – Aegean Sea	37°35'	22°45'	Rami Arafeh & Erik Westberg
	=	Lehena – Ionian Sea	37°59'	21°15'	Rami Arafeh & Erik Westberg
	=	Igoumenitsa – Ionian Sea	39°31'	20°11'	Rami Arafeh & Erik Westberg
	Croatia	Split – Adriatic Sea	43°31'	16°27'	Rami Arafeh & Erik Westberg
	Italy	Marina Romea – Adriatic Sea	44°31'	12°16'	Rami Arafeh & Erik Westberg
	=	Marina de Lisina – Adriatic Sea	41°53'	15°26'	Rami Arafeh & Erik Westberg
	=	Egnazia – Adriatic Sea	40°54'	17°22'	Rami Arafeh & Erik Westberg
	=	Punta Alicia – S. Italy	39°24'	17°09'	Rami Arafeh & Erik Westberg
	=	Locri – S. Italy	38°20'	16°24'	Rami Arafeh & Erik Westberg
	=	Foce di Simeto - Sicily	37°24'	15°06'	Rami Arafeh & Erik Westberg
	=	Villamare – S. Italy	40°04'	15°35'	Rami Arafeh & Erik Westberg
	=	Albigna – W. Italy	42°30'	11°11'	Rami Arafeh & Erik Westberg
	=	Olmaia – W. Italy	43°09'	10°31'	Rami Arafeh & Erik Westberg
	=	St. Salvador – W. Mediterranean	41°11'	1°32'	Rami Arafeh & Erik Westberg
	Spain	Playa el Serradal – W. Mediterranean	40°00'	-0°01'	Rami Arafeh & Erik Westberg
	=	Playa de Olivia – W. Mediterranean	38°55'	-0°04'	Rami Arafeh & Erik Westberg
	=	Playa la Cabana – W. Mediterranean	37°23'	-1°37'	Rami Arafeh & Erik Westberg
	=	Playa la San Miguel – W. Mediterranean	36°42'	-2°48'	Rami Arafeh & Erik Westberg
	=	Cueta – N. Africa	35°54'	-5°17'	Rami Arafeh & Erik Westberg
	=	Playa de las Lanches	36°01'	-5°37'	Rami Arafeh & Erik Westberg
=	Playa el Hoyo	37°12'	-7°17'	Rami Arafeh & Erik Westberg	

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	Portugal	Cabadelo	41°41'	-8°50'	Rami Arafah & Erik Westberg
	Spain	Ailla d'Arrousa	42°32'	-8°52'	Rami Arafah & Erik Westberg
	=	Playa la Espasa	43°29'	-5°13'	Rami Arafah & Erik Westberg
	=	Playa de Layda	43°24'	-2°39'	Rami Arafah & Erik Westberg
	France	Les Jars	46°21'	-1°22'	Rami Arafah & Erik Westberg
	=	Bourgneut en Rete	47°02'	-2°02'	Rami Arafah & Erik Westberg
	=	St. Efflam	48°44'	-3°16'	Rami Arafah & Erik Westberg
	=	St. Benoit des Ondes	48°37'	-1°51'	Rami Arafah & Erik Westberg
	=	Le Crotoy	50°14'	1°37'	Rami Arafah & Erik Westberg
	England	Sandwich	51°16'	1°20'	Rami Arafah & Erik Westberg
	=	Mappelton	53°52'	-0°08'	Bernhard von Hagen – Uni. Halle
	=	St. Andrews	56°20'	-2°47'	H. Peter Comes – Uni. Mainz
	=	Romney Sand	50°58'	0°58'	Rami Arafah & Erik Westberg
	=	Wittering	50°46'	-0°55'	Rami Arafah & Erik Westberg
	=	Exmouth	50°35'	-3°23'	Rami Arafah & Erik Westberg
	=	Braunton Burrows	51°04'	-4°12'	Rami Arafah & Erik Westberg
	=	Sandy Bay	51°22'	-2°56'	Rami Arafah & Erik Westberg
	Holland	Hoofplaat	51°22'	3°39'	Rami Arafah & Erik Westberg
	Germany	St. Peter Ording	54°18'	8°38'	J. W. Kadereit & G. Kadereit
	=	Heligen Hafen	54°22'	10°58'	J. W. Kadereit & G. Kadereit
	=	Fehrman	54°30'	11°13'	J. W. Kadereit & G. Kadereit
	=	Boinsdorf	54°01'	11°32'	J. W. Kadereit & G. Kadereit
	=	Prerow	54°26'	12°34'	J. W. Kadereit & G. Kadereit
	=	Zinnowitz	54°04'	13°54'	J. W. Kadereit & G. Kadereit
	=	Gonsenheim	50°00'	8°11'	Natalie Schmalz
	=	Budenheim	50°01'	8°10'	Natalie Schmalz
	Sweden	Nyehusen	55°50'	14°13'	Erik Westberg
	=	Lernacken	55°33'	12°53'	Erik Westberg
	=	Viken	56°09'	12°34'	Erik Westberg
	Denmark	Løgstør	56°58'	9°15'	Erik Westberg
	=	Jerup strand	57°31'	10°25'	Erik Westberg
	Hungary	Soroksar	47°24'	19°07'	Maria Höhn - Corvinus University of Budapest
	=	Tahito	47°54'	19°06'	Maria Höhn - Corvinus University of Budapest
	Iran	Isfahan	32°00'	57°30'	Iraj Mehregan

## Appendix I

Species	Country	Locality name	Geographical coordinates		Collector / vendor
			North/ East-	West	
<i>Calystegia soldanella</i>	Turkey	Akpinar	41°18'	28°49'	Rami Arafah & Erik Westberg
	=	Sahilköy	41°12'	29°23'	Rami Arafah & Erik Westberg
	=	Yeniköy Plage	40°23'	28°24'	Rami Arafah & Erik Westberg
	=	Gonen	40°19'	27°37'	Rami Arafah & Erik Westberg
	=	Ezine	39°45'	26°07'	Rami Arafah & Erik Westberg
	Greece	Erasmio Beach	40°52'	24°50'	Rami Arafah & Erik Westberg
	=	Igoumenitsa	39°31'	20°11'	Rami Arafah & Erik Westberg
	=	Lehena	37°59'	21°15'	Rami Arafah & Erik Westberg
	=	Olimbiada	40°35'	23°50'	Rami Arafah & Erik Westberg
	=	Evia – Oreio – Aegan Sea	38°57'	23°06'	Rami Arafah & Erik Westberg
	Italy	Foce di Simeto	37°24'	15°06'	Rami Arafah & Erik Westberg
	=	Laguna di Vinicia / Cavallino	45°28'	12°34'	J. W. Kadereit & G. Kadereit
	=	Porto Garibaldi	44°40'	12°14'	J. W. Kadereit & G. Kadereit
	=	Macchia di Migliorino	43°49'	10°16'	Rami Arafah & Erik Westberg
	=	Marina di Lesina	41°54'	15°21'	Rami Arafah & Erik Westberg
	=	Marina di Pisticci	40°18'	16°47'	Rami Arafah & Erik Westberg
	=	Marina Romea	44°31'	12°17'	Rami Arafah & Erik Westberg
	=	Mondragone	41°07'	13°53'	Rami Arafah & Erik Westberg
	=	Parco Nazionale del Circe	41°23'	12°55'	Rami Arafah & Erik Westberg
	=	Porto Civitanova	43°18'	13°44'	Rami Arafah & Erik Westberg
	=	San Ferdinando	38°3'	15°54'	Rami Arafah & Erik Westberg
	France	Bourgneuf	47°02'	-2°02'	Rami Arafah & Erik Westberg
	=	Labenne Ocean	43°37'	-1°27'	Rami Arafah & Erik Westberg
	=	Las Castellas	43°21'	3°33'	Rami Arafah & Erik Westberg
	=	Le Crotoy	50°14'	1°37'	Rami Arafah & Erik Westberg
	=	Les Jars	46°21'	-1°22'	Rami Arafah & Erik Westberg
	=	Les Sables d'Or	48°39'	-2°25'	Rami Arafah & Erik Westberg
	=	N. Parc de la Joie	48°4'	-3°39'	Rami Arafah & Erik Westberg
	=	Piscarrose Plage	44°27'	-1°15'	Rami Arafah & Erik Westberg
	=	Plaga N. Hourtin Ocean	45°13'	1°10'	Rami Arafah & Erik Westberg
	=	Ars en Re	49°23'	-1°00'	Rami Arafah & Erik Westberg
	=	Plage l'Aber	48°14'	-4°26'	Rami Arafah & Erik Westberg
	=	Port Louis	47°41'	-3°17'	Rami Arafah & Erik Westberg
=	Agon Countin Ville	49°03'	-1°36'	Rami Arafah & Erik Westberg	
=	Utah beach museum	49°25'	-1°10'	Rami Arafah & Erik Westberg	
Spain	Ailla d' Arrousa	42°32'	-8°51'	Rami Arafah & Erik Westberg	

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	=	Lieneres	43°26'	-3°58'	Rami Arafah & Erik Westberg
	=	Menio	43°21'	-8°12'	Rami Arafah & Erik Westberg
	=	Cadiz – S. Spain	36°10'	-6°00'	Rami Arafah & Erik Westberg
	=	Platja de Olivia	38°55'	-0°04'	Rami Arafah & Erik Westberg
	=	Playa la Espasa	43°29'	-5°13'	Rami Arafah & Erik Westberg
	=	Playa de Navia	43°34'	-6°43'	Rami Arafah & Erik Westberg
	=	Rio oka (Playa de Layda)	43°24'	-2°41'	Rami Arafah & Erik Westberg
	Portugal	Armacao de Pera	37°06'	-8°21'	Rami Arafah & Erik Westberg
	=	Foz do Arelho	39°26'	-9°13'	Rami Arafah & Erik Westberg
	=	Cabeledo	41°41'	-8°50'	Rami Arafah & Erik Westberg
	=	Torreira	40°45'	-8°42'	Rami Arafah & Erik Westberg
	=	Santa Andrea	38°07'	-8°48'	Rami Arafah & Erik Westberg
	=	Praia de Vieira	39°53'	-8°59'	Rami Arafah & Erik Westberg
	England	Studland	50°39'	-1°57'	Rami Arafah & Erik Westberg
	=	Sandwich	51°16'	1°20'	Rami Arafah & Erik Westberg
	=	Widmouth	51°13'	-4°04'	Rami Arafah & Erik Westberg
	New Zealand				

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Species	Country	Locality name	Geographical coordinates		Collector / vendor
			North/ East-	West	
<i>Halimione portulacoides</i>	Turkey	Gömec	39°24'	26°46'	Rami Arafeh & Erik Westberg
	=	Küçükkuyu	39°31'	26°26'	Rami Arafeh & Erik Westberg
	=	Cardak	40°23'	26°24'	Rami Arafeh & Erik Westberg
	=	Gallipoli	40°36'	38498	Rami Arafeh & Erik Westberg
	=	Bandirma Bay	40°23'	27°23'	Rami Arafeh & Erik Westberg
	=	Yeniciftlik	40°59'	27°51'	Rami Arafeh & Erik Westberg
	Greece	Evia – Oreoi	38°57'	23°05'	N. A.
	=	Githio	36°46'	22°34'	Rami Arafeh & Erik Westberg
	=	Nei Poroi	39°59'	22°40'	Rami Arafeh & Erik Westberg
	=	Nei Anchialos	39°15'	22°49'	Rami Arafeh & Erik Westberg
	=	Makri	40°51'	25°41'	Rami Arafeh & Erik Westberg
	=	Potamos	40°23'	22°55'	Rami Arafeh & Erik Westberg
	=	Strimonas	40°47'	23°51'	Rami Arafeh & Erik Westberg
	=	Erasmio	40°52'	24°50'	Rami Arafeh & Erik Westberg
	=	Aktio	38°56'	20°45'	Rami Arafeh & Erik Westberg
	=	Lehena	37°59'	21°15'	Rami Arafeh & Erik Westberg
	=	Nafplio	37°35'	22°45'	Rami Arafeh & Erik Westberg
	=	Iqoumenitsa	39°31'	20°11'	Rami Arafeh & Erik Westberg
	Croatia	Split	43°31'	16°27'	Rami Arafeh & Erik Westberg
	=	Zadar	44°06'	15°15'	Rami Arafeh & Erik Westberg
Italy	Riva Longa	45°45'	13°31'	Rami Arafeh & Erik Westberg	
=	Marina Romea	44°31'	12°16'	Rami Arafeh & Erik Westberg	
=	Porto Civitiana	43°18'	13°44'	Rami Arafeh & Erik Westberg	
=	Gargano	N. A.	N. A.	Dr. W. Licht – Uni. Mainz	
=	Egnazia	40°51'	17°24'	Rami Arafeh & Erik Westberg	
=	Albinia	42°30'	11°11'	Rami Arafeh & Erik Westberg	
=	Parco Nazionale del Circe	41°23'	12°55'	Rami Arafeh & Erik Westberg	
Spain	Ruimar Platja	40°43'	-0°50'	Rami Arafeh & Erik Westberg	
=	Mar Menor	37°43'	-0°44'	Rami Arafeh & Erik Westberg	
=	Playa de la Serradal	40°00'	-0°01'	Rami Arafeh & Erik Westberg	
=	El Pinet	38°09'	-0°37'	Rami Arafeh & Erik Westberg	
=	Playa de la Barrosa	36°21'	-6°12'	Rami Arafeh & Erik Westberg	
=	Isla Christina	37°12'	-7°19'	Rami Arafeh & Erik Westberg	
=	Algeciras River	36°10'	-5°27'	Rami Arafeh & Erik Westberg	
=	Playa de Navia	43°33'	-6°43'	Rami Arafeh & Erik Westberg	
=	La Algarda	36°49'	-6°20'	Rami Arafeh & Erik Westberg	

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	=	Puerto del Rev	37°12'	-1°48'	Rami Arafeh & Erik Westberg
	=	Ailla d'Arrosa	42°32'	-8°52'	Rami Arafeh & Erik Westberg
	=	Miño	43°21'	-8°12'	Rami Arafeh & Erik Westberg
	=	Playa de Layda	43°24'	-2°40'	Rami Arafeh & Erik Westberg
	Portugal	Linecres	43°26'	-3°58'	Rami Arafeh & Erik Westberg
	=	Armacao de Pera	37°06'	-8°21'	Rami Arafeh & Erik Westberg
	=	Bestiada	40°46'	-8°41'	Rami Arafeh & Erik Westberg
	=	Foz de Arelho	39°27'	-9°13'	Rami Arafeh & Erik Westberg
	France	Port Loius	47°49'	-3°17'	Rami Arafeh & Erik Westberg
	=	Charron	46°18'	-1°08'	Rami Arafeh & Erik Westberg
	=	Le Daurdoff	48°37'	-3°50'	Rami Arafeh & Erik Westberg
	=	Le Greves	48°30'	-2°41'	Rami Arafeh & Erik Westberg
	=	St. Mare de la Mer	43°27'	4°27'	Rami Arafeh & Erik Westberg
	=	Berriaud Plage	43°06'	6°12'	Rami Arafeh & Erik Westberg
	=	Agde	43°17'	3°26'	Rami Arafeh & Erik Westberg
	=	Canet	42°40'	3°02'	Rami Arafeh & Erik Westberg
	=	Road D-92 / Saille	47°18'	-2°26'	Rami Arafeh & Erik Westberg
	=	Neyran	45°30'	-1°05'	Rami Arafeh & Erik Westberg
	=	Avdenge	44°41'	-1°00'	Rami Arafeh & Erik Westberg
	=	Mont St. Micheal	48°37'	-1°30'	Rami Arafeh & Erik Westberg
	=	Plage de Alber	48°14'	-4°26'	Rami Arafeh & Erik Westberg
	=	Grd. Fort Phillip	51°00'	2°04'	Rami Arafeh & Erik Westberg
	England	Little Hampton	50°47'	-0°34'	Rami Arafeh & Erik Westberg
	=	Walpersweck	52°18'	1°39'	Bernhard von Hagen – Uni. Halle
	=	Braunton Burrows	51°06'	-4°13'	Rami Arafeh & Erik Westberg
	=	Lullworth	50°37'	-2°14'	Rami Arafeh & Erik Westberg
	=	River Otter's mouth	50°37'	-3°18'	Rami Arafeh & Erik Westberg
	=	Sandwich	51°16'	1°20'	Rami Arafeh & Erik Westberg
	Germany	St. Peter Ording	54°18'	8°38'	J. W. Kadereit and G. Kadereit
	=	Langeness	54°38'	8°35'	J. W. Kadereit and G. Kadereit
	=	Norderney	53°43'	7°15'	J. W. Kadereit and G. Kadereit
	=	Harlesiel	53°42'	7°48'	J. W. Kadereit and G. Kadereit
	Israel	Yerekon River	34°54'	32°08'	Ori Fragman – Hebrew University

## Appendix II

### - List of primers and adapter molecules used in the AFLP analysis:

**Table 1** Primers coding, fluorescent label and sequences used in the AFLP analysis: (A) = adapter, (PS) = Preselective primer, (S) = Selective primer. Underlined letters indicate selective site.

Primer	Florescent labeled	Sequence (5'-3')
A-M (-)	-----	GAC GAT GAG TCC TGA G
A-M (+)	-----	TAC TCA GGA CTC AT
A-E (-)	-----	CTC GTA GAC TGC GTA CC
A-E (+)	-----	AAT TGG TAC GCA GTC
PS-E01	-----	GAC TGC GTA CCA ATT CA
PS-M02	-----	GAT GAG TCC TGA GTA AG
S-E40	Yellow / NED	GAC TGC GTA CCA ATT CA <u>GC</u>
S-E37	Yellow / NED	GAC TGC GTA CCA ATT CA <u>CG</u>
S-E38	Blue / 6-FAM	GAC TGC GTA CCA ATT CAC <u>T</u>
S-E39	Blue / 6-FAM	GAC TGC GTA CCA ATT CA <u>GA</u>
S-E45	Green / HEX	GAC TGC GTA CCA ATT CA <u>TG</u>
S-E33	Green / HEX	GAC TGC GTA CCA ATT CA <u>AG</u>
S-M54	-----	GAT GAG TCC TGA GTA AC <u>CT</u>
S-M55	-----	GAT GAG TCC TGA GTA AC <u>GA</u>
S-M57	-----	GAT GAG TCC TGA GTA AC <u>GG</u>
S-M61	-----	GAT GAG TCC TGA GTA AC <u>IG</u>

### I) AFLP template preparation:

DNA template (5.0µl ≈150-200ng) was simultaneously restricted and ligated in 10.0µl reaction mixture. The mixture components were split into two parts: Restriction Reaction Mixtures 1 and 2 (RLM-1 and RLM-2; Table 2). Afterwards they mixed with template DNA in microtiter plates (96 wells). Plates were incubated for 15 hours at 22°C then restricted-ligated reaction mix was diluted by adding 30 µl of DEPC-H<sub>2</sub>O.

**Table 2** Restriction Ligation Mixtures 1 (RLM-1) and (RLM-2). Amounts include 10% pipetting error.

<b>RLM-1</b>		
		Amount / sample
1	DS H <sub>2</sub> O	0.6575 µl
2	T <sub>4</sub> DNA ligase buffer 10X	0.88 µl
3	0.5 M NaCl	0.88 µl
4	BSA (10 mg/ml) 100X	0.088 µl
5	<i>MseI</i> Adapter Pair 50 pmol/µl	0.525 µl
6	<i>EcoRI</i> Adapter Pair 5.0 pmol/µl	0.525 µl
		<b>3.2 µl</b>
<b>RLM-2</b>		
1	DS H <sub>2</sub> O	1.388 µl
2	T <sub>4</sub> DNA ligase buffer 10X	0.18 µl
3	0.5 M NaCl	0.18 µl
4	BSA (10 mg/ml) 100X	0.0180 µl
5	<i>MseI</i> (10 U/µl)	0.0838 µl
6	<i>EcoRI</i> (20 U/µl)	0.11 µl
7	T4 DNA Ligase (400 U/µl)	0.001 µl
		<b>1.8 µl</b>

### II) Preselective amplification:

After diluting the RLM mix, only 5.0µl were used as a template for the next step (preselective amplification). Preselective amplification components and amounts are listed in Table 3.

## Appendix II

**Table 3** Components and amounts of the preselective amplification (in  $\mu\text{l}$ /reaction)

1	DS H <sub>2</sub> O	15.4 $\mu\text{l}$
2	PCR-Puffer (10X)	2.5 $\mu\text{l}$
3	MgCl <sub>2</sub> (50 mM)	1.25 $\mu\text{l}$
4	dNTPs (20 mM)	0.25 $\mu\text{l}$
5	Primer M02 (50 ng/ $\mu\text{l}$ )	0.25 $\mu\text{l}$
6	Primer E01 (50 ng/ $\mu\text{l}$ )	0.25 $\mu\text{l}$
7	Taq polymerase (5 u/ $\mu\text{l}$ )	0.1 $\mu\text{l}$
8	DNA from RLM reaction	5.0 $\mu\text{l}$
	<b>Total</b>	<b>25.0 <math>\mu\text{l}</math></b>

PCR reaction protocol: The primers PS-E01 and PS-M02 were designed in complementary pattern in order to anneal at their proper adapters in addition to one extra base at the end to provide selectivity (amplifying part of the restricted-ligated DNA fragments). PCR reactions (Table 4) were performed in the thermal cycler (Biotherm-Biometra–Thermogradiend cycler) or (PTC-100, MJ Research Inc.).

**Table 4** PCR protocol implemented in the Preselective amplification

Temp	Time	No of cycles
72°C	2 min.	1
94°C	10 sec.	20
56°C	30 sec.	
72°C	2 min.	
60°C	30 min.	
4°C	$\infty$	-

PCR product was then diluted in 1:10 v/v with DEPC-H<sub>2</sub>O for the next reaction.

### III) Selective amplification:

In order to get a moderate amount of amplified products (bands), selective amplification protocol is based on labeling primers that anneal to the rare-cut ends only. Fluorescent primers (S-E) were labeled from ABI Prism-Applied Biosystems, Applera GmbH, Germany. Selective primers were also designed to contain two extra bases to provide further selectivity (Table 1.2). The reaction was performed three times independently using different fluorescent-colored primers; green (HEX), blue (6-FAM), and yellow (NED). The Selective amplification reaction was performed in 15 $\mu\text{l}$  volumes. Components and amounts of the reaction are listed in table 5 and PCR details are listed in table 6.

**Table 5** Components of the selective amplification ( in  $\mu\text{l}$ /reaction)

1	DS H <sub>2</sub> O	7.12 $\mu\text{l}$
2	PCR-Puffer (10X)	1.5 $\mu\text{l}$
3	MgCl <sub>2</sub> (50 mM)	0.75 $\mu\text{l}$
4	dNTPs (20 mM)	0.15 $\mu\text{l}$
5	Msel primer Mxx (50 ng/ $\mu\text{l}$ )	0.25 $\mu\text{l}$
6	EcoRI Dye primer Exx (50 ng/ $\mu\text{l}$ )	0.18 $\mu\text{l}$
7	Taq polymerase (5 u/ $\mu\text{l}$ )	0.05 $\mu\text{l}$
8	DNA from Preselective reaction	5.0 $\mu\text{l}$
	<b>Total</b>	<b>15.0 <math>\mu\text{l}</math></b>



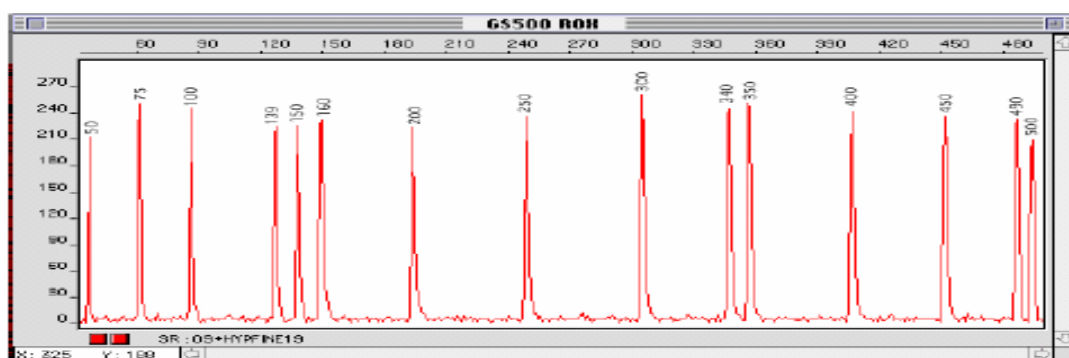
## Appendix II

**Table 6** PCR protocol of the selective amplification

1	94°C X 2min		
2	94°C X 10 sec	64°C X 30 sec	72°C X 2 min
3	94°C X 10 sec	63°C X 30 sec	72°C X 2 min
4	94°C X 10 sec	62°C X 30 sec	72°C X 2 min
5	94°C X 10 sec	61°C X 30 sec	72°C X 2 min
6	94°C X 10 sec	60°C X 30 sec	72°C X 2 min
7	94°C X 10 sec	59°C X 30 sec	72°C X 2 min
8	94°C X 10 sec	58°C X 30 sec	72°C X 2 min
9	94°C X 10 sec	57°C X 30 sec	72°C X 2 min
10	94°C X 10 sec	56°C X 30 sec	72°C X 2 min
11	60°C X 30 min		
12	4°C X ∞		

**- Preparation of reaction mixture for Acrylamide gel and gel run conditions:**

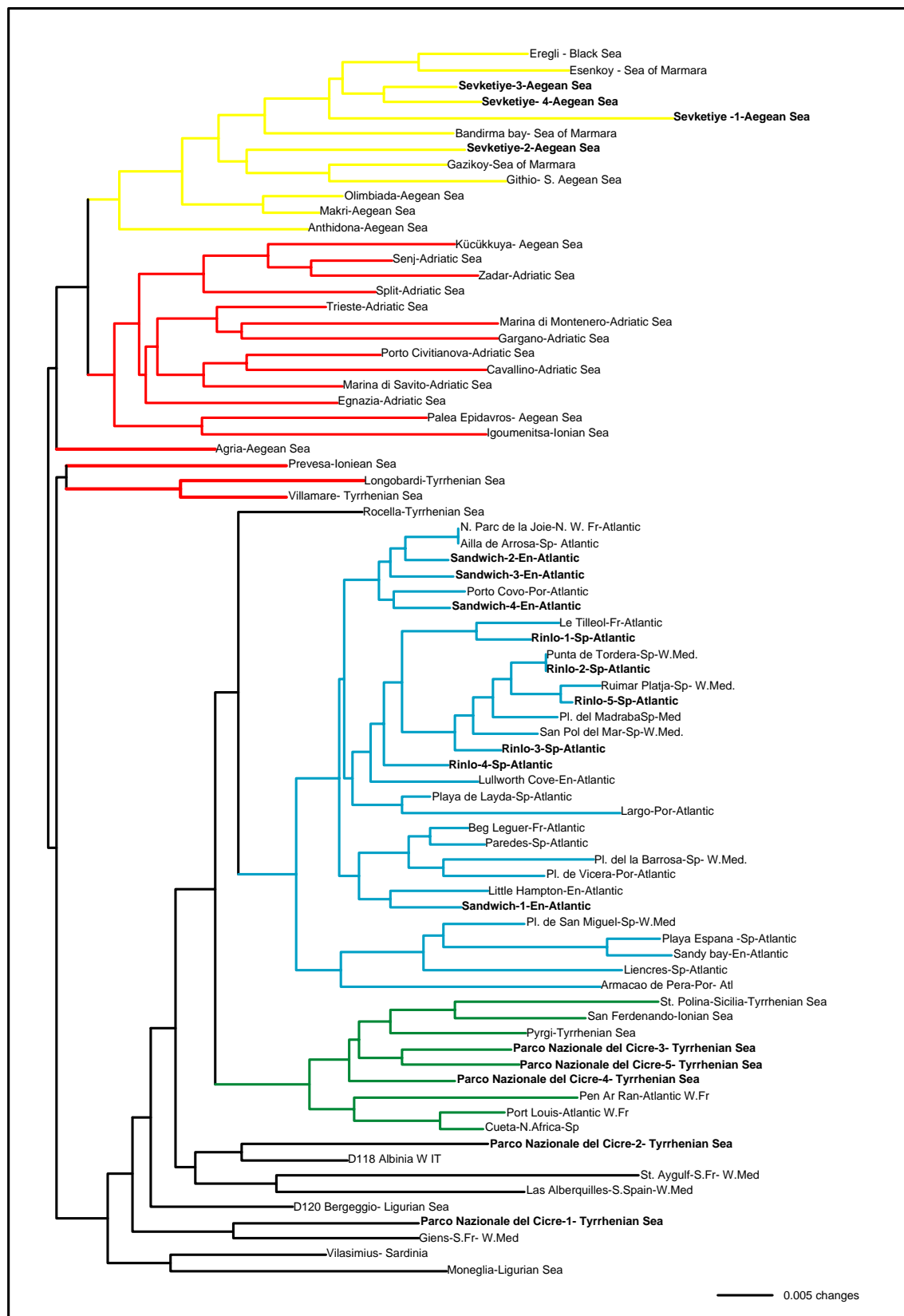
For each individual, the three selective amplification reactions of the three colors were mixed at 1.5, 2.25, and 2.25µl from blue, yellow and green, respectively, then only 2.0µl of the mixture were loaded on the Acrylamide gel. Mixture of formamide, ABI loading buffer, and GS ROX-500 standard (500 bp) were added to the reaction multiplex. ABI sequencing machine was set to fragment analysis setting and sampled loaded and run under the following conditions: Voltage: 3000 volts, 60.0 mA. Gel Temp: 51°C. Laser Power: 40.0 mW. Run Time: 8.00 hours.



**Figure 1** ROX-500 standard used to adjust the size of AFLP fragment in each slot. Numbers on peaks are fragment size in bases.

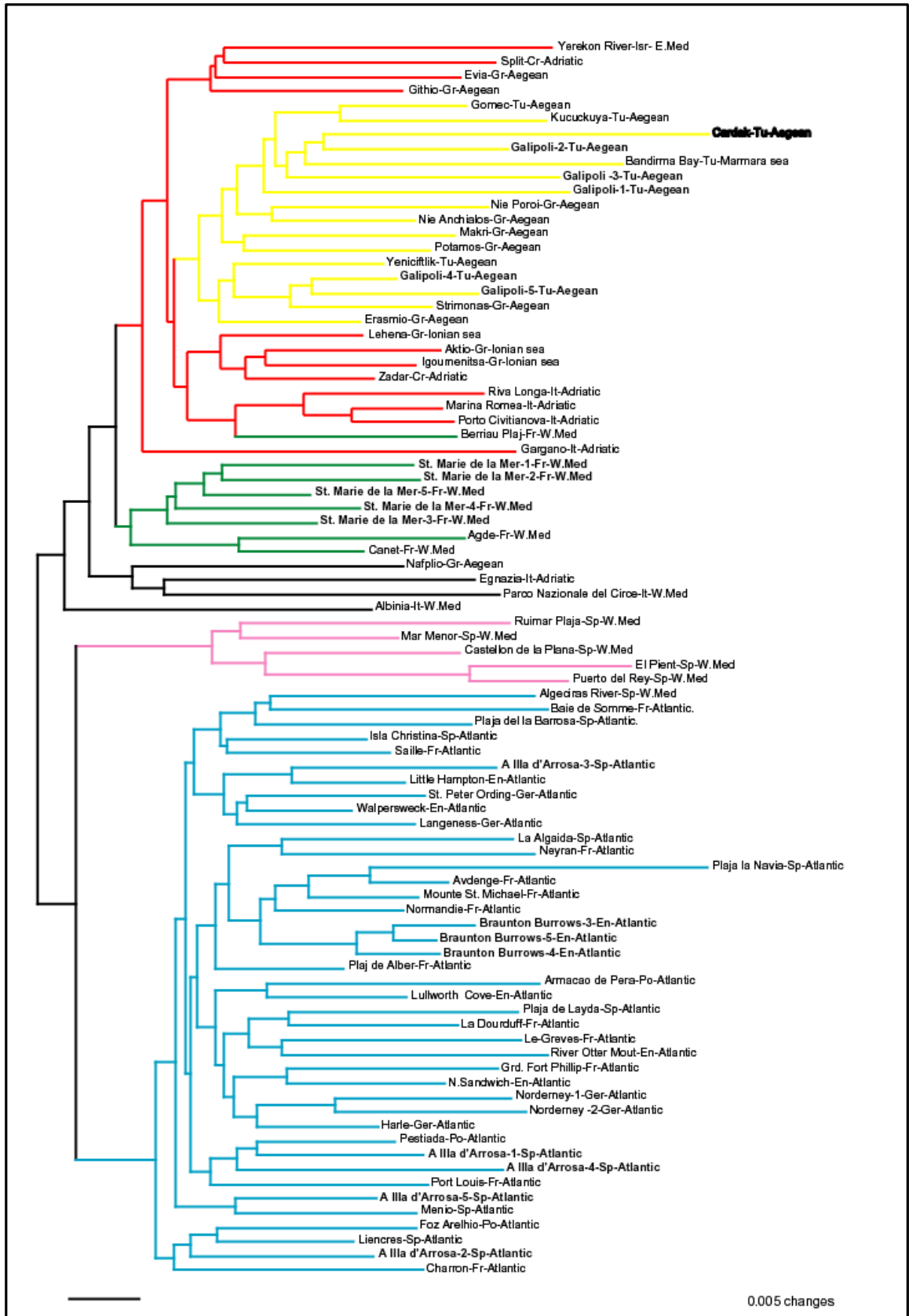
Peak amplitude thresholds were for BLUE (60), GREEN (30), YELLOW (40), and RED (40).

## Appendix III



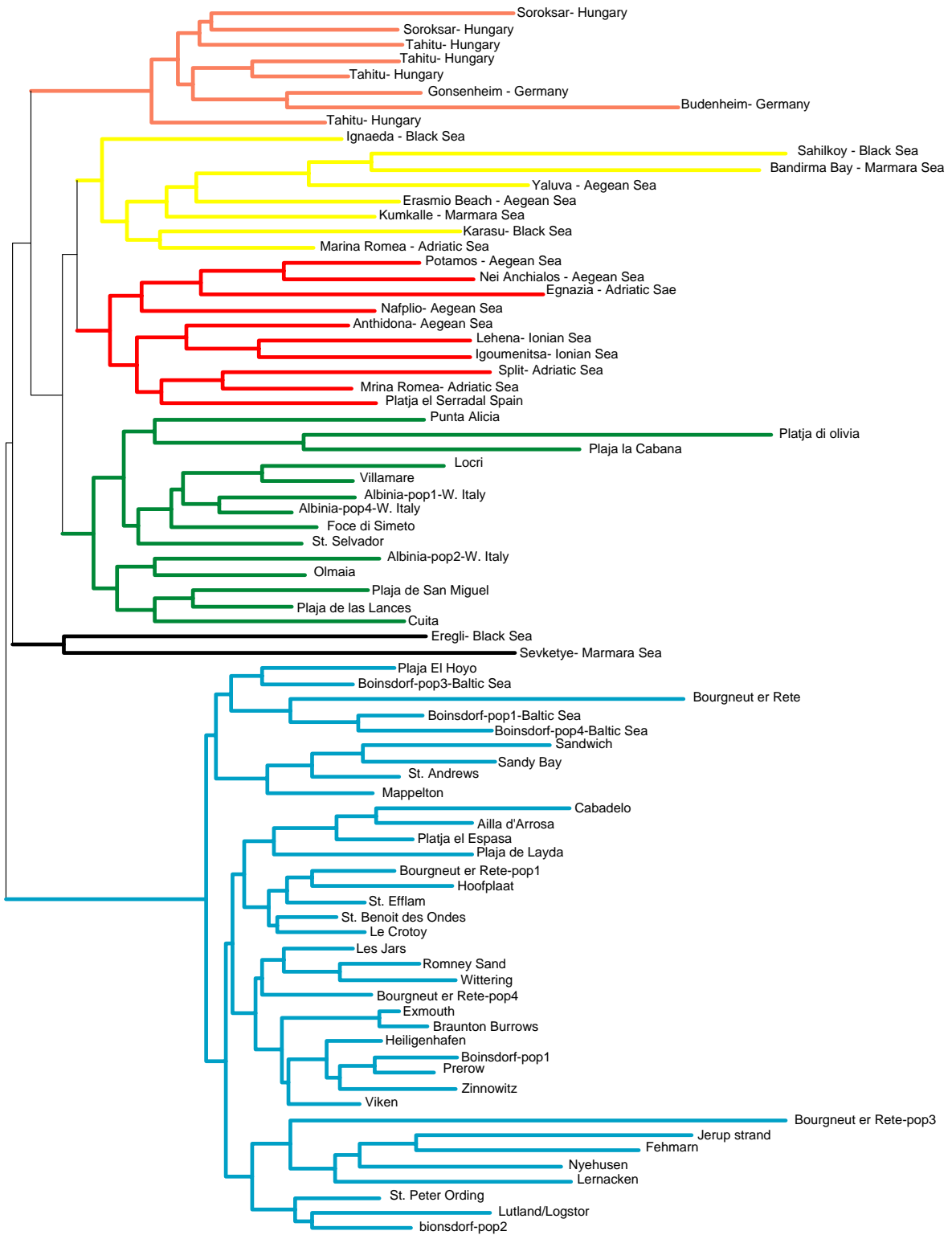
**Figure 1.** Mid-point rooted neighbor-joining NJ phenogram of *Crithmum maritimum*

## Appendix III



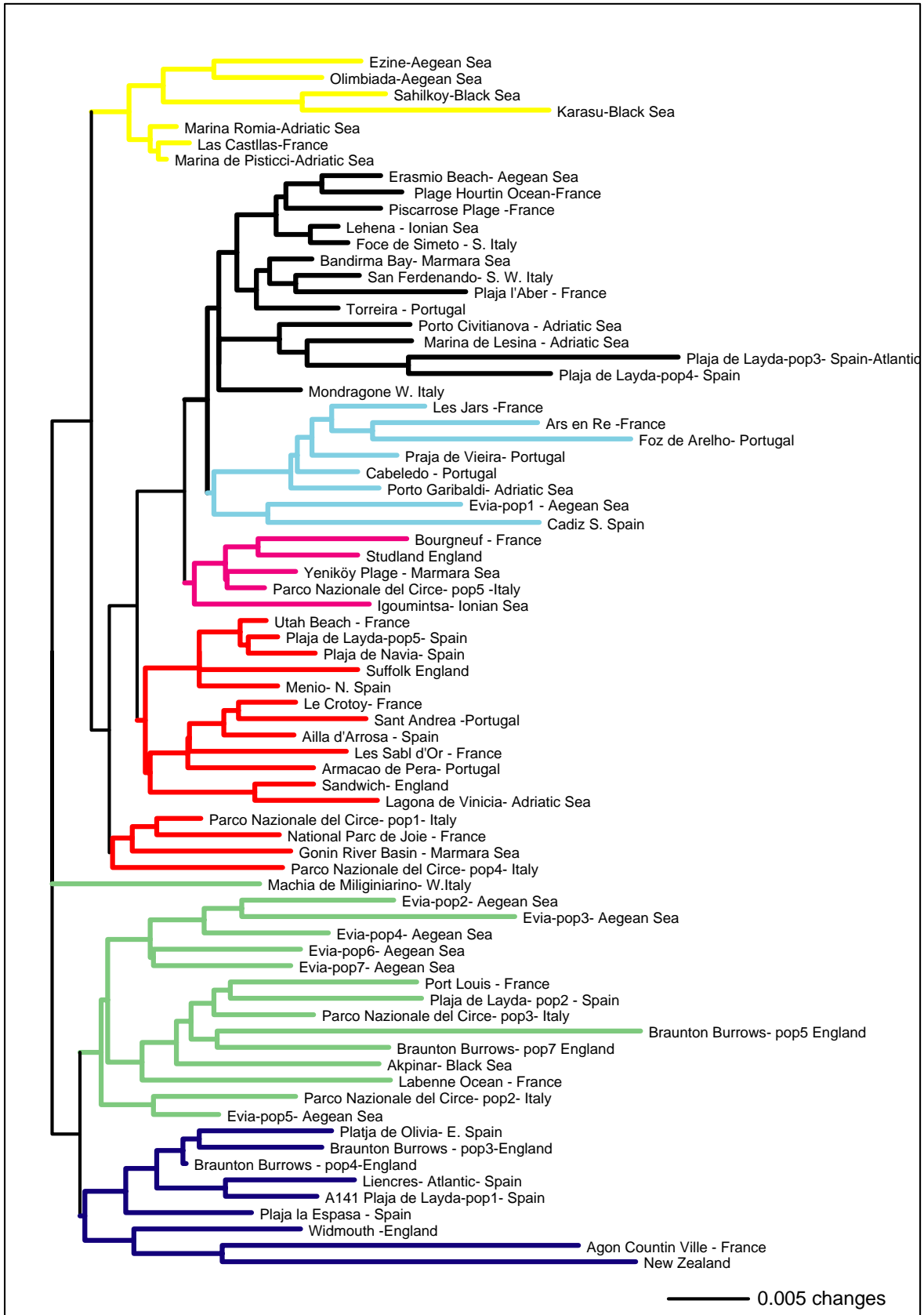
**Figure 2.** Mid-point rooted neighbor-joining NJ phenogram of *Halimione portulacoides*

## Appendix III



**Figure 3.** Mid-point rooted neighbor-joining NJ phenogram of *Salsola kali*.

## Appendix III



**Figure 4.** Mid-point rooted neighbor-joining NJ phenogram of *Calystegia soldanella*.

## Appendix IV

-The complete ITS sequence of *Crithmum maritimum*

```
1   TCGAA GCCTG CAACA GCAGT ACGAC CCGCT AACTC GTAAA CACAT TGGGC
51  AAGGT AATGG GGATT TGGTT CCTCG TTTGC GAACC CCTGG CAGGT GGCCC
101 CTTTC CAGTG GCCAC CGGCC TATGA AATCA TTCGG GCGCG GAATG CGCCA
151 AGGAA TATAA AACTG AATTG ACGTT CGCTT CCGGT AAGCG GGCAG TGGCG
201 TCATT CCGAA ACACA AACGA CTCTC GACAA CGGAT ATCCC GGCTC TCGCA
251 TCGAT GAAGA ACGTA GCGAA ATGCG ATACT TGGTG TGAAT TGCAG AATCC
301 CGTGA ACCAT CGAGT CTTTG AACGC AAGTT GCGCC CGAAG CCACT AGGCT
351 GAGGG CACGT CTGCC TGGGT GTCAC GCATC GTGTT GCCCC CGACC ACTCA
401 CTCCT AGAGG AGATC CGGTT TGGGG GCGGA AATTG GCCTC CCGTG CATAT
451 TATCG CGCGG TTGGC GCAAA AGCGA GTCTC CGGCG ACGGA CGTCG CGACA
501 TCGGT GGTGG TAAAA AGACC TTGTC TTGTC GCGCG AATCC GGGTC AGGTT
551 GGTGA GCTCG AGGAC CCTTA GCGCG CACAC ATTGT GTGCG CTTCG ATTGT
601 GACCC CAG
```

-The complete ITS sequence of *Calystegia soldanella*

```
1   TCGAA ACCTG CTAAG CAGAA CGACC GCGGA ACAAG TTTAA TTTTC ACTAT
51  TCTCG AACGG GTTGC GTCCT TTCCT TCGGG AAGGG GCTCG TCCCG AGCGA
101 ACAAT GAACC CCCGG CGCAG AACGC GTCAA GGAAT ACCAT AACGA GACTG
151 CCTGC TGCC TCGCT CCGTT CGCGG AATGC GCGGG TGGTG TTGGC GTCTT
201 ACTTA ACAAA AAATG ACTCT CCGCA ACGGA TATCT CGGCT CTCGC ATCGA
251 TGAAG AACGT AGCGA AATGC GATAC TTGGT GTGAA TTGCA GAATC CCGTG
301 AACCA TCGAG TCTTT GAACG CAAGT TGCGC CCAA GCCGT CAGGC CGAGG
351 GCGCG TCTGC CTGGG CGTCA CGCAT CGCGT CGCCC CCCTC CTCGA TCTTG
401 TGCCG AGCCG TGGGG AGCGG AAGAT GGCC TCCGT GCTCC TCCC GGTGC
451 GTGGT TGGCC CAAAT GCGAG TTCCT GACGA CGGAC GTCAC GGCGA GTGGT
501 GGTGC TACCC TCGGT GCATA TCGTC GCGCT GTGCC CCCGT CGTCC GCGGG
551 ACTAA TGACC CTTTT TGAGC CCCTC TACCG AGTCC GGCTC TTCGA CCGGG
601 ACCC CA
```

-The complete ITS sequence of *Halimione portulacoides*

```
1   TCGAA ACCTG CCCAG CAGAG CGACC AGAGA ACATG TTTAT CATGA ACGGG
51  GATGA GGTGA AAGCC CCTTC CCTAA GCCGG GGAGT CGCGA CCGCC TTGGC
101 GGGCG GTCAT TTTCG GCCCA ATAAC AAACC CCGGC GCGGT CTGCG CCAAG
151 GAACA TGGAT ACAAG TGTGC CTTT CGGAA CCGGT TTGCC GGTGG CGGAT
201 GTGGC ACCAA GTCGT ATATA ACATT AAACG ACTCT CCGCA ACGGA TATCT
251 CCGCT CTCGC ATCGA TGAAG AACGT AGCGA AATGC GATAC TTGGT GTGAA
301 TTGCA GAATC CCGTG AACCA TCGAG TCTTT GAACG CAAGT TGCGC CCGAA
351 ACCAT TAGGT CGAGG GCACG CCTGC CTGGG CGTCA CGCAT CGCGT CTCCC
401 CCCCC CACCT CTCGT GGGAG GGCGG AGGAA GATGG CCTCC CATGC CTCAC
451 CGGCG GTGGA TGGCC TAAAT AAGGA GCCCC CGGTT ACGAA GTGCC GCGGC
501 AATTG GTGGA ATACA ACGCC TAGCC TAGGA TGCAT CGTAG TCGCG CACAT
551 TGTAG CTGTT GAGGA CTCGC AGGAC CCTTA GCTGT TTGCC CTTAG GGGCG
601 GCAAA ACCGT TCGA CCCCA GG
```

-The complete ITS sequence of *Halimione verrucifera*

```
1   TCGAA ACCTG CCCAG CAGAG CGACC AGAGA ACATG TTTAT CATGA ACGGG
51  GGTGA GGCGA GAGCC CCTTC CCTAA GCCGG GGAGT CGCAC CGCCT TGGTG
101 GGGCG TCATC TTTCG CACAA TAACG AACCC CCGCG CGGTC TGCGC CAAGG
151 AACAT GGATA CAAGT GTGCC CTTT GCAAC CCGTT TGCCG GTCGC GGATG
201 TGGCA CCAAG TCGTA TATAA CATTA AACGA CTCTC GGCAA CGGAT ATCTC
251 GGCTC TCGCA TCGAT GAAGA ACGTA GCGAA ATGCG ATACT TGGTG TGAAT
301 TGCAG AATCC CGTGA ACCAT CGAGT CTTTG AACGC AAGTT GCGCC CGAAG
351 CCAC TAAAT GAGGG CACGC CTGCC TGGGC GTCAC GCATC GCGTC TCCCC
401 CCACC ACCTC TCGTG GGAGG GCGGA GGAAG ATGAC CTCCC ATGCC TCACC
451 GGGCG TGGAT GGCCT AAATA AGGAG CCCCC GGTTA CGAAG TGCCG CGGCA
501 ATTGG TGGAA TACAA GGCCT AGCCT AGGAT GCATC GTAGT CGCGC ACATT
551 GTAGC TCTTG AGGAC TCGCA GGACC CCTAG TTGTT TGCCC TAAGG GGCGG
601 CAAAA CCGTT GCGAC CCCCG G
```

## Appendix IV

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-The complete ITS sequence of *Salsola kali*

```
1   TCGAA ACCTG CCCAG CAGAT TGA CT AGCGA ATGCG TTTTA CATGC ATGGG
51  GTGGC TGGTG TCCTG ACTTG TCATG CCATC TCCCA TCCTG TGGCC TGGGT
101 AATGT GCACT TGTGT GCTTT GTCCA GGCAA ATTAA CGAAC CCCGA CGCGT
151 CATGC GTCAA GGAAC ATCAA TACAG TGTGT GCCCA TTCCT TGCTC GGTTA
201 TTCGG CGCAA GGATG CGGCA CCTAG TCTAA AATAA ATTAA AACGA CTCTC
251 GGCAA CGGAT ATCTC GGCTC TCGCA TCGAT GAAGA ACGTA GCGAA ATGCG
301 ATACT TGGTG TGAAT TGCAG AATCC CGTGA ACCAT CGAGT CTTTG AACGC
351 AAGTT GCGCC CGAAG CCTTC TGGCC GAGGG CACGT CTGCC TGGGC GTCAC
401 GCATC GCGTC TCCCC CAATC CACCT ACTTG TAGGT AGGAT GTGGG GGAGG
451 AGGAT GGCTT CCCGC GCCTC ACCGG GCGTG GATGG CCTAA ATTAG GAGCC
501 TCAGG TCACG ACTGC TGC GG CGATT GGTGG TCATC ATTTT TTTCC GTGCA
551 GTGCG TGACC AAAGT GGACT CGTAG GACCC TGTGT TGT TT CCTTT TGGAG
601 ACAA CCGTG CGACC CCAGT
```

-The complete ITS sequence of *Salsola tragus*

```
1   TCGAA ACCTG CCCAG CAGAT TGA CT AGCGA ATGCG TTTTA CATGC ATGGG
51  GTGGG TGGTG TCGTG ACTTG TCATG CCATC TCCCA TCCTG TGGCC TGGGT
101 AATGT GCACT TGTGT GCTTT GTCCA GGCAA ATTAA CGAAC CCCGA CGCGT
151 CATGC GTCAA GGAAC ATCAA TACAG TGTGT GCCCA TTCCT TGCTC GGTTA
201 TTCGG CGCAA GGATG CGGCA CCCAG TCTAA AATAA ATTAA AACGA CTCTC
251 GGCAA CGGAT ATCTC GGCTC TCGCA TCGAT GAAGA ACGTA GCGAA ATGCG
301 ATACT TGGTG TGAAT TGCAG AATCC CGTGA ACCAT CGAGT CTTTG AACGC
351 AAGTT GCGCC CGAAG CCTTC TGGCC GAGGG CACGT CTGCC TGGGC GTCAC
401 GCATC GCGTC TCCCC CAACC CACCT ACTTG TAGGT AGGAT GTGGG GGAGG
451 AGGAT GGCTT CCCGC GCCTC ACCGG GTGTG GATGG CCTAA ATTAG GAGCC
501 TCAGG TCACG ACTGC TGC GG CGATT GGTGG TCATC ATTTT TTTCC GTGCA
551 GTGCG TGACC AAAGT GGACT CGTAG GACCC TGTGT TGTCC CCTTT TGGAG
601 ACAA CCGTG CGACC CCAG
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