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Molecular markers for the assessment of genetic variability in threatened plant species

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1. INTRODUCTION

1. GENETIC VARIABILITY AND MOLECULAR MARKERS IN THREATENED PLANT SPECIES

The genetics of threatened species have been of great interest to both evolutionary biologists and conservation managers for long time (Avise and Hamrick 1996, Young and Clarke 2000, Hedrick 2001).

Analysis of the genetic structure is necessary not only to fully evaluate the impact of the endangered status on genetic variation of the population, but also because knowledge on the genetic structure of the species can be applied to the preservation of the evolutionary potential of species, which is one of the conservation goals (Godt and Hamrick 1998). Thus, molecular tools can be valuable means for investigating the pattern of genetic diversity in threatened species, and clarifying demographic and ecological issues early in species management in order to plan long-term conservation or restoration projects (Kim et al. 2005).

As predicted by population genetic theory, loss of genetic variation is a major threat to endangered species with small populations or located in narrow geographic areas. A low level of genetic variability often results in minor fitness of individuals (Oostermeijer et al. 1994, Fischer and Matthies 1998, Luijten et al. 2000, Hansson and Westerberg 2002), reduces the viability or adaptability of populations in changing environments (Young et al.1996), and in extreme cases causes the extinction of species. These effects may be most pronounced in species that are self-compatible and/or have limited seed dispersal ability. However, some endemic species exhibit highly levels of diversity compared to their common congeners (Torres et al. 2003, Conte et al. 2004, Ellis et al. 2006). Ellis et al. (2006), for istance, used nuclear and chloroplast microsatellites to investigate the population genetics of an extremely rare sunflower, *Helianthus verticillatus* Small., which is known from only three locations in North America. Despite its rarity, *H. verticillatus* possesses significantly higher levels of genetic diversity than the more common *H. angustifolius* at nuclear loci and equivalent levels of chloroplast diversity.

Moreover, a low level of genetic diversity is also expected in clonal species where, theoretically, the clonal propagation has similar effects for population genetic structure as strict selfing (inbreeding) reproductive system. However, as observed in *Prunus ssiori*, clonal populations can maintain considerable genetic diversity, comparable to sexually reproducing species (Nagamitsu 2004).

Thus, although it is possible to find generalizations in published literature that link pattern of genetic diversity to factors such as reproductive system or geographic range (Hamrick and Godt 1989, Karron 1991, Ellstrand and Elam 1993, Gitzendanner and Soltis 2000), predicting the amount and distribution of genetic variability in plant species on the basis of distribution size and mating system is often not reliable and each case should be independently investigated.

In last decades, genetic issues have gone from relative obscurity to a significant emphasis in conservation research as modern molecular techniques revolutionized our ability to delineate relationships among individuals, populations, and species. Despite some researchers have questioned the relative importance of genetic information, stating that ecological or demographic issues may be more pressing (e.g. Lande 1988, Schemske et al. 1994), molecular markers have become part of a repertoire of tools needed to assess the amount of genetic variation in populations of endangered species and to address the everincreasing loss of biodiversity.

An outstanding advantage of molecular approach is the immense amount of potential data they provide (Petersen and Seberg 1998). Furthermore rates of evolution of different parts of the genome are extremely variable, allowing molecular data to be applicable at any taxonomic level.

Both dominantly (e.g. AFLP, RAPD, and ISSR) and codominantly inherited markers (e.g. allozymes and microsatellites) have been used to study population genetics and life history traits in many species. Among these, polymerase chain reaction (PCR)-derived markers obtained with nonspecies specific primers have become exceedingly popular since they do not request sequence information for the target species. Consequently, these methods are especially suited to situations where little or no molecular genetics research has been conducted previously, which is true for the majority of wild plant species, in particular for endangered species.

The first and so far most commonly used method in this group is RAPD (random amplified polymorphic DNA) which was introduced in 1990 (Williams et al. 1990). A few years later, the relatively similar ISSR (intersimple sequence repeats) (Zietkiewicz et al. 1994) and the somewhat more technically demanding AFLP (amplified fragment length polymorphism) (Vos et al. 1995) were introduced. In spite of the obvious advantages of these methods related to the efficient and quick PCR amplification of polymorphic DNA fragments starting from small amounts of template, however, they share some limitations in the interpretation of the multi-band profiles produced: heterozygotes cannot be detected because of their dominant nature; homology of comigrating bands cannot be assigned certainly; from a technical point of view, competitive priming (Halldén et al. 1996), and the occurrence of artefactual bands produced by nested primer annealing or interactions within and between DNA strands during PCR (Rabouam et al. 1999) still remain potential problems. The difficulty of achieving robust profiles, particularly in RAPDs, may make the reliability of these markers somehow questionable, but the reproducibility of RAPD analysis can be enhanced through improved laboratory techniques and band scoring procedures (Skroch and Nienhuis 1995, Weising et al. 1995) while AFLP and ISSR are less affected by the problem of reliability than RAPD (Zietkiewicz et al. 1994, Vos et al. 1995, Palacios et al. 1999) because longer primers and higher annealing temperatures are employed.

From the 90ies, SSR markers, based on microsatellite DNA loci with tandem repeats of one to six nucleotides, became increasingly popular in plant population genetics due to their hypervariability. These loci are detected with PCR, and sequence information is necessary for primer design. As opposed to previous multilocus-based approaches, SSR analysis provides locus-specific and codominant markers. The major drawback with microsatellite DNA analysis has been attributed to time and cost involved in developing species-specific primers from genomic libraries or sequence databases (Squirrell et al. 2003).

The use of degenerate primers (primers developed for a particular species and applicable to related taxa) offers an exciting prospect since avoids the laborious and time-consuming process of cloning new microsatellites. However, in many instances, heterologous primers do not lead to amplification products at all or introduce

artefacts since the structure and/or average length of the actual microsatellite locus can differ considerably between different taxa (van Treuren et al. 1997). Microsatellite performance may be sometimes hampered by apparent heterozygote deficiency due to the occurrence of null alleles (Callen et al. 1993) or short allele dominance (Wattier et al. 1998).

The different molecular techniques hitherto examined have become the genetic markers of choice for many applications in biodiversity studies. They differ in the way that they evaluate DNA sequence variation without sequencing and in the type of data that they generate, but the common rationale behind their development has been the search for polymorphic and "easy-to-handle" markers. Their abundance and ubiquitous distribution have made them very valuable genetic markers.

In data compilations, estimates of genetic variation obtained with different types of dominant markers (AFLP, RAPD, ISSR) proved to be quite similar in magnitude, both for within and among populations (Zawko et al. 2001). In contrast, microsatellite-derived estimates of within-population diversity were at least twice higher than values from non-SSR loci (Nybom et al. 2004). This difference may be attributed to the hypervariability of SSR loci, up to four orders of magnitude higher than the mutation rate at diverse loci (O'Hanlon et al. 2000). In contrast, population differentiation estimated by microsatellites was found lower than that measured by codominant markers (Nybom et al. 2004) but the discrepancy in this case may be apparent and attributable to the use of traditional statistics that underestimate measures of differentiation from highly polymorphic SSRs (Hedrick 1999).

As example of feasibility of using molecular markers for accurate fingerprinting of endemic taxa some recent case studies will be mentioned in detail. Palop-Esteban et al. 2007 used microsatellite markers to investigate the levels and distribution of genetic diversity within and among populations of *Limonium dufourii* (Girard) Kuntze. It is a highly endemic, triploid species (2n=3x=27) from the coasts of eastern Spain (Castellón and Valencia provinces) whose distribution range has been greatly reduced along with urban development (Crespo and Laguna 1993). Given its critical status, *L. dufourii* has been considered a priority species for conservation and has been included as Critically Endangered in the Spanish catalogue of endangered plants (VVAA 2000; Crespo 2004).

In this study, sixty-five alleles from 13 microsatellite regions were amplified in a sample of 122 individuals collected from the six extant populations. *Limonium dufourii* showed moderate to high levels of genotypic diversity within populations in the analyzed microsatellite regions, with most genotypes restricted to one or a few populations, a common pattern in asexually reproducing plants. *L. dufourii* presented also a strong population differentiation with a high proportion of the genetic variance distributed among populations (72.06%) and significant isolation by distance. This pattern can be explained by restricted gene flow between populations, founder events produced by a limited number of individuals, absence of recombination and spread of single asexual clones within populations. In order to preserve extant genetic variation of *L. dufourii*, *in situ* strategies such as the preservation of its habitat are suggested.

Heptacodium miconioides Rehd., the only species in genus Heptacodium, is an endangered plant, endemic to China (Jin et al. 2007) that has declined in recent decades to such an extent that it is limited to small isolated areas and eventually fragmented into island-like small populations. To characterize genetic diversity and genetic differentiation within and among populations of *H. miconioides,* 12 ISSR primers were tested on 180 adult trees. Diversity indices revealed low genetic variation at population level and high genetic variation at species level, indicating that *H. miconioides* populations are endangered for ecological reasons and long-term deforestation rather than for scarce genetic variation. Two-thirds of the total variation was attributed to differences among populations by the AMOVA analysis. This highly structured pattern may be correlated to small number of remnant individuals and consequent genetic drift in the small population isolated by distance, while significant correlation between geographical distance and genetic distance reflects the population distribution pattern of isolation-by-distance. It appear evident that, to maintain the most of the genetic diversity accumulated by *H. miconioides*, conservation *in situ* by preserving as many populations as possible is the best way to protect this threatened species.

12 RAPD primers were used to assess genetic variation between- and within populations of *Anisodus tanguticus* Wu & C. Chen., an endangered perennial species endemic to the Qinghai Tibetan Plateau (Zheng et al. 2008). *A. tanguticus* is a medicinal plant and, as a result of extensive collection and habitat over-exploitation,

the number and size of the extant populations have decreased greatly in the last years. Also in this case, analysis of molecular variance (AMOVA) showed that among-population genetic variation accounted for about two-thirds of the total genetic variation. The differentiation among isolated populations may be a consequence of anthropic factors or a natural effect: in fact this species is distributed in the Qinghai Tibetan Plateau where high mountains and deep valleys are abundant. The complex topography of the region may have hindered gene flow via both pollen and seeds among populations, and thus promoted population differentiation. The observed genetic variations suggest that as many populations as possible should be considered in any planned *in situ* or *ex situ* conservation programs for this species.

In conclusion, both dominant multi-locus (RAPD and ISSR) markers and codominant single locus (microsatellites) markers are able to estimate levels and partioning of genetic variability in threatened plant species, providing appropriate information in relation to the conservation of genetic resources.

1.1.1 DISTRIBUTION, MORPHOLOGY AND ECOLOGY OF THE PUTATIVE PARENTAL SPECIES OF *QUERCUS CRENATA* LAM.: *QUERCUS CERRIS* L. AND *QUERCUS SUBER* L.

Despite *Quercus suber* L. and *Quercus cerris* L. belong to the same taxonomic group, subgenus *Cerris* (Schwarz 1993, Manos et al. 1999), they are well distinct morphologically, and have different geographical and ecological ranges.
The natural distribution ranges of *Q. cerris* from central and southern Europe to Asia Minor, although it has been planted extensively north of its native range over the last 500 years (Stone et al. 2001) (Figure 1a). In Italy it is widely distributed in all regions, but is rare in Sicily and absent in Sardinia.
Q. cerris (Turkey oak) is a large deciduous tree growing to 25-40 m tall with a trunk up to 2 m diameter. The bark is dark grey and deeply furrowed. The leaves are 7-14 cm long and 3-5 cm wide, with 6-12 triangular lobes on each side; the regularity of the lobing varies greatly, with some trees having very regular lobes, others much less regular. The flowers are wind-pollinated aments, maturing about 18 months after pollination; the fruit is a large acorn, 2,5-4 cm long and 2 cm broad, bicoloured with an orange basal half grading to a green-brown tip; the acorn cup is 2 cm deep, densely covered in soft 4-8 mm long 'mossy' bristles. It prefers basic soils and prevails between 100 and 800 m asl. Its range includes submontane and montane vegetation belts, but can reach 1200 m on sunny slopes (Bellarosa et al. 2003a). The species is fairly resistant to thermal extremes, and is moderately tolerant of summer drought; its fully deciduousness and biennial reproductive cycle are well suited to overcome stress due to winter cold, and allow the species to reach the slopes of the Alps (Bellarosa et al. 2005).

Q. suber has a narrow geographical range, restricted to discontinuous areas located exclusively in the western part of the Mediterranean Basin and along the Atlantic coast of North Africa and of south-western Europe, including the main west Mediterranean islands as well as the coastal belts of Maghreb (Algeria and Tunisia), Provence (France) and Catalonia (Spain). In Italy it is distributed mainly along the Tyrrhenian coast; it is absent along the Adriatic coast, except in Apulia, where there

are small stands, while it is present in central Sicily and widespread in Sardinia (Bellarosa et al. 2003a) (Figure 2a).

Q. suber (cork oak) is a sclerophyllous evergreen oak. It may reach about 20 m in height, with massive branches forming a round crown. Its thick and soft bark is the source of cork, which is stripped every 10–12 years from the outer layer of the bark along the lower portion of the trunk (Gellini and Grossoni 1997). The leaves are 4-7 cm long, weakly lobed or coarsely toothed, dark green above, paler beneath, with the leaf margins often downcurved. The acorns are 2-3 cm long, in a deep cup fringed with elongated scales, 5 to 7 veins. It is a monoecious wind-pollinated species with a protandrous system to ensure cross pollination (Figure 2b).

Cork oak avoids limestone substrates and usually grows in warm stands of the humid and sub-humid Mediterranean areas with at least 450 mm mean annual rainfall and >4–5°C mean temperature for the coldest month.

In natural conditions cork oak usually occurs in pure stands or in mixed stands together with *Q. ilex* L.and *Q. pubescens* Willd. However, the surface area covered by the species is rapidly and progressively declining, mainly due to human activities connected with fires, overgrazing, ploughing, indiscriminate extraction of cork, and insect or pest attacks (Careddu and Vogiatzakis 2003). The different strategies for drought resistance and the absence of competition for water, documented by Nardini et al. (1999) in a mixed natural stand of *Q. cerris* and *Q. suber* growing in Sicily, attest that the two species experience different degrees of water stress in their natural environments, and resist drought to different extents.

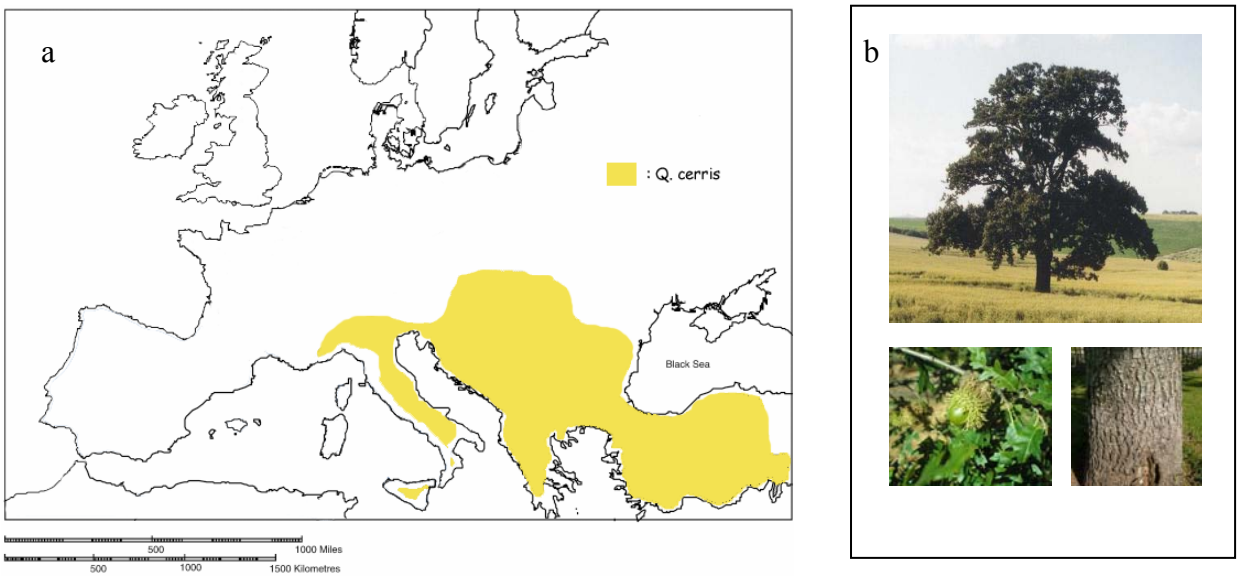


Figure 1: (a) natural distribution range of *Quercus cerris* L.; (b) tree, acorns, leaves and bark of *Quercus cerris* Lam.

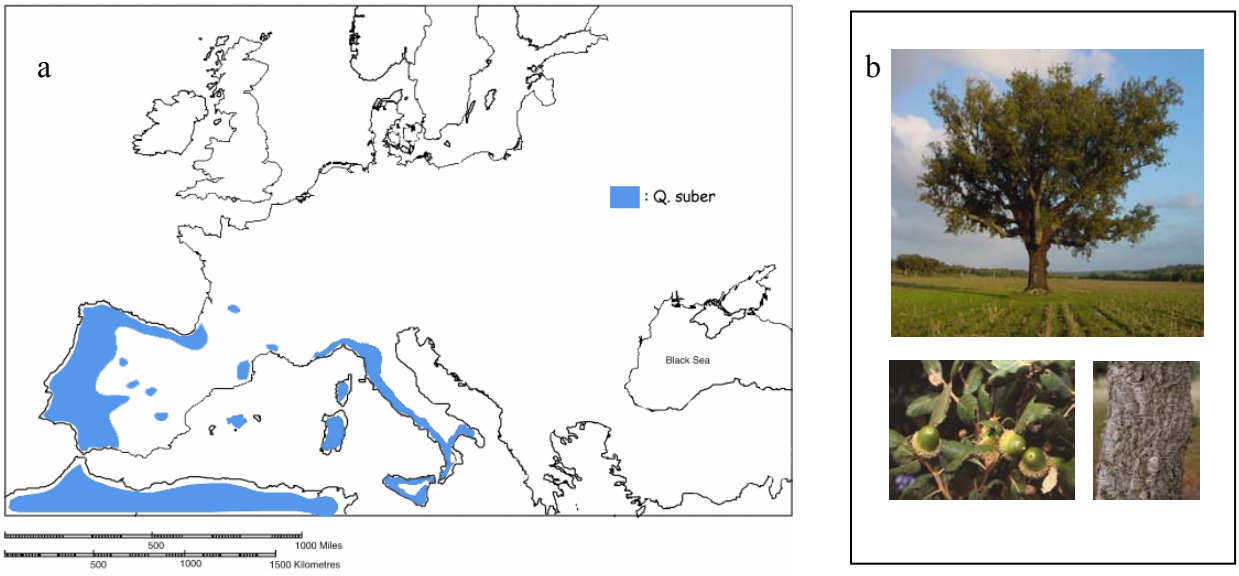


Figure 2: (a) natural distribution range of *Quercus suber* L.; (b) tree, acorns, leaves and bark of *Quercus suber* Lam.

1.1.2 *QUERCUS CRENATA* LAM. : TAXONOMY, DISTRIBUTION, MORPHOLOGY AND ECOLOGY

Quercus crenata Lam. is a species with a controversial taxonomic status: in most floras (Flora Europaea 1964, Pignatti 1982, Schwarz 1993) it is hyphothesized to be a hybrid between *Quercus suber* L. and *Quercus cerris* L.; some Authors considered instead it as a fixed species (Hegi 1957, Odasso and Prosser 1996).

Besides the unresolved taxonomic position of this species, nomenclatural ambiguity results from the fact that Santi ("Viaggio al Monteamiata" 1795) described a plant of Tuscany which closely resembled the plant of Lamarck which he named *Quercus pseudo-suber*, whereas Gussone (1825, 1844) used the name *Q. fontanesii* for a plant of Sicily, also morphologically similar to *Q. crenata* (Cristofolini and Crema 2005).

The species, assuming the three names are synonyms (Schwarz 1993), occurs as rare and scattered individuals, from southern France (Alpes Maritimes) to all continental Italy (without Apulia) and Sicily, western Slovenia and western Croatia (Cristofolini and Crema 2005) (Figure 3a). However, if in peninsular Italy and in Sicily it occurs where the ranges of *Q. cerris* and *Q. suber* overlap, its presence in northern Italy, Alpes Maritimes and Slovenia is puzzling since *Q. suber*, one of the presumed parents, is not present in these regions, and long distance pollen dispersal from the nearest stands of *Q. suber* (on the Thyrrhenian coast) is highly unlikely.

As a possible explanation for this paradox, Goiran (1897, 1899) proposed that the *Q. crenata* specimens surviving at this time in the north-eastern Italy may be considered the offspring of hybridization events prior to local extinction of *Q. suber*. On the other hand, considering the ecological and morphological differentiation among *Q. crenata* individuals growing in different regions, Barbero et al. (1972) suggested that the name *Q. crenata* may be referred to a heterogeneous complex including two different entities, i.e. a "good" species, growing in the northern part of the distribution range, and a swarm of hybrids *inter parentes* in the southern part. More recently, Cristofolini and Crema (2005) examined the morphology of 91 specimens of supposed *Q. cerris* x *Q. suber* hybrids and grouped them into two taxa: *Quercus crenata* Lam., applied to plants considered relicts of ancient hybridization and growing in northern Italy, southern France and Slovenia, and *Quercus* x *pseudosuber* Santi, referred to plants growing in peninsular Italy and Sicily, which are considered

inter parentes hybrids. In addition to morphological intermediacy, biochemical and molecular additivity have been used to document the hybrid status of *Q. crenata* in peninsular Italy, where *Q. cerris* and *Q. suber* occur sympatrically. Seed proteins and rDNA restriction fragments of both putative parental species were found in *Q. crenata* (Bellarosa et al. 1996). Nuclear rDNA ITS sequences from *Q. cerris* and *Q. suber* did not clearly exhibit codominance in *Q. crenata*, however, the three species clustered together in the "Cerris s.s." group and the most parsimonious analysis determined the position of *Q. crenata* as more closely related to *Q. suber* (Bellarosa et al. 2005).

Q. crenata (Figure 3b) is a semi-evergreen tree of medium height (10 to 20-25 m) with bark deeply furrowed and moderately corky. Leaves are circular to oblong, usually ovate, with base truncate, little foliar lobes which are rounded and slightly incised and apex acute; surface adaxially is glabrous and coloured brilliant green, while abaxially is grey-white due to the abundance of hairs, they are coriaceous resembling those of *Q. suber* but their general shape is intermediate between those of *Q. suber* and *Q. cerris* (Figure 3c). They persists throughout the winter and fall in spring, shortly before the new leaves develop. It is a monoecious plant, the male aments are sessile, 3-6 cm long, with connate sepals; stamens (4-6), surrounding tuft of silky hairs. The female flowers are tiny, solitary and occur at the leaf axils, with 5-6 connate sepals and long styles (4-6). The flowering period is between May and early June and the pollination is anemophilous. The fruits have a biennial maturation, and the warty cupules have linear and reflexed scales resulting similar to those of *Q. cerris*.

The plant, as *Q. cerris*, prefers heavy, sandy or clay soils, neutral or slightly acid. It grows between 150 m and 1,000 m, at south, south-east or south-western exposure. It is mostly found in open places, in meadows, more rarely in woods, usually *Q. cerris* coppice.

Q. crenata is considered protected in Piemonte (L.R. n°32 – 2/11/1982), Veneto and Emilia-Romagna (L.R. n°2 – 1970). It has been proposed for preservation in Liguria (Cresta and Salvidio 1991) and Trentino.

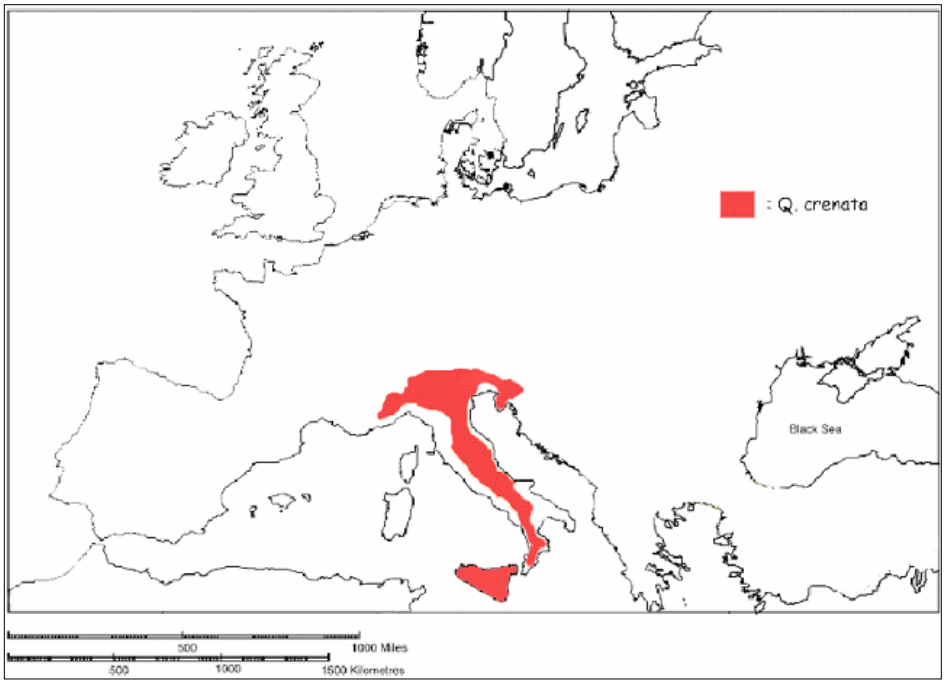


Figure 3a: distribution range of *Quercus crenata* Lam.

Figure 3b: *Quercus crenata* Lam. individual in Acqui –AL. (11cr).

Figure 3c: acorns, leaves and bark of *Quercus crenata* Lam.

1.1.3 HYBRIDIZATION AND INTROGRESSION IN THE GENUS *QUERCUS*

Hybridization is considered to be widespread among plants and has been estimated to be involved in the differentiation of up to 70% of the Angiosperms (Whitham et al. 1991). As recently reported (Rieseberg and Wendel 2004), phylogenetic studies conducted over the past decade in several plant genera have shown that the 'marks' of past hybridization are considerably more frequent than previously believed (Cronn and Wendel 2004, Doyle et al. 2004). Hybridization is therefore a prominent factor in plant evolution which may trigger the differentiation of new lineages (Arnold 1997).

Hybrids usually grow intermingled with one or both of the original species. The introgression, the movement of genes (gene flow) from one species into the gene pool of another by backcrossing between the interspecific hybrid and one of its parents, is one of the principal evolutionary consequences of reproduction in the wild plants. It may readily occur if viability and fertility of the hybrids are sufficiently high and is expected to increase levels of genetic diversity, to provide new gene combinations on which selection can act, and may also lead to speciation if reproductive isolation is established. In any case, introgression is difficult to identify. In polymorphic populations, introgression may go unnoticed because appropriate methods for its detection are not used or few diagnostic features separate parental taxa. Futhermore, differences between introgressed individuals and the parent species follow a decay function with time since first introgression occurred (Rieseberg and Wendel 1993).

Although morphology, comparative anatomy and physiology have provided primary evidence for plant hybridization and introgression in hundreds of studies, during the last three decades classical strategy for the confirmation of a hybrid origin of a species have increasingly been complemented by molecular techniques.

Morphological traits and molecular data have been used jointly to verify and explore hybridization: in *Iris fulva* Ker-Gawl and *Iris haxagona* L. (Arnold 1994), *Salix sericea* Marshall and *Salix eriocephala* Michaux (Hardig et al. 2000), *Helianthus anomalus* Blake, *Helianthus annuus* L. and *Helianthus petiolaris* Plains

(Schwarzbach et al. 2001), *Cardamine pratensis* L. and *Cardamine raphanifolia* Pourr. (Lihová et al. 2007).

Oaks (*Quercus* genus, Fagaceae) consist of several hundred diploid species (2n=2x=24), which are known for their high propensity to interbreed. Interspecific hybridization makes the task of identifying oaks - already a challenge because of the large number of taxa, phenotypic plasticity, and juvenile versus adult character differences - especially difficult. Indeed, oaks represent a classic example of a taxonomic group in which individual species maintain distinct morphological and ecological identities despite extensive hybridization and introgression (Stebbins 1950, Burger 1975, Grant 1981, Whittemore and Schaal 1991), as observed in cross breeding species: *Q. suber* L. and *Q. ilex* L. (Belhabib et al. 2001), *Q. gambelii* Nutt. and *Q. grisea* Liebm. (Williams et al. 2001), *Q. crassifolia* Humb. & Bonpl. and *Q. crassipes* Humb. & Bonpl. (Tovar-Sánchez and Oyama 2004), *Q. affinis* Scheidw. and *Q. laurina* Humb & Bonpl. (González-Rodríguez et al. 2004).

According to Ishida et al. (2003), several intercrossing oak species are more distinctly discriminated by morphological or ecological (i.e., adaptive) traits than by isozyme or DNA markers because the firsts might have differentiated faster than the seconds during relatively rapid and recent speciation processes. In addition, isozymes or DNA markers, which are probably not affected by natural selection, might have been transferred from species to species through hybridization, while alleles responsible for differential adaptation might not have been transferred despite hybridization.

Because oaks appear to have weak internal barriers to hybridization, they have been proposed as a model taxon for species concept that rely on ecological criteria, rather than reproductive isolation, in delimiting species boundaries (Van Valen 1976). Oaks, along with other wind-pollinated species, are seen as bearing the “cost” of hybridization, producing many hybrid offsprings that are destined either to fail or to be restricted to narrow or ephemeral habitats (Stebbins et al. 1947, Hardin 1975).

Jiggins and Mallet (2000) suggested that such hybrid zones are more effectively maintained by ecological divergence between parental species than by their genetic incompatibility. As a result, most pairs of *Quercus* species which remain distinct

despite hybridization differ in ecological niches (Kleinschmit et al. 1995, Howard et al. 1997, Bruschi et al. 2000, Tomlinson et al. 2000, Williams et al. 2001).

Hybridization in oaks was initially detected based on morphological characters (Stebbins et al. 1947, Hardin 1975, Cottam et al. 1982, Rushton 1993). Leaf morphology, in particular, has been useful to demonstrate hybridization between two distantly related Mexican black oaks *Quercus conzattii* and *Quercus eduardii* (Bacon and Spellenberg 1996) and to evaluate the pattern of relationships among populations and the change of particular foliar traits across the geografical gradient in a complex consisting of two species of Mexican red oaks, *Quercus affinis* Scheidw., and *Quercus laurina* Humb. & Bonpl. (González-Rodríguez et al. 2005).

When morphological characters alone did not confirm unequivocally the existence of hybridization (Bacilieri et al. 1995, Manos et al. 1999, Mayol and Rossellò 2001), other methods were employed (Crawford et al. 1993, Rieseberg and Ellstrand 1993).

Quercus afares Pomel, an endemic North African species which combines morphological, physiological and ecological traits from *Quercus suber* L. and *Quercus canariensis* Willd., was investigated using both nuclear (allozymes) and chloroplastic markers (cpDNA) (Mir et al. 2006). The study showed that *Q. afares* Pomel originated from a *Quercus suber* x *Quercus canariensis* hybridization. At most loci, *Q. afares* predominantly possesses alleles from *Q. suber*, suggesting that the initial cross between *Q. suber* and *Q. canariensis* was followed by backcrossing with *Q. suber*.

Craft et al. (2002) applied DNA microsatellite markers to study hybridization between *Quercus lobata* L. and *Quercus douglasii* L., two widely overlapping species growing in a mixed stand in central coastal California, and concluded that hybrids of *Q. douglasii* x *Q. lobata* were actually rare that and morphological plasticity of the two species might have led to overestimates of crossbreeding events. These results stress that apparently intermediate phenotypes between *Q. lobata* and *Q. douglasii* are not necessarily hybrids and that true hybrids are not necessarily intermediate in phenotype.

High correspondence between morphological variables and RAPD markers was found in a hybrid zone between *Q. gambelii* and *Q. grisea* in New Mexico (Howard et al. 1997), while different rates of evolution in different characters may explain the

partial congruence between morphology and molecular markers observed some times in plant hybrid analyses where, in general, introgression of morphological characters is more restricted than introgression of molecular markers (Rieseberg and Wendel 1993).

To characterize and discriminate *Quercus crispula* Blume and *Quercus dentata* Thunberg and their hybrids, Ishida et al. (2003) used several morphological traits, the composition of *Phyllonorycter* species (leafmining insects) and AFLP data. Morphological traits and *Phyllonorycter* composition differ enough in these two oaks species and resulted to be useful for identification of species and hybrids. AFLP data instead were less informative for the identification of hybrids, because the degree of molecular differentiation between the two species was low. Then, the two species were similar genetically and this similarity contrasts rather sharply with their morphological distinctness. Although there are several possible explanations for the incongruence between molecular and phenotypic patterns (Rieseberg and Ellstrand 1993), it seems likely that if foliar morphology has experienced restricted introgression despite interspecific gene flow and exchange of neutral markers, it is probably due to selective factors operating against the recombination of genomic regions controlling adaptively relevant traits, while considerable gene flow can still occur at the rest of the genome, as suggested by Wu (2001). The same Author states that that if reproductive isolation has once developed between species or populations to some degree, genes responsible for that isolation and submitted to differential selection might not transfer across species even if hybridization occurs.

1.1.4 *PRIMULA APENNINA* WIDMER: TAXONOMY, MORPHOLOGY, DISTRIBUTION AND ECOLOGY

Primula apennina Widmer is a member of section *Auricula* subsection A*uricula* (Primulaceae).

Primula L. comprises c. 500 species distributed largely in the Sino-Himalayan region (Hu and Kelso 1996) which, together with the adjacent ranges in Central Asia, account for some 78 per cent of all *Primula* species. Members of *Primula* distributed outside of this Asian highland center of diversity occupy the mountains or high latitudes of North America, Europe, and Asia; a few species also extend into South America, Ethiopia, Java, and Sumatra (Richards 1993).

Section *Auricula* is endemic to the central and southern European mountains and is one of the few endemics of the European alpine system with a comparatively large number of species (Ozenda 1995). Its distributional range encompass the Cantabrian Mountains, Pyrénées, Alps, Sudetan Mountains, Carpathians (including Tatra Mountains), Apennines, and Balkans. The highest species diversity, however, is clearly found in the Alps, where 21 species occur, including 12 local or regional endemics (Zhang and Kadereit 2004).

The majority of species of sect. *Auricula* likely originated from an Asian ancestor in the earlier Quaternary (Zhang et al. 2004). A molecular clock analysis based on ITS sequences suggests that sect. *Auricula* originated approximately 3.6 Mya, i.e. in the Pliocene, and that the two major clades of this section (*Auricula, Cyanopsis*) shared a common ancestor at about 2.4 Mya, i.e. at the Plio-Pleistocene boundary (Zhang et al. 2004). Thus, the ancestor of this section must have reached Europe between 3.6 and 2.4 Mya (Zhang and Kadereit 2004).

P. apennina, included in the section *Auricula*, is an endangered species endemic to the mountain tops of the Tosco-Emilian Apennines. It is hexaploid (Zhang and Kadereit 2004), with a chromosome number of 2n=62 derived on the basis of x=11.

Its range is a narrow corridor of approximately 45 km x 5 km between Monte Prado (Reggio Emilia) and Monte Orsaro (Parma), where *P. apennina* grows in isolated populations on the mountain tops (Figure 4a).

P. apennina Widmer is a hemicryptophyte plant (Figure 4b), with small bracts, 1-3 (-5) mm long. The leaves are wedge-shaped, rather square-ended usually with entire margins or occasionally very shallowly wavy-toothed. The most important characters for species identification include red-tipped glandular hairs shorter than 0.2 (-0.3) mm along margin and the absence of a farinose flavonoid exudate on the leaves. New leaves are produced in May and June. The scape exceeds the leaves, forming a more clustered head of flowers with a hint of blue in the pink to red perianth. It has one inflorescence stalk, each with 2-18 flowers with 2-6 (-7) mm long calyces, which emerge in spring. All flowers in a population open fairly synchronously from May to June and the flowering continues for 2-3 weeks.

Like all species of section *Auricula*, *P. apennina* is a heterostylous and self-incompatible plant (Richards 1993) with two distinct mating types: long and short-styled morphs, requiring insect pollinators for reciprocal pollination. This structural difference is often accompanied by a sporophytically controlled, diallelic incompatibility system which makes intermorph crosses more successful than intramorph crosses. Production of fruits is concentrated from June to July.

It proliferates both by sexual and vegetative reproduction. Each genet of the species is composed of various numbers of physiologically independent ramets, which are clonally propagated by woody short rhizomes which grow a few centimeters each year.

It is more frequently found in acidic grassland, in stabilized scree or peaty turf, but also on shaded volcanic cliffs (Richards 1993), mostly in north-facing ledges and sandstone crevices at 1,500-1,800 m.

It is included in the Regional Red List of the endangered vegetal species (Conti et al. 1997), in the Appendix II of Bern Convention and in Appendices II and IV of the 92/43/CEE Directive ("Habitats" Directive).

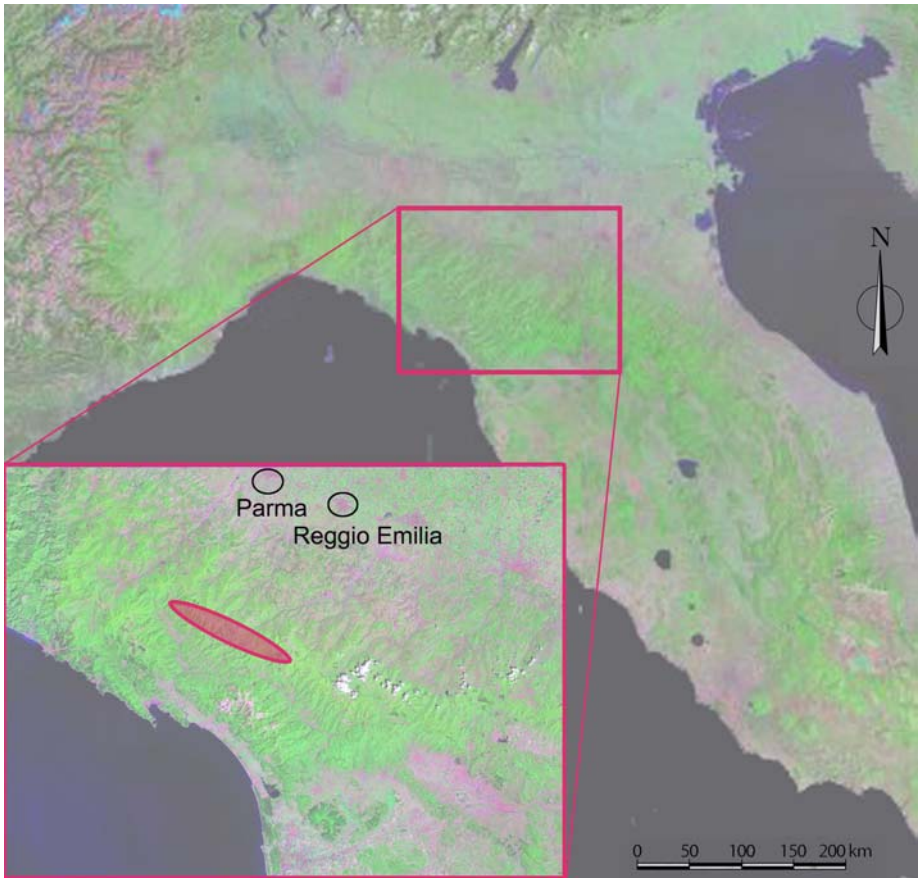


Figure 4a: distribution range of *Primula apennina* Widmer.

Figure 4b: *Primula apennina* Widmer

1.2.1 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

Since their introduction, Random Amplified Polymorphic DNA (RAPD) markers (Williams et al. 1990) have become very popular and have been used for a variety of purposes in plant genetics: cultivar identification (Cabrita et al. 2001, Martins-Lopes et al. 2007, Rasul et al. 2007), parentage determination (Elisiàrio et al. 1999), genetic relationships evaluation (Nicese et al. 1998, Rout et al. 2006), estimation of population genetic variability (Sales et al. 2001, Medraoui et al. 2007), identification of interspecific hybrids (Neuffer et al. 1999, Caraway et al. 2001, Koontz et al. 2001, Lee et al. 2006, Saitou et al. 2007) and estimation of clonality (Esselman et al. 1999, Haangelbroek et al. 2002, Albert et al. 2003, Chen et al. 2006).

The basic principles of the method have been presented by three independent groups in the early 1990s, each suggesting a different protocol. The standard RAPD technology (Williams et al. 1990) utilizes short synthetic oligonucleotides (10 bases long - GC content of at least 50%) of random sequences as primers to amplify anonymous PCR fragments from genomic template DNA under relaxed stringency conditions. Amplification products are generally separated on agarose gels and stained with ethidium bromide. Welsh and McClelland (1990) independently developed a similar technique using primers about 15-20 nucleotides long and called it the arbitrarily primed polymerase chain reaction (AP-PCR) technique. Two cycles with low stringency (allowing for mismatches) are followed by 30 to 40 cycles with high stringency. Radiolabeled nucleotides are included in the last 20 to 30 cycles only. PCR are separated by polyacrylamide gel electrophoresis and made visible by autoradiography. PCR amplification with primers shorter than 10 nucleotides [DNA amplification fingerprinting (DAF)] has also been used producing more complex DNA fingerprinting profiles (Caetano-Annoles et al. 1991). Although the three approaches are different with respect to the length of the random primers, amplification conditions and visualisation methods, they all relies on the fact that whereas the standard PCR requires two different oligonucleotides whose base composition is fixed by the sequence of the fragment to be amplified, RAPDs require only the presence of a single “randomly chosen” oligonucleotide, without any prior

knowledge of the genome subjected to the analysis. Under the annealing conditions used, this single oligonucleotide acts as both a forward and reverse primer.

There is, therefore, a theoretically infinite number of oligonucleotides which could be chosen as RAPD primers. Individual RAPD primers are able to hybridize to several hundred sites within the target DNA, however, not all of these hybridizations lead to the production of a PCR fragment. In order for this to happen, it is necessary for the primer to anneal at two sites on opposite strands of the DNA within 2 kb of each other, i.e. the approximate maximum size of a PCR fragment.

According to the model propesed by Caetano-Annolés et al. (1992) RAPD amplification is modulated at two levels.

First, primer target sites are selected in a template screening phase. The selectivity at this stage is determined by primer sequences and influenced by reaction conditions. Bona fide as well as mismatch annealing may occur, resulting in a complex family of primary amplification products.

In subsequent rounds of amplification, the newly formed molecules may interact in diverse ways, in the sense that competition may occur among single-stranded template DNA, primers, and terminal palindromic sequences of amplified ssDNA molecules to form double-stranded template DNA, primer-target DNA complexes, intra-molecular hairpin loops in the ssDNA. The model suggests that the different types of molecules tend to reach an equilibrium, and only a subset of potential target sites is amplified to high copy numbers. Given that RAPD primer sequences are arbitrarily chosen, the genome is expected to be sampled randomly.

The ability of RAPDs to produce multiple bands using a single primer means that a relatively small number of primers can be used to generate a very large number of fragments. These fragments are usually generated from different regions of the genome and hence multiple loci may be examined very quickly.

SOURCE OF VARIABILITY AND POLYMORPHISM

RAPD polymorphisms can theoretically result from several types of events:

- insertion of a large piece of DNA between the primer binding sites may exceed the capacity of PCR, resulting in fragment loss
- insertion or deletion of a small piece of DNA will lead to a change in size of the amplified fragment
- the deletion of one of the two primer annealing sites results in either the loss of a fragment or an increase in size
- a nucleotide substitution within one or both primer target sites may affect the annealing process, which can lead to a presence *versus* absence polymorphism or to a change in fragment size.

Polymorphisms resulting from insertions or deletions between mutated primer binding sites are codominant. They represent, however, only a small portion of the polymorphisms identified: 5% according to 1546 463 bibia

Polymorphisms between individuals mainly result from sequence differences in one or both of the primer binding sites and are visible as the presence or absence of a particular amplification product. These polymorphisms behave, therefore, as dominant genetic markers (Sperisen and Bücher 1998).

High sensitivity of the technique to changes in experimental conditions, artifactual bands produced in the PCR process, and the dominant inheritance of RAPD markers are obvious limitations in applicability and in interpretation of results.

However, with proper attention and study, RAPD markers have proved to be a highly effective and efficient method for genetic analyses.

An important measure to enhance reproducibility is keep reaction conditions perfectly constant within each set of experiments. Qualitative changes in banding pattern have been obtained increasing the annealing temperatures (Weising et al. 2005) and using slow transition from the annealing to the extension steps (Schweder et al. 1995). Moreover, since both heterozygous- and homozygous-dominant individuals at a certain locus have the same “band present” phenotype, adequate approaches based on phenetic relations and disregarding allele frequencies should be used in performing data analyses.

1.2.2 INTER-SIMPLE SEQUENCE REPEATS (ISSR)

One of the variant following the utilization of microsatellites as molecular marker is based on using single primers complementary to part of SSR sequences in the PCR-amplification.

The successful application of microsatellite-specific oligonucleotides as PCR primers was first described by Meyer et al. (1993), who amplified DNA from different strains of the human fungal pathogen *Cryptococcus neoformans* with the primers $(CA)_8$, $(CT)_8$, $(CAC)_5$, $(GTG)_5$, $(GACA)_4$, and $(GATA)_4$. The technique was subsequently applied to numerous plant genetics studies: identification of cultivars (Raina et al. 2001, Arnau et al. 2003), genetic mapping (Sankar and Moore 2001), assessment of genetic diversity (Zhao et al. 2007, Rizza et al. 2007), biogeographical studies (Meekins et al. 2001), detection of somaclonal variation (Leroy and Leon 2000, Leroy et al. 2001) and molecular systematics (Raina et al. 2001, Mort et al. 2003, Dogan et al. 2007).

Several acronyms were proposed, including single primer amplification reactions (SPAR), inter-simple sequence repeat PCR (ISSR-PCR), and microsatellite-primed PCR (MP-PCR), all referring to semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Gupta et al. (1994) used 23 primers complementary to di-, tri-, tetra-, and pentanucleotide repeats to amplify genomic DNA across a panel of eukaryotes. They found that tetranucleotide repeat primers were most efficient in amplifying polymorphic patterns. GC- as well AT-rich primers worked equally well. Primers representing a combination of two tetranucleotide repeats, or compound microsatellites, were also effective. Single base permutations produced different PCR fingerprints. Banding patterns of higher complexity were observed when radiolabeled PCR products were separated on denaturing polyacrylamide gels and detected by autoradiography. Bands mapped as dominant markers in a segregating maize population. These results were in part confirmed by Weising et al. (1995a) who used a variety of di-, tri-, and tetranucleotide repeats as PCR primers for the analysis of plant species. Distinct and

polymorphic banding patterns were only obtained with tri- and tetranucleotide repeat-specific primers containing a minimum of 25% GC.

ISSRs sample a large portion of the genome, because microsatellites are abundant throughout the genome and evolve rapidly; consequently, ISSRa may reveal a high number of polymorphic fragments per primer. Meyer et al. (1993) stressed that MP-PCR combined some advantages of RAPD analysis (i.e., no need for sequence information) and microsatellite analysis (i.e., use of high-stringency annealing conditions, leading to more reproducible banding patterns). According to Tikunov (2003) the repeatibility of ISSR is better than RAPDs because ISSR primers are longer (15 to 20 bp) and hence have higher annealing temperature.

Both the use of ISSRs and RAPDs has been objected because bands of the same size may not be homologous, but this problem is considered minimal at the infraspecific level, where homologous bands represent about 90% of comigration products (Schrader and Graves 2004). If carefully optimized, both RAPD and MP-PCR are expected to yield reliable and reproducible results within the same laboratory.

Some times complex fingerprintings are produced by ISSRs due to incidental annealing of primers within SSRs during PCR amplification. Initial priming in fact may occur in different registers within the microsatellite target region or the average product size may be continuously reduced by internal priming in sucessive cycles so that the final product is expected to be primed from the extreme 3'-end of each flanking microsatellite.

The more sophisticated anchored ISSR variant developed by Zietkiewicz et al. (1994), also coined anchored microsatellite-primed PCR (AMP-PCR) uses 5'- or 3'-anchored di- or trinucleotide repeats as single PCR primers. The anchor is composed of nonrepeat bases and ensures that the amplification is initiated at the same nucleotide position in each cycle. AMP-PCR has several advantages over unanchored variants of microsatellite-primed PCR. First, primer design ensures annealing of the primer only to the ends of a microsatellite, thus circumventing internal priming and smear formation. Second, the anchor allows only a subset of the targeted inter-repeat regions to be amplified, thereby reducing the overwhelming number of PCR products

sometimes produced from interepeat regions to sets of easily resolvable bands. Third, functional 5'-anchors ensure that the targeted microsatellite is part of the product.

SOURCE OF VARIABILITY AND POLYMORPHISM

Since the primer is a SSR motif the frequency and distribution of the microsatellite repeat motifs influence the generation of bands.

In general, primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism than primers with other di-, tri- or tetra-nucleotide repeats. (AT) repeats are the most abundant di-nucleotides in plants but the primers based on (AT) would self- anneal and not amplify. Tri and tetra-nucleotides are less frequent and their use in ISSRs is lesser than the di-nucleotides. The (AG) and (GA) based primers have been shown to amplify clear bands in rice (Blair et al. 1999, Joshi et al. 2000, Reddy et al. 2000, Sarla et al. 2000), trifoliate orange (Fang et al. 1997) and Douglas fir and sugi (Tsumura et al. 1996), whereas primers based on (AC) di-nucleotide repeats were found more useful in wheat (Nagaoka and Ogihara 1997, Kojima et al. 1998) and potato (McGregor et al. 2000).

Usually di-nucleotide repeats, anchored either at 3' or 5' end reveal high polymorphism (Blair et al. 1999, Joshi et al. 2000, Nagaoka and Ogihara 1997).

The evolutionary rate of change within microsatellites is considerably higher than most other types of DNA, so the likelihood of polymorphism in these sequences is greater. The source of variability in the ISSRs can be attributed to any one of the following reasons or any combination of these. The extent of polymorphism also varies with the nature (3'-anchored, or 5'-anchored) of the primer employed. The primers anchored at 3' end give clearer banding pattern as compared to those anchored at 5' end (Tsumura et al. 1996, Nagaoka and Ogihara 1997, Blair et al. 1999). When 5' anchored primers are used, the amplified products include the microsatellite sequences and therefore variability in number of nucleotides within the sequence would result in length polymorphisms.

ISSR markers are theoretically inherited in a dominant or codominant Mendelian fashion (Gupta et al. 1994, Wu et al. 1994, Tsumura et al. 1996, Wang et al. 1998,

Sankar and Moore 2001), however, they are interpreted as dominant markers similar to RAPD data (Wolfe et al. 1998b).

Polymorphism may relate to mutations at the priming site that prevent amplification giving a presence/absence pattern while insertion/deletion events within the SSR region or the amplified region would result in the absence of a product or, more rarely, in length polymorphism, depending on the amplifiability of the resulting fragment size.

1.2.3 MICROSATELLITES

SSRs are among the most commonly used molecular markers in population and evolutionary biology and are widely used to evaluate the genetic diversity within species (Sangiri et al. 2007, Ali et al. 2007, Varshney et al. 2007, Zhan et al. 2008), to investigate phylogenetic relationships (Goldstein and Pollock 1997, Zhan et al. 2008), to identify and test the paternity of cultivars (Diaz et al. 2007), to study population structure and gene flow (Schueler et al. 2006, Edh et al. 2007), to develop a gene mapping (Hayden et al. 2006), to reveal the clonal status of a species and to determine the extent of clonality (Nagamitsu et al. 2004, Iketani et al. 2007).

SSR polymorphism is reflects differences in simple repetitive sequences of defined regions of the genome. With the advent of polymerase chain reaction (PCR) technology this property was converted into a highly versatile genetic marker (Litt and Luty 1989, Tautz 1989, Weber and May 1989) and became the basis for SSR-based DNA fingerprinting. Products of different length can be amplified with primers flanking the variable microsatellite region and single loci are typically amplified, resulting in one or two bands, depending on the homo- or heterozygous state in diploid organisms. Therefore, microsatellites are considered locus-specific and codominant markers.

The popularity of microsatellites stems from a unique combination of several important advantages: the relatively abundance with uniform genome coverage, the enormous extent of allelic diversity, the hypervariability, the codominant inheritance, the ease of detection by PCR using pair of flanking primers, and requirement for only a small amount of starting DNA. Numerous molecular marker strategies have been developed, but the most common employs sequence information of repeat-flanking regions to design locus-specific PCR primer pairs. The necessity of sequence information for primer design is the more serious obstacle of this technique, in addition to the possible presence of undetected null alleles, which can interfere with the interpretation of inheritance data. Null alleles can be due to mutations in one or both primer binding sites (Weber and May 1989) and these mutations can prevent PCR amplification. Homozygous individuals for a null allele do not show any band at all, whereas heterozygotes have only one band and therefore mimic a homozygote on

the electrophoresis gel. Erroneous interpretations due to null alleles may be solved by redesigning primer pairs for the locus, avoiding the mutated primer binding site and by examining multiple microsatellite loci, reducing the influence of null alleles.

Microsatellites, or Simple Sequence Repeats (SSRs), or Simple Tandem Repeats (STRs), are very short motifs (about 1 to 6 base pairs) usually characterized by a high degree of repetition and occur at many thousand loci in the nuclear genome.

They are widely dispersed through the genomes of eukaryotes and some prokaryotes (Weber 1990, Field and Wills 1996). They tend to occur in non-coding DNA, most frequently in introns and intergenic regions. However, the tandem-repetitive organization is also exhibited by some genes, such as the transcription units for histone mRNA and ribosomal RNA, and, in some plants, they are demonstrated to be associated to non-repetitive DNA (Morgante et al. 2002). As a general rule, trinucleotide repeats are the predominant type of microsatellite found in exons, whereas repeats consisting of multiples of one, two, four, and five base pairs are rare in genes.

In plants, the presence of microsatellites was first demonstrated by RFLP fingerprinting with $(GATA)_4$ and $(GACA)_4$ oligonucleotide probes in the genome of chickpea (*Cicer arietinum*) and barley (*Hordeum vulgare*) in 1989 (Weising et al. 1989). In 1992 Akkaya et al. first used PCR primers complementary to flanking regions of six SSR loci to ascertain the presence and degree of simple sequence repeat (SSR) DNA length polymorphism in the soybean.

These initial studies suggested a lower abundance of microsatellites in plants as compared with animals. However, more recent surveys based on large data sets from *Arabidopsis*, rice, maize, soybean, and wheat genome demonstrated that microsatellites frequencies in plants are higher than previously anticipated (Morgante et al. 2002, Cardle et al. 2000). Estimates of the frequency of SSR occurrence in plant genomes range from average inter-SSR distances of less than 10kb considering all repeat motifs in database sequences (Becker and Heun 1995, Lagercrantz et al. 1993), to over 1,2 Mb considering only GA/CT and GT/CA repeats in genomic libraries (Broun and Tanksley 1996).

The repeated sequence is often simple, consisting of two, three or four nucleotides (di-, tri-, and tetranucleotide repeats respectively), although tri-, tetra- and

pentanucleotide motifs are generally less common than mono- and dinucleotide repeats. $(A)_n$, $(AT)_n$, $(GA)_n$ and $(GAA)_n$ repeats are the most frequent motifs in plants. Over 25% of identified loci may belong to compound and/or interrupted families, which tend to be less polymorphic than perfect families (Jarne 1996).
Weber (1990) recognized three microsatellite classes which relate to the degree of perfection of the arrays: perfect repeats, which consist of a single, uninterrupted array of a particular motif, imperfect repeats, in which the array is interrupted by one or several out-of-frame bases, and compound repeats, with intermingled perfect or imperfect arrays of several motifs.

SOURCE OF VARIABILITY AND POLYMORPHISM

Microsatellites are subject to mutations during evolution. Microsatellite mutation rates proved to vary considerably depending on the locus, on the number, the length and the type of repeat motif, the flanking sequence, the recombination rate, the organism, and sometimes the alleles (Schlötterer 2000).
Despite microsatellites being widely used as genetic markers (Schlötterer 2004), the processes underlying microsatellite genesis are still not fully understood. In principle three different, not mutually exclusive, processes have been described; DNA replication slippage, 3'-extension of retrotranscipts and transposable elements containing a proto-microsatellite.
DNA replication slippage, is the predominant mutation mechanism of microsatellites, causing the gain and loss of repeat units. Slipped-strand mispairing involves local denaturation and displacement of the strands of a DNA duplex followed by mispairing of complementary bases at the site of an existing short tandem repeat. Following replication or repair can then lead to insertions or deletions of one or several of the short repeat units.
Levinson and Gutman (1987) proposed that short proto-microsatellites are generated by mutation. Once a sufficient number of repeats are generated, DNA replication slippage can operate and expand the repeat. Rose and Falush (1998) suggested that a

minimum number of repeats is required before DNA slippage can extend the proto-microsatellite, but not all the Authors agree (Pupko and Graur 1999).

This simple model of microsatellite genesis is contrasted with experimental evidence showing high frequency of microsatellites in the proximity of interspersed repetitive elements, such as short interspersed repeats (SINEs) and long interspersed elements (LINEs). Nadir et al. (1996) suggested that they originated from the 3'poly(A) tails of reverse transcribed RNA which has been inserted into the genome. Nevertheless, it is unlikely that the majority of microsatellites originated through this mechanism. Firstly, mainly A-rich microsatellites are associated with the 3'poly(A) tails (Nadir et al. 1996). Secondly, at least in some plants, microsatellites are abundant throughout the genome (Schlötterer 2000, Morgante et al. 2002). Ultimately, microsatellite genesis has been associated with proto-microsatellite sequences embedded in transposable elements. Consistent with the hypothesis that proto-microsatellites in transposable elements can be one important factor in microsatellite genesis, most microsatellites associated with repetitive elements were expansions of transposable elements sequences in barley (Ramsay et al. 1999). Nevertheless, it is not clear to what extent (proto-) microsatellites contained in repetitive DNA contribute to the genomic microsatellite repertoire.

PRIMERS TRANSFERABILITY BETWEEN SPECIES AND PRIMERS ISOLATION

The cross-species transfer of nuclear microsatellite markers in plants has been demonstrated in numerous taxa. This consists of the use, in the species under investigation, of microsatellite primers isolated in a closely related species.

The chance of a successful cross-species (heterologous) amplification of any DNA sequence by polymerase chain reaction certainly depends on the source and characteristics of the genomic library and on the evolutionary distance of the species sampled (Dayanandan et al. 1997). Given that primer binding sites are expected to be more conserved when the microsatellite flanking sequences are maintained under selective constraints and that microsatellites are surprisingly common in the vicinity of genes (Morgante et al. 2002), microsatellite within genes provide good chances to

design primer pairs which are more broadly applicable. Nevertheless, it is yet unclear why microsatellites and their flanking DNA are relatively conserved in some taxa, but not in others.

For a considerable number of economically important plant species, including potato, tomato and rice, computer-assisted cloning provides a valuable source of marker generation, futhermore database mining has already resulted in hundreds of microsatellite markers. For the majority of species, however, since database entries are still limited or even nonexistent and/or cross-transferability is not applicable, microsatellites have to be cloned and their flanking regions sequenced for every species under study. The labour-intensive procedures needed to develop microsatellite markers for a new species have discouraged the use of SSR markers on a large scale. Nevertheless, elaborate enrichment cloning techniques have been developed which facilitate the isolation of SSR locus-specific primers. The most popular class of isolation methods is based on selective hybridization. The basic protocol as proposed by Karagyozov et al. (1993), Armour et al. (1994), Kijas et al. (1994), is relatively straightforward, although several modifications have been independently suggested by various authors in an attempt to further optimize crucial steps or to remove unnecessary procedures. Zane et al. (2002) have presented a modification to the classic hybridization selection which results in a faster and simpler method. This protocol, called FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats) will be described in Material and Methods.

1.3.1 ASSESSMENT OF HYBRIDIZATION AND INTROGRESSION IN THE GENUS *QUERCUS* USING RAPD AND ISSR MARKERS

Methods involving examination of DNA have proven to be most conclusive in identifying plant hybrids (Crawford et al. 1993, Rieseberg and Ellstrand 1993) because molecular markers can provide a large number of neutral and independent characters that are extremely useful in the genetic analysis of hybrid zones (Riesenberg and Ellstrand 1993).

In particular, both random amplified polymorphic DNA - RAPD - (Samuel et al. 1999, Scheepers et al. 2000, Caraway et al. 2001, Ho et al. 2002, González-Pérez et al. 2004), and inter-simple sequence repeat - ISSR - (Wolfe et al. 1998 A-B, Wolfe and Randle 2001, Ruas et al. 2003, Archibald et al. 2004), have been extensively used.

RAPD markers were employed in the assessment of genetic diversity and the documentation of hybridization in three species of *Casuarina* grown in Taiwan showing that most plants of *Casuarina* currently grown in Taiwan are the result of introgressive hybridization involving C. *equisetifolia*, *C. glauca*, and, at a less extent, *C. cuninghamiana* (Ho et al. 2002).

González-Pérez et al. (2004) used RAPD primers to differentiate unambiguously *Phoenix canariensis*, a Canarian endemic palm species, from its widespread congener *Phoenix dactylifera* and to detect hybridization events between the two species.

Wolfe et al. (1998) used genetic markers generated from ISSR primers to examine patterns of hybridization and purported examples of hybrid speciation in a hybrid complex involving *P. centranthifolius*, *P. grinnellii*, *P. spectabilis* and *P. clevelandii*. The study revealed patterns of introgression involving *P. centranthifolius* and demonstrated the hybrid origin of *P. clevelandii* from *P. centranthifolius* and *P. spectabilis*. Morphological traits and ISSR markers were used to test the hybrid status of intermediate individuals found in sympatric populations of *Zaluzianskya microsiphon* (O.Kuntze) K. Schum. and *Zaluzianskya natalensis* Krauss (Archibald et al. 2004). Putative hybrids had intermediate band frequencies relative to the two species for most of the loci, lending support to the hypothesis of occurring hybridization followed by asymmetrical backcrossing to *Z. microsiphon*.

Markers such as AFLP, ISSR or RAPD have usually provided better discrimination also between closely related, hybridizing oak species (Bodénès et al. 1997, Samuel 1999, Coart et al. 2002), compared to other techniques.

The patterns of variation of randomly amplified polymorphic DNA (RAPD) markers and several foliar traits were studied to assess the structure of genetic and morphological variation between *Quercus affinis* Scheidw. and *Quercus laurina* Humb., two closely related Mexican red oaks with partially overlapping distributions (González-Rodríguez et al. 2004). Because none of the markers was completely diagnostic, a maximum likelihood estimate of hybrid index scores was used. It indicated a shift in the genetic composition of populations from one species to the other along a macrogeographic gradient, with genetically intermediate populations situated in the area of overlap. Foliar variation was also continuous between the two species, but only a comparatively small fraction of the individuals was intermediate, and a particular morphology predominated in most populations (i.e., *Q. affinis*-like or *Q. laurina*-like individuals). The observed patterns were interpreted as consistent with the original hypothesis of an a origin for the individuals located in the area of intergradation through hybridization between the two oak species and subsequent introgression.

To estimate genetic diversity in the same species and to analyze genetic differentiation among populations, and between morphologically defined groups of populations representing the two species (i.e., *Q. affinis*-like and *Q. laurina*-like populations) a larger, random sample of molecular markers constituted by RAPD bands was used (González-Rodríguez et al. 2005). A large proportion of the total genetic variation was found within populations of these two Mexican red oaks. The genetic relationships among the populations were largely noncongruent with their morphological classification of populations as *Q. affinis*-like or *Q. laurina*-like, suggesting that interspecific genetic exchange has affected the morphological differentiation between *Q. affinis* and *Q. laurina* to a lesser extent. A significant association between geographic and genetic distances among populations was confirmed by a Mantel Test suggesting that the distribution of nuclear genetic variation among populations in the *Q. affinis* - *Q. laurina* complex is firstly a

function of geography and secondly, but also significantly, a reflection of the morphologically based taxonomic subdivision of populations. This results implies that gene flow and isolation by distance are the predominant forces shaping the population structure of neutral, nuclear genetic variation within this complex.

In contrast, RAPD markers and morphological characters were highly coincident and supported the hypothesis of hybridization between *Quercus crassifolia* H. & B. and *Quercus crassipes* H. & B. in Mexico (Tovar-Sánchez and Oyama 2004). The Authors used diagnostic primers which demonstrated geographic pattern in support of the morphological evidence, indicating that each species is distinct and that each has some degree of genetic cohesiveness.

To estimate the consequences of interspecific hybridization on the genetic structure of kermes oak (*Quercus coccifera* L.) populations in the Iberian Peninsula, ITS sequences and ISSR banding patterns were investigated (Rubio de Casas et al. 2007). ISSR results gave clear evidence of recent and recurrent gene-flow across *Quercus* taxa and ITS results demonstrated extensive hybridization in *Q. coccifera* populations of the Iberian Peninsula with holm oak *Q. ilex*. Ongoing gene-flow and interspecific introgression may be responsible for limited molecular divergence of kermes oak populations and *Quercus* species. In spite of molecular evidence for widespread hybridization between kermes and holm oaks, morphologically identifiable hybrids are rare.

The reported examples show that RAPD and ISSR analyses may provide adequate tools to unambiguously characterize hybrid individuals and to estimate the possible incidence of introgression between closely related *Quercus* species.

In spite of the limitations imputed to RAPD and ISSR techniques, these markers can become immensely useful if complete patterns, comprising information from many loci randomly distributed across the genome, are considered (Wu et al. 2004, Archak et al. 2003).

1.3.2 DEVELOPING OF MICROSATELLITE MARKERS IN THE GENUS *PRIMULA*

Despite the great advances in genomic technology observed in the last years, the availability of molecular tools such as microsatellite markers has been limited in the genus Primula. Thus, genomic microsatellite enriched libraries can be an efficient alternative for marker development in this species.

Microsatellite markers for the genus Primula were first developed by Isagi et al. (2001) in a clonal herb, *Primula sieboldii* E. Morren which occurs in moist habitats in northeastern Asia (Richards 2003). Among the 75000 clones screened, approximately 200 (0.27%) were identified as positive. Seventyfive positives were randomly chosen out of the 200 clones for the DNA sequencing and repeated structures were found in 56 clones. Among the 56 microsatellites, there were thirty, three, two and two identical clones, resulting in 23 distinct microsatellite sequences. Of these, 13 contained flanking regions of suitable length to design primers. Among 13 designed primer pairs, eight amplified target microsatellite loci. Allelic variation was examined for about 20 samples of *P. sieboldii*; of the eight primer sets which amplified the target microsatellite, seven were polymorphic. The seven polymorphic loci had 2–7 alleles and expected heterozygosities ranged from 0.23 to 0.81. The relatively small number of alleles observed for each locus may be caused by limited sampling area for the present study or by the loss of genetic diversity in this species as a consequence of habitat fragmentation.

Some of these primers were subsequently used in the same species to estimate the gene dispersal distance, the magnitude of inbreeding depression (Ishihama et al. 2005), paternity analysis (Ishihama et al. 2006) and genetic restoration (Honjo et al 2007).

In 2003 Ueno et al. developed a genetic enriched library in *Primula sieboldii* using a Digoxigenin-labeled $(CT)_{20}$ probe. 1728 clones were sequenced and 193 were suitable for primer design; seven of these primer pairs were subsequently selected for the clear banding patterns and used to examine variability of the loci in 30-32 samples. The seven polymorphic loci had 2–9 alleles and expected heterozygosities ranged from 0.067 to 0.808. This library was expanded with nine additional primer

pairs (Ueno et al. 2005) with 4–13 alleles per locus, and expected heterozygosity values of 0.269–0.838.

Five of these primer pairs developed by Ueno et al. (2003-2005), other three and a trnT-trnL cpDNA primer pair, were used by Kitamoto et al. (2005) to identify clones and to reveal spatial genetic structure among and within populations of *P. sieboldii*. It grows in the University Forest of Tsukuba, located on the side of Mt. Yatsugatake (Nagano Prefecture-Central Japan) along seven streams, each composed of anything from several to hundreds of genets. This study reveals that, in this species, the dispersal of clonal propagule is a rare effect, given that of the 380 samples, only two pairs of ramets with identical multilocus genotypes for the eight SSR loci were distantly distributed along the same streambank. Moreover, the genetic differentiation among streamside population at SSR loci was low compared with that in cpDNA, suggesting that seed dispersal among streams was restricted, and pollen was the primary agent of gene flow among streamside populations while, the differentiation among subpopulations within streams, low at both markers, suggests that seed dispersal occurs along the stream probably during flooding.

Most of these primer pairs developed by Ueno et al. (2003-2005) were also used in paternity analysis (Ishihama et al. 2003, Ishihama et al. 2006) and in genetic restoration (Honjo et al. 2007).

In 2004, Shimono et al. characterized 11 polymorphic loci for *Primula modesta* Bisset et Moore, a common alpine plant distribuited throughout Japan, whose populations are expected to serve as a model monitoring system of influences of anthropogenic climate change on Japanese alpine habitats. Using two biotinylated probes $(CT)_{15}$ and $(GT)_{15}$, fragments contaning microsatellite were selected with streptavidin coated magnetic beads, ligated into plasmid vectors and cloned into competent cells. To verify the presence of a microstellite, 2550 recombinant clones were transferred to nylon membrane and screened by colony hybridization with Digoxigenin-labelled $(CT)_{20}$ and $(GT)_{20}$ probes. A total of 1056 positive clones were detected and 670 were selected for sequencing. Using the criterion of at least 10 repeat units in the target sequence, 48 primer pairs were designed and specific amplification and polymorphism were achieved for 11 primer sets. A total 31-35 samples were genotyped for allelic diversity and from three to fourteen alleles were

scored. The observed heterozygosity ranged from 0.161 to 0.828 and no evidence for linkage disequilibrium was found.

Kreivi et al. (2006) developed polymorphic microsatellite markers for *Primula nutans*, a seshore plant, endangered in Finland and near threatened in Sweden. The genomic DNA was amplified using DOP (degenerate oligonucleotide-primed) – PCR and DNA fragments of 650-1300bp in size were ligated to a vector and screened with γ^{32}P end-labelled dinucleotide probes $(AG)_{10}$, $(TC)_{10}$, $(GT)_{10}$, and $(CA)_{10}$. Seven primer pairs were designed; three loci showed nonspecific amplification or were monomorphic and, using 378 samples, the number of alleles was low, ranging from two to four with observed heterozygosity of 0.003-0.229 and expected heterozygosity of 0.016-0.527.

Five of the microsatellite primers developed by Ueno et al. (2003-2005) for *Primula sieboldii* were then used as cross-species microsatellite markers in a clonal herb, *Primula kisoana* (Ohtani et al. 2005) endemic to Mount Narukami and the surrounding area in the northern Kanto region of Honshu Island in Japan. In order to evaluate the extent of clonality and the genetic variation within the species in seven local populations, comprising of less than 600 ramets, also eight non-coding spacers of chloroplast DNA were amplified and sequenced in this study. Six of eight non-coding spacers of cpDNA were polymorphic and four different haplotypes were distinguished. Only ten genotypes were found for the five microsatellite loci, each of which was likely to represent a unique genet. In total, the number of genets surviving in the wild would be at most twenty, suggesting a rapid and severe bottleneck of this species. These markers also reveled that the species still maintains relatively high levels of genetic diversity even after a rapid decline in its population. However, because genetic relationships between haplotypes are not close, only a small part of the past gene pool may now exist, demonstrating the top priority to preserve all remaining populations of *P. kisoana*.

1.4 GOALS

ARBITRARY AND SEMI-ARBITRARY MARKERS

In the present study RAPD and anchored ISSR markers were employed to analyze the molecular differentiation among morphologically defined groups of individuals representing the three oak species *Q. cerris*, *Q. suber* and *Q. crenata.*

Further objectives were: 1) to document the hybrid status of *Q. crenata* grown in Northern Italy, a part of its distribution range where its putative parents do not overlap at present. 2) to detect possible introgressive patterns related to the relative frequency of parental taxa, 3) to evaluate the suitability and congruence of RAPD and anchored ISSR markers and compare the discriminating power of these different markers in order to assess genetic diversity and hybridization among closely related species.

SPECIFIC MARKERS

No previous microsatellite markers have been published, and no study of genetic population structure have been made for *Primula apennina* Widmer. Therefore, the aim of this investigation was to characterize polmorphic microsatellite loci for ongoing and subsequent population genetic studies.

2. MATERIALS AND METHODS

2.1 ARBITRARY AND SEMI-ARBITRARY MARKERS (RAPD AND ANCHORED ISSR)

2.1.1 PLANT SAMPLING

Leaves from *Q. crenata* plants were collected at all the known and accessible sites in northern Italy (Table 1-Figure 1). All the *Q. crenata* sampled individuals occur as solitary plants in open areas or interspersed with *Q. cerris*. *Q. crenata* specimens were identified by possessing a combination of parental characters, in particular the leaf persistence in winter, which makes it easily identifiable; given that the sampling was conducted preferably in the autumn and winter periods.

In order to provide material from presumed parent species the sample included from one to six *Q. cerris* individuals close to *Q. crenata*, and a small sample of *Q. suber* from localities beyond the Tosco-Emilian Apennine; *Q. crenata* plants were also collected in the latter area, when present and standing in close proximity. When more than one tree was tested at the same site, the specimens chosen were separated from each other by at least 100 m to avoid collecting related individuals. In all, 37 *Q. cerris*, 21 *Q. suber* and 27 *Q. crenata* individuals were considered. Voucher specimens are preserved in the herbarium at Bologna University (BOLO).

At each site, undamaged leaves were collected from the plants and kept in silica gel. After lyophilization, the leaves were stored at -20°C until required.

Sampling Sites	Locality	Region	Number of samples /species and abbreviations					
			Quercus cerris		*Quercus suber*		*Quercus crenata*	
1	Ca' Carbonaro (BG)	Lombardia					1	**1cr**
2	Cugno (BS)	Lombardia	3	**2cA, 2cB, 2cC**			1	**2cr**
3	La Santa (BS)	Lombardia	3	**3cA, 3cB, 3cC**			1	**3cr**
4	Cugnolo (BS)	Lombardia					1	**4cr**
5	Sarezzo (BS)	Lombardia	3	**5cA, 5cB, 5cC**			1	**5cr**
6	Gussago (BS)	Lombardia					1	**6cr**
7	Monte Baldo (VR)	Veneto	3	**7cA, 7cB, 7cC**			1	**7cr**
8	Cerro Veronese (VR)	Veneto					1	**8cr**
9	Casalborgone (TO)	Piemonte	3	**9cA, 9cB, 9cC**			1	**9cr**
10	Tassarolo (AL)	Piemonte					1	**10cr**
11	Acqui (AL)	Piemonte					1	**11cr**
12	Cimaferle (AL)	Piemonte					2	**12crA, 12crB**
13	Bedonia (PR)	Emilia	3	**13cA, 13cB, 13cC**			1	**13cr**
14	Guiglia (MO)	Emilia	3	**14cA, 14cB, 14cC**			1	**14cr**
15	Gaggio Montano (BO)	Emilia	3	**15cA, 15cB, 15cC**			1	**15cr**
16	Zocca (MO)	Emilia	1	**16c**			1	**16cr**
17	Montese (MO)	Emilia	6	**17cA, 17cB, 17cC, 17cD, 17cE, 17cF**			2	**17crA, 17crB**
18	Monte Romano (RA)	Romagna	3	**18cA, 18cB, 18cC**			1	**18cr**
19	Monte Gamberaldi (FI)	Romagna	3	**19cA, 19cB, 19cC**			1	**19cr**
20	Tirrenia (PI)	Toscana			1	**20s**		
21	Tenuta del Tombolo (PI)	Toscana			3	**21sA, 21sB, 21sC**		
22	Calambrone (PI)	Toscana			1	**22s**		
23	Colognole (LI)	Toscana					1	**23cr**
24	Val Benedetta (LI)	Toscana					1	**24cr**
25	Monterotondo marittimo (GR)	Toscana			3	**25sA, 25sB, 25sC**	4	**25crA, 25crB, 25crC, 25crD**
26	Castiglione della Pescaia (GR)	Toscana			1	**26s**		
27	Alberese (GR)	Toscana			3	**27sA,27sB, 27sC**		
28	Capo d'Arco-Isola d'Elba (LI)	Toscana			3	**28sA, 28sB, 28sC**		
29	Luogosanto (SS)	Sardegna			3	**29sA, 29sB, 29sC**		
30	Nuraghe Majori (SS)	Sardegna			3	**30sA, 30sB, 30sC**		
			37		*21*		*27*	

Table 1: collecting data for samples of *Quercus cerris*, *Q. suber* and *Q. crenata*. Site numbers correspond to those in Figure 1. Shown are the locality name, region, number of individuals sampled at each site and abbreviations used for single plants.

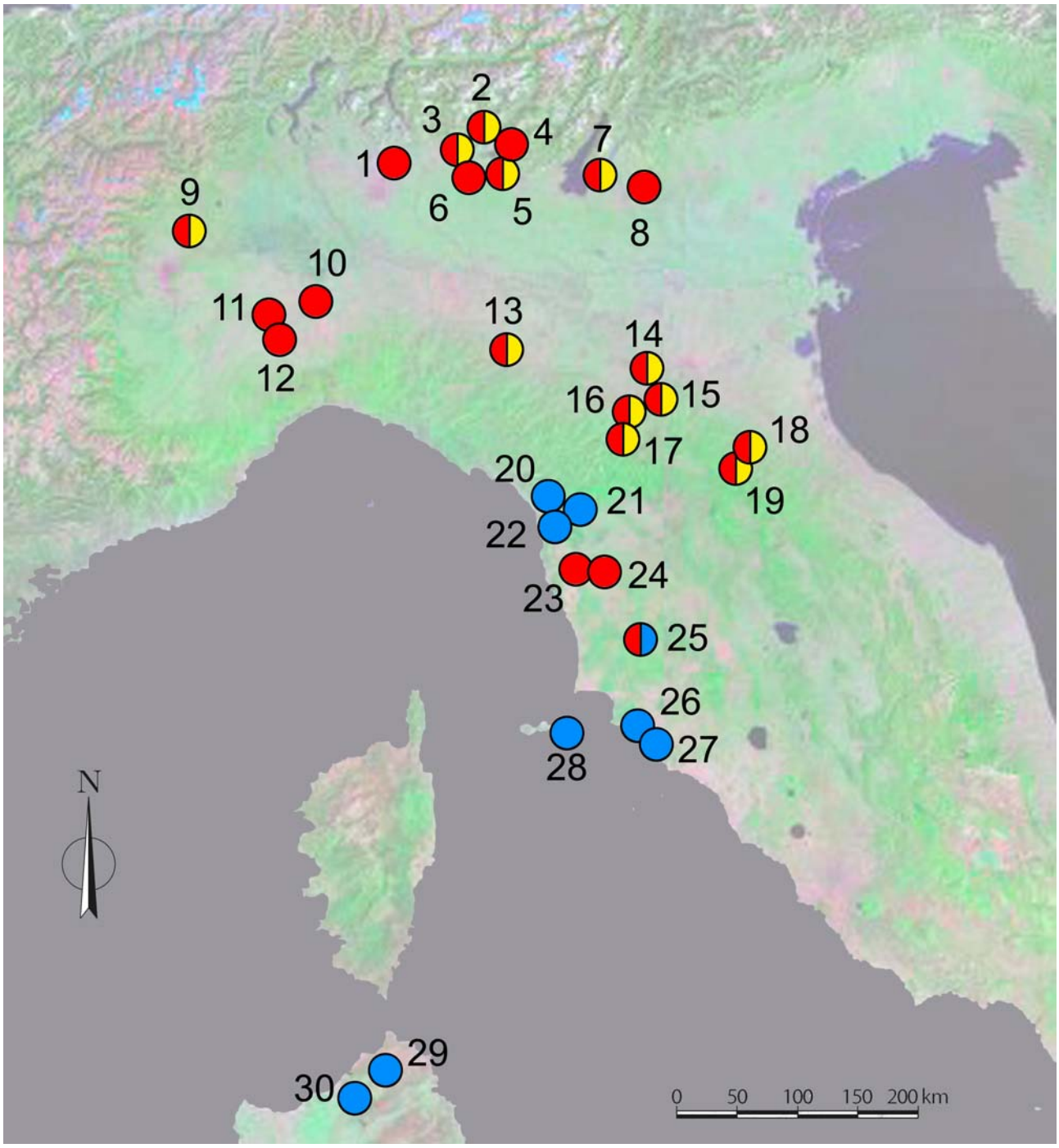


Figure 1: oak sampling sites in northern Italy (locations from 1 to 19) and central Italy (locations from 20 to 30). Numbers correspond to the list given in Table 1. ● symbol correspond to *Quercus crenata* samples, ● symbol to *Quercus cerris* samples and ● symbol to *Quercus suber* samples.

2.1.2 DNA EXTRACTION

The protocol of Dumolin et al. (1995) for genomic DNA extraction was followed, with only minor modifications. Specifically, 3-4 mg of frozen leaf tissue per plant was ground to a fine powder in liquid nitrogen with 0,1 M Tris-HCl, 0,02 M EDTA (ethylenediaminetetraacetic acid), 1,4 M NaCl, 1% PVP (polyvinyl-pyrrolidone), 2% ATMAB (alkyltrimethyalammonium bromide), 0,2% 2-mercaptoethanol. The omogenate was transferred to a Beckman tube (2 ml) and place inside the shaking incubator for 1 h at 55°C to provide the cellular lysis. To facilitate the precipitation of the fragments 400 µl of dichloromethane was added to the cooled tube and mixed gently. The emulsion was centrifugated at 13000 rpm for 10' (4°C) and, to avoid pipetting the inter phase, only 600 µl of upper phase was collected in a new labelled Eppendorf tube. To ensure the complete separation of the genomic DNA and the cellular waste this sequence was repeated again. To promote the DNA isolation and precipitation 270 µl of cooled isopropanol (-20°C) was added to the supernatant. The tube was put in a freezer (-20°C) and stored overnight.

To cause the DNA separation the tube was centrifugated at 13000 rpm for 10' (4°C) and the supernatant was removed carefully to avoid the pellet loss. The DNA pellet was dried by leaving it upside down on filter paper for 15'. The DNA was washed by adding 1 ml of ethanol 76% and centrifugated again at 13000 rpm for 10' (4°C). The ethanol was removed carefully and the DNA pellet was dried at room temperature for 1h 30'. The final suspension was made in 50 µl of pure water.

The DNA concentration was determined for each sample by the absorbance read at 260 nm (BioPhotometer, Eppendorf) and adjusted to 5-10 ng/µl for working solution. The tube was then stored at -20°C until required.

Purity determination of DNA interference by contaminants can be recognized by ratio calculations in the Eppendorf BioPhotometer. The ratio A_{260}/A_{280} is used to estimate the purity of nucleic acid since protein absorbs at 280 nm. Pure DNA should have a ratio of approximately 1,8. Absorption at 230 nm reflects contamination of the sample by substances such as carbohydrates, peptides, phenols or aromatic compounds. In the case of pure samples, the ratio A_{260}/A_{230} should be >2,0.

2.1.3.1 RAPD (RANDOM AMPLIFIED POLYMORPHIC DNA) AMPLIFICATION

The "Ready-To-Go RAPD Analysis Beads" kit (GE Healthcare Life Sciences) was used for the RAPD reactions. It provides the reagents for RAPD reactions in a convenient ambient-temperature-stable bead. The beads are manufactured using a proprietary technology licensed to GE Healthcare and are available predispensed into 0,5 ml PCR tubes. Ready-To-Go RAPD Analysis Beads have been optimized for RAPD reactions and contain thermostable polymerases (AmpliTaq DNA polymerase and Stoffel fragment), dNTPs (0,4 mM each dNTP in 25 µl reaction volume), BSA (2,5) and buffer [3 mM $MgCl_2$, 30 mM KCl and 10 mM Tris, (pH 8,3)] in a 25 µl reaction volume. The two different thermostable polymerases, combined in a proprietary ratio, produce a more complex RAPD fingerprinting pattern than either of the polymerases alone. The only reagents that must be added to the reaction are an arbitrary primer and template DNA. The Ready-To-Go bead format significantly reduces the number of pipetting steps, thereby increasing the reproducibility of the RAPD technique and minimizing the risk of contamination.
In addition to the RAPD Analysis Primer included in the kit, a primer set consisting of six primers of arbitrary sequence, with a GC content at least 60% and containing no hairpin structures, was used. Each primer is a 10-mer of arbitrary sequence that is specifically designed and tested for use in RAPD analysis. The RAPD Analysis Primer Set contains 2,5 nmol of each of the following primers:

RAPD Analysis Primer 1 - (5'-d[GGTGCGGGAA]-3')
RAPD Analysis Primer 2 - (5'-d[GTTTCGCTCC]-3')
RAPD Analysis Primer 3 - (5'-d[GTAGACCCGT]-3')
RAPD Analysis Primer 4 - (5'-d[AAGAGCCCGT]-3')
RAPD Analysis Primer 5 - (5'-d[AACGCGCAAC]-3')
RAPD Analysis Primer 6 - (5'-d[CCCGTCAGCA]-3')

The primers were reconstituted with 500 µl of sterile distilled water to give a final concentration of 5 pmol/µl and stored at -20°C. Then 10 µl (50 pM) of the primer of

choice and 10 ng of the required template DNA were added to each reaction mixture along with sterile distilled water to make up the total volume of 25 µl.

Several annealing temperatures were tested in a series of preliminary amplifications and the best results were obtained with higher values than those routinely used. Very stringent conditions at the annealing stage are known to give the most efficient reproducibility of the RAPD fingerprints because at high temperatures only perfectly matching primer/template hybrids are stable and contamination by products does not appear (Linz et al. 1990).

The PCR Express thermal cycler (Hybaid) was programmed as follows (primer-specific annealing temperatures given in Table 2, pag. 48): an initial melting step of 4 min at 94°C; 40 cycles each of 1 min at 94°C (denaturation), 1 min at 50°C–55°C (annealing), 2 min at 72°C (extension); a final extension step of 72°C for 5 min was performed after the 40 cycles and samples were maintained at 4°C after the completion of the cycles, until they could be used, or stored at -20°C. In order to ensure reproducibility of the results, each primer-sample combination was repeated at least twice.

2.1.3.2 ANCHORED ISSR (INTER SIMPLE SEQUENCE REPEAT) AMPLIFICATION

Three UBC (University of British Columbia) primers (synthesized from Operon Biotechnologies, Germany) were selected for the ISSR analysis based on that they could give reproducible bands: $(AG)_8T$, $(AG)_8C$, $(GA)_8C$.

The "PuReTaq Ready-To-Go PCR Beads" kit (GE Healthcare Life Sciences) was used for the ISSR reactions. The beads contain room temperature-stable beads with stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2,5 units of recombinant PuReTaq DNA polymerase and reaction buffer. When a bead is reconstituted to a 25 µl final volume, the concentration of each dNTP is 200 µM in 10 mM Tris-HCl, (pH 9,0 at room temperature), 50 mM KCl and 1,5 mM $MgCl_2$.

For each reaction was added to a tube containing a PCR bead 30 ng template DNA, 4 µl (40 pM) of the primer and sterile high-quality water to a final volume of 25 µl.

Amplifications were done using the same thermocycler with the following settings: 1 cycle of 5 min at 94°C followed by 45 cycles of 1 min at 94°C, 45 sec at 56°-58°C (Table 2, pag. 48), and 2 min at 72°C; a final extension step of 72°C for 7 min was performed. The effects of DNA concentrations and different temperatures during the annealing stage of amplification were examined for each primer in a series of preliminary amplifications in order to ensure reproducibility and polymorphic amplification patterns.

2.1.4 AUTOMATIZED PAGE

The amplification products were separated using polyacrylamide Phast-Gel minigels and visualized using the Amersham Biosciences PhastSystem high speed electrophoresis system.

The Amersham Biosciences PhastSystem consists of a Separation-Control unit for system control and electrophoresis, and a Development Unit for gel staining.

Separation is performed on a thermostatic plate capable of maintaining temperatures accurately from 0 to +70°C. The gel was positioned on the separation bed and the PhastGel buffer strip holder was placed over it. The buffer strips were inserted into the compartments in the buffer strip holder; one in the anode (+) and one in the cathode (-) compartment; they are made of 3% agarose IEF and serve as buffer reservoirs to generate discontinuous buffer systems in the gel during a run. Approximately 1 µl of each sample was loaded onto the gel by using the sample applicator 8/1 with 8 sample wells. The electrodes rest on the strips during electrophoresis and transfer current and voltage to the gel to promote the bands separation.

For RAPD fragments a PhastGel gradient 10-15 with a continuous gradient from 10 to 15% polyacrylamide was used, whereas PhastGel gradient 8-25 was used for ISSR fragments. These minigels consist of a 13 mm stacking zone and a 32 mm gradient gel zone. The buffer system in the gels is 0,1 M acetate (leading ion) and 0,1 M Tris, pH 6,4. The buffer system in the buffer strips is of 0,2 M tricine (trailing ion), 0,2 M

Tris and 0.55% SDS (analytical grade), pH 7,5.

The gel ran according to the protocol for PhastGel in the Phast System Owner's Manual:

First step (sample applicator lowered onto the gel) **1.1**:	100W 7mA 1,0W 15°C 10Vh
Second step (sample applicator raised from the gel) **1.2**:	250W 7mA 2,0W 15°C 100Vh

The duration (volthours) of the separation runs for each RAPD and ISSR primer was different, in order to clearly visualize the size range of the amplified products (Table 2, pag. 48).

At the separation end the gel was transferred to the development chamber and visualized with silver stain according to a procedure adapted from Bassam et al. (1991):

1^{th} step - fixing solution: 20% trichloroacetic acid for 5 min at 25°C

2^{th} step - sensitizer solution: 5% glutaraldehyde 50% for 6 min at 40°C

3^{th} and 4th step - washing solution: distilled water for 2 min at 40°C

5^{th} step - staining solution: 0,137% silver nitrate, 0,04% formaldehyde for 10 min at 30°C

6^{th} step - washing solution: distilled water for 2 min and 30 sec at 25°C

7^{th} step - washing solution: distilled water for 30 sec at 25°C

8^{th} step - washing solution: distilled water for 30 sec at 30°C

9^{th} developing solution – 2,5% sodium carbonate, 0,06% formaldehyde, $1,10^{-4}$% sodium thiosulphate

10^{th} step – stopper solution: 10% acetic acid for 20 min at room temperature

11^{th} step - preserving solution: 10% glycerol and 10% acetic acid for 20 min at room temperature.

The presence of formaldehyde in the silver staining solution and in the developing solution improves both sensitivity and contrast, reducing development time, while the sodium thiosulphate is useful for dissolving insoluble silver salts by complex

formation, removing silver ions from the minigel surface, which in turn decreases non specific and background staining.

The concentration of silver nitrate, formaldehyde and sodium thiosulphate is focal to ensure a reproducible and clear band pattern; therefore a preliminary screening was conducted on 21 samples using the six RAPD primers and the three ISSR primers. In order to establish the best balance between these reagents, different concentrations were tried:

-in the staining solution the silver nitrate was tested from 0,100% to 0,212% and formaldehyde between 0% and 0,05%

-in the developing solution the sodium thiosulphate was varied from 0% to $1,10^{-4}$% and the formaldehyde between 0,05% and 0,075%.

The development was carried out automatically up to the 8^{th} step and manually completed to assess the optimal developing time, which was different for each primer (Table 2, pag. 48).

Following staining, the gel was dried and analyzed.

Primer type	Sequence (5'-3')	Annealing temperature (°C)	Separation run (Vh)	Developing time (min)
RAPD primers				
1	GGTGCGGGAA	50	71-72	4'30"-6'
2	GTTTCGCTCC	51	66-67	6'-8'
3	GTAGACCCGT	51	60-62	5'-7'
4	AAGAGCCCGT	54	69-71	5'-6'30"
5	AACGCGCAAC	52,5	66-70	4'30"-7'
6	CCCGTCAGCA	55	70-76	5'-7'
ISSR primers				
UBC 807	$(AG)_8T$	56	74-81	5'30"-6'30"
UBC 808	$(AG)_8C$	58	76-83	4'30"-6'30"
UBC 811	$(GA)_8C$	57	79-85	3'30"-5'30"

Table 2: list of RAPD and ISSR primers used and optimal annealing temperature for each of them; duration of the separation run and developing time are also indicated.

2.1.5 DATA SCORING AND STATISTICAL ANALYSIS

DATA SCORING

Digital images of the gels were analyzed and fragment sizes were determined by comparison with the known fragments of the standard marker lane pBR322 DNA-BstN I Digest (121 bp, 383 bp, 929 bp, 1058 bp, 1857 bp) using the TotalLab image analysis software (Fotodyne Inc.), which permits the fast and consistent analysis of 1D electrophoresis gel images performing precise band edge detection.

To assess the reproducibility of PCR products, the DNA from the 85 individuals was amplified independently twice with each RAPD and ISSR primer. Only intensely stained, unambiguous, polymorphic bands were used in the analysis.

Amplified fragments, with the same mobility according to the molecular weight (bp), were clustered in weight ranges and scored as discrete variables, using 1 to indicate presence and 0 for absence of homologous bands. Since RAPD and ISSR are dominant markers, each amplification product was considered to represent the molecular phenotype at a single bi-allelic locus and it was assumed that similarity of fragment size was an indicator of homology (O'Hanlon and Peakall 2000). Although the frequency of the two alleles at each locus can be inferred from the frequency of presence and absence of the band (e.g. Lynch and Milligan 1994), analyses that do not rely on knowing these frequencies were preferred in this study to avoid the uncertain assumption of Hardy-Weinberg equilibrium.

Two binary qualitative data matrices of the different RAPD and ISSR molecular phenotypes were assembled; the rows corresponded to the samples and the columns corresponded to the weight ranges (bp).

These two matrices were then used for the following statistical analysis.

STATISTICAL ANALYSIS

In order to assess the molecular diversity within each species sample, the percentage of polymorphic fragments (PPB) and the Simpson diversity index (S) were calculated by the software PAST, version 1.63 (Hammer et al. 2001). Simpson's Index (S) measures the probability that two individuals randomly selected from a sample will

belong to the same species.

The ISSR and RAPD binary matrices were processed in the PAUP* version 4.0b10 (Swofford 2000) package and converted into similarity matrices using the Nei and Li index (Nei and Li 1979).

Many statistical analyses are based on genetic distances and rely on estimates of phenotypic diversity, thereby obviating the need for locus-specific data. As a first step in these analyses, multilocus band patterns are subjected to one of various strategies to quantify pairwise similarity of the genotypes represented in the different lanes. Most commonly a similarity index is calculated from band sharing data of each pair of fingerprints. These indices can be used to construct matrices of pairwise similarity which are used as an input file for various subsequent multivariate analyses.

The Nei and Li coefficient is one of the most commonly used similarity indices, which is also known as Dice's coefficient:

$D= 2\ n_{ab}/(\ n_a + n_b)$

Here, n_a and n_b represent the numbers of bands present in lanes a and b, respectively, and n_{ab} represents the number of bands shared by both lanes. D can acquire any value between 0 and 1, where 0 means no bands in common, and 1 means patterns are identical. This index takes only positive matches (both bands are present) into account and, placing a weight of 2 on shared bands, purportedly permits a better differentiation of individuals with low levels of similarity. Given that the absence of an RAPD or ISSR band may have several different causes, it has been argued that using the mutual absence of bands is improper for calculating similarity. The similarity matrices serve as a starting point for the subsequently multivariate analyses.

The main purpose of multivariate statistics is to condense the differences between the entries for many characters into fewer characters and to visualize these entries in a multidimensional space.

2.1.5.1 CLUSTER ANALYSIS

Cluster analysis, also referred to as distance method or phenetic method, is the most used type of classification analysis, whose goal is to group similar objects into identifiable and interpretable classes that can be distinguished from neighboring classes and to resume these relationships in a dendrogram (tree-diagram). Cluster analysis takes *m* observations, each of which has associated with *n* continuous numerical variables, and segregates the observation into groups. It can be used to group observations on the basis of species abundances or presences-absences, or to group organisms on the basis of similarity in measured characteristics such as morphology or DNA fragments.

There are several methods available for clustering data but the most commonly used by environmental scientists are agglomerative and hierarchical clustering. These methods proceed by taking many separate observations and grouping them into successively larger clusters until one cluster is obtained.

The UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) (Sneath and Sokal 1973) is an algorithmic method which uses a specific series of calculations to estimate a tree. The starting point is the ISSR or the RAPD matrix of pairwise similarity, calculated from the primary data by the Nei and Li algorithm using the program PAUP. This program first finds the pair of taxa with the smallest distance between them and defines the branching between them as half of that distance placing a node at the midpoint of the branch. It then combines the two taxa into a "cluster" and rewrites the matrix with the distance from the cluster to each of the remaining taxa. Since the "cluster" serves a substitute for two taxa, the number of entries in the matrix is now reduced by one. That process is repeated on the new matrix and reiterated until the matrix consists of a single entry. That set of matrices is then used to build up the tree by starting at the root and moving out to the first two nodes represented by the last two clusters. The resulting dendrograms express phenetic similarities among the taxa and are therefore called phenograms. They do not necessary reflect phylogenetic relationships.

Strict consensus trees, containing only those clusters found in all the trees and based on the two similarity matrices, were constructed with the UPGMA procedure. In order to evaluate the topological consistency, the consensus fork index (CFI) (Colless 1980, Swofford 1991) and the Mickevich (1978) consensus information index (MCI) were also calculated. The consensus fork Index (CFI) measures tree similarity by calculating the number of shared clades between two trees divided by the total number of possible clades, which is the total number of taxa minus two.

2.1.5.2 PRINCIPAL COMPONENT ANALYSIS

A principal component analysis was performed in order to highlight the resolving power of the ordination.

Ordination techniques are used to order multivariate data. Ordination creates a few key variables, each of which is a composite of many of the original variables. It constructs a new set of orthogonal coordinate axes such that the projection of points onto them has the maximum variance. These new variables characterize as fully as possible the variation in a multivariate dataset and they are not correlated with one another. Used in this way, ordination is a data-reduction technique: beginning with a set of *n* variables, the ordination generates a smaller number of variables that still illustrate the important patterns in the data.

Among the ordination methods, Principal Component Analysis (PCA) is one of the most important techniques. PCA is a procedure for finding hypothetical variables (components) which account for as much of the variance in the multidimensional data as possible. PCA has several applications, the most important of them are:

- reduction of the data set to only three variables (the three most important components), for plotting and clustering purposes
- determination of a correlation between the three most important components and some other underlying variables.

The PCA takes the cloud of data points, and rotates it such that the maximum variability is visible. The first stage in rotating the data cloud is to standardize the data by subtracting the mean and dividing by the standard deviation.

The first component extracted in a Principal Component Analysis accounts for a maximal amount of total variance in the observed variables where the "total variance" in the data set is simply the sum of the variances of the observed variables. Because of this, the total variance in a Principal Component Analysis will always be equal to the number of observed variables being analyzed. Then, under typical conditions, the first component will be correlated with at least some of the observed variables. It may be correlated with many.

The second component extracted will have two important characteristics. First, this component will account for a maximal amount of variance in the data set that was not accounted for by the first component. Again under typical conditions, this means that the second component will be correlated with some of the observed variables that did not display strong correlations with component 1.

The second characteristic of the second component is that it will be uncorrelated (or orthogonal) with the first component. Literally, if the correlation between components 1 and 2 is computed, that correlation would be zero.

The remaining components that are extracted in the analysis display the same two characteristics: each component accounts for a maximal amount of variance in the observed variables that was not accounted for by the preceding components, and is uncorrelated with all of the preceding components. A Principal Component Analysis proceeds in this way, with each new component accounting for progressively smaller and smaller amounts of variance (this is why only the first few components are usually retained and interpreted). When the analysis is complete, the resulting components will display varying degrees of correlation with the observed variables, but are completely uncorrelated with one another.

The Principal Component Analysis was conducted in this study using the program NTSYS-pc vers. 2.2 (Rohlf 1996). Variables chosen for any analysis are usually measured in different units and are generally not additive. Hence, it is necessary to convert them in some standard comparable units such that the initial scale chosen for

measuring them do not bias the results. The binary matrices of RAPD and ISSR data were standardized using the default options of the STAND program. Standardized data were converted into symmetric correlation matrices with the SIMINT function. Three eigenvectors were extracted from the correlation matrices using the EIGEN function. The RAPD and ISSR standardized data were projected onto the resulting eigenvectors using the PROJ function and two three-dimensional plots of the RAPD and ISSR objects were achieved using the MOD3D function.

2.1.5.3 THE HYBRID INDEX

In order to quantify the genetic contribution of the two hybridizing parental species to *Q. crenata* individuals, a hybrid index was computed using the software program HINDEX (Buerkle 2005) version for Linux (x86) systems. The hybrid index (*h*) is based on information from molecular markers and uses maximum-likelihood (ML) to estimate the proportion of alleles that were inherited from one of two parental species; for this reason does not require to identify diagnostic loci for the two parental species *Q. suber* and *Q. cerris*. Allele frequencies in the parental populations were used as the end points for calculating a plant's *h*. Plants with an *h* of zero were genotypically similar to plants from *Q. suber*-like parental populations, whereas those with an *h* of 1 were genotypically similar to plants from *Q. cerris*-like parental populations. The index is defined for dominant and codominant molecular markers.

The likelihood function is determined by the unknown individual's genotype and the frequencies of alleles within each of the parental species at each of the loci. For dominant loci the index h_j is the probability that the *j*th diploid genotype is from one of the two parental species. Suppose that each locus consists of two alleles, with the A allele dominant to the a allele. RAPDs and ISSRs are assumed to be dominant, with genotypes AA or Aa corresponding to band presence. The A allele is present in a parental species (*Q. cerris*) at frequency *p*, and in the other parental species (*Q. suber*) at frequency *q*.

The probability of observing a band from an individual from *Q. cerris* is Pr[AA or Aa| *Q. cerris*] =1-Pr[aa] = $1-(1-p)^2 = p(2-p)$. Similarly, the probability of observing a band, given membership in *Q. suber*, is $1-(1-q)^2 = q(2-q)$. On the other hand, the probability of not observing a band from an individual of *Q. cerris* is Pr[aa| *Q. cerris*] = $(1-p)^2$. Likewise for *Q. suber*. The likelihood of hybrid index h_j is $L_{j|b} = h^{p(2-p)}(1-h)^{q(2-q)}$ when a band is observed, and $Lj|n = h^{(1-p)2}(1-h)^{(1-q)2}$ when no band is observed. Maximizing the likelihood with respect to the hybrid index, dLog $L_{j|b}$/dh= 0, provides a maximum likelihood estimator of the index.

Suppose a band is observed. The maximum likelihood estimator of the hybrid index is then $h_{j|b} = p(2-p)/[p(2-p) + q(2-q)]$. This index takes on value 1 when an observed band is present only in *Q. cerris*, 0 when an observed band is present only in *Q. suber*, and intermediate values when both species contain the band. When a band is not observed, the maximum likelihood estimate of the hybrid index is $h_{j|n} = (1-p)^2/[(1-p)^2 + (1-q)^2]$. Again, the index ranges between 0 and 1, with extreme values occurring when only one species is polymorphic for band presence.

This method extends to multiple loci (RAPD and ISSR bands), assuming loci are independent of one another. Suppose an individual has dominant phenotype at 1, …, m loci and recessive phenotype at m + 1, …, n loci. The likelihood of the hybrid index h_j is $L_j = \Pi^m{}_{i=1}\, L_{i,j|b} \times \Pi^n{}_{i=m+1}\, L_{i,j|n}$.

The maximum likelihood estimate of the hybrid index for the jth individual is:

$$hj = \frac{\Sigma^m{}_{i=1}\, p_i(2-p_i)}{\Sigma^m{}_{i=1}\, p(2-p_i)+q_i(2-q_i)} + \frac{\Sigma^n{}_{i=m+i}(1-p_i)^2}{\Sigma^n{}_{i=m+i}\,(1-p_i)^2+(1-q_i)^2}$$

where p_i indicates allele frequency of the dominant allele at the ith locus in *Q. cerris* species.

The results were represented in two frequency histograms and the distributions were tested for normality by the Shapiro-Wilk W-test which calculates a W statistic that tests whether a random sample, x_1, x_2, ..., x_n comes from a normal distribution . The null hypothesis for this test is that the data are normally distributed. Small values of

W are evidence of departure from normality. The Prob.< W value listed in the output is the p-value. If the chosen alpha level is 0.05 and the p-value is less than 0.05, then the null hypothesis that the data are normally distributed is rejected. If the p-value is greater than 0.05, then the null hypothesis has not been rejected.

The distributions were then compared by Pearson correlation coefficient (ρ) using PAST version 1.40 (Hammer et al. 2001).

The correlation between two variables reflects the degree to which the variables are related. The most common measure of correlation is the Pearson Product Moment Correlation (ρ). Correlation is a technique for investigating the relationship between two quantitative, continuous variables. Pearson's correlation coefficient (ρ) is a measure of the correlation of two variables X and Y measured on the same object or organism, that is, a measure of the tendency of the variables to increase or decrease together. It is defined as the sum of the products of the standard scores (which are a dimensionless quantity derived by subtracting the population mean from an individual raw score and then dividing the difference by the population standard deviation) of the two measures divided by the degrees of freedom.
The result obtained is equivalent to dividing the covariance between the two variables by the product of their standard deviations.

The coefficient ranges from −1 to 1. A value of 1 shows that a linear equation describes the relationship perfectly and positively, with all data points lying on the same line and with Y increasing with X. A score of −1 shows that all data points lie on a single line but that Y increases as X decreases. A value of 0 shows that a linear model is inappropriate – that there is no linear relationship between the variables.

2.1.5.4 THE MANTEL TEST

In order to estimate the congruence among dendrograms, cophenetic matrices for each marker was computed and compared using the Mantel test. The Mantel Test was conducted in this study using the program NTSYS-pc vers. 2.2 (Rohlf 1996). The SIMQUAL program was used to calculate the Nei and Li index and the two similarity matrices obtained for the two markers was processed with the COPH program in order to compute the two cophenetic matrices from the two tree matrices. The MXCOMP program performs the comparisons taking the two cophenetic matrices and plot one matrix against the other element by element. It computes the cophenetic correlation, r, and the Mantel test statistic, Z (Mantel 1967) to measure the degree of relationship between the two matrices and it can be used as a measure of validity of fit for a cluster analysis.

Mantel's statistic is based on a simple cross-product term:

$$z = \sum_{i=1}^{n} \sum_{j=1}^{n} x_{ij} y_{ij}$$

and is normalized:

$$r = \frac{1}{(n-1)} \sum_{i=1}^{n} \sum_{j=1}^{n} \frac{(x_{ij} - X)}{s_x} \cdot \frac{(y_{ij} - Y)}{s_y}$$

where x and y are variables measured at locations i and j and n is the number of elements in the distance matrices (= $m(m\text{-}1)/2$ for m sample locations), and the s_x and s_y are standard deviations for variable x and y. The degree of fit can be interpreted subjectively as follows:

$0.9 < r$ Very good fit.

$0.8 < r < 0.9$ Good fit.

$0.7 < r < 0.8$ Poor fit.

$r < 0.7$ Very poor fit.

2.2 SPECIFIC MARKERS

2.2.1 PLANT MATERIAL AND DNA EXTRACTION

The sampling involved 16 individuals collected from Monte Prado site, 4 individuals from the Lago Santo site, and 4 individuals from Lago Verde site.
Genomic DNA was extracted from 4 mg of leaf tissue of *Primula appenina* Widmer using the Invisorb Spin Plant Minikit for 250 extractions with slight modifications. The starting material was omogenized in a mechanical tissue homogenizer (Ribolyser, Hybaid) using thirty small glass balls and four medium glass balls to help powdering. In order to induce lysis of membranes and liberation of DNA from nuclei, it was added 400 µl of Lysis buffer P and 20 µl of proteinase K, to remove RNA. After a incubation at 65°C for 45 min under continuous shaking, the lysate was centrifugated at 12000 rpm for 10 min (4°C) and transferred onto a Spin Filter by a blu tip. After a centrifugation of 1 min at 12000 rpm (4°C) the Spin Filter was removed and 200 µl of Binding buffer P was added. The suspension was transferred onto a new Spin Filter and incubated for 1 min and then 550 µl of Wash Buffer I was added to the binded DNA in order to wash it. The washing step was repeated adding 550 µl of Wash Buffer II. After a centrifugation of 5 min at 12000 rpm (4°C) to remove residual ethanol the Spin Filter was placed into a 1,5 ml Receiver Tube and 100 µl of prewarmed Elution Buffer D was added to obtain the DNA elution. The DNA concentration was determined by the absorbance read at 260 nm (BioPhotometer, Eppendorf) and the tube was then stored at -20°C until required.

2.2.2 CROSS-SPECIES TRANSFERABILITY WITHIN THE GENUS *PRIMULA*

All the primer pairs isolated for the genus Primula and available in the GenBank database are used: 16 primer pairs developed for *Primula sieboldii* (Ueno et al. 2003, 2005), 11 primer pairs for *Primula modesta* (Shimono et al. 2004), 7 primer pairs for *Primula nutans* (Kreivi et al. 2006) and 3 primer pairs for *Primula vulgaris*

(http://www.ncbi.nlm.nih.gov/) were tested in 32 sample individuals of *Primula apennina* Widmer.

PCR reactions were performed in a final volume of 25 µl. The reaction buffer contained 25 ng of DNA template, 0,02 µmol/l of each primer, 0,4 mM of each dNTP, 4 mM of $MgCl_2$ and 0,05 units/µl Taq-DNA polymerase (Fermentas). One cycle of 3 min at 94°C, was followed by 35 cycles of 30 sec at 94°C, 30 sec at the annealing temperature chosen for each primer in a precedent stage, and 1 min at 72°C, with a final elongation step of 7 min at 72°C.

In order to isolate only the band of the expected size the "crush and soak" method was used. This method provided that the portion of the silver-stained polyacrilamide gel containing the band of interest was cut out, the gel slice was crushed against the wall of the tube with a pipette tip and, after centrifugation (13000 g for 2 min), the supernatant was used as template in a PCR.

PCR was performed as descrived above, and the products were loaded onto a polyacrylamide gel in order to control the size of the amplified fragment. After verification of the size, the PCR products were sequenced (ABI PRISM® 310 Genetic Analyzer – Applera).

2.2.3 MICROSATELLITE MARKERS ISOLATION BY FIASCO PROTOCOL

Microsatellite markers were isolated using the modified genomic DNA enrichment protocol of fast isolation by AFLP of sequences containing repeats (FIASCO) (Zane et al. 2002).

This protocol involves the following steps:

- Genomic DNA is simultaneously fragmented with *MseI* and ligated to *MseI* AFLP adaptor MseAdU (5'-GAC GAT GAG TCC TGA G -3') e MseAdD: (5'- TAC TCA GGA CTC AT -3') to facilitate later cloning steps.

- Ligation products are amplified in PCR with adapter-specific primers. The number of cycles in the PCR amplification needs to be optimized because over-amplification was found to change the average size of amplified fragments. The PCR conditions producing a visible product on agarose gel (in the form of a smear) are considered optimal and are selected for further use.

- In order to obtain several hundred nanograms of amplified DNA, PCR amplification under optimal conditions is replicated 10 times. The resulting PCR products are mixed and controlled by an agarose gel.

- DNA is then hybridized with single-stranded, microsatellite-specific oligonucleotides attached to streptavidin-coated magnetic particles as target for hybridization selection. DNA molecules hybridized to biotinylated $(AC)_{17}$ probe . The beads-probe-DNA complex is then separated by a magnetic field from the hybridization buffer, which is then discarded.

- After washing off unbound DNA, hybridizing fragments, which should be enriched with microsatellites, are eluted from beads-probe complex by two denaturation steps and reamplified using adapter-complementary primers. These PCR products are the best candidates for producing a highly enriched microsatellite library, because they are likely to contain the largest proportion of repeat-containing fragments.

- The enriched, PCR-amplified DNA fraction is then ligated into a vector and transformed into *Escherichia coli* competent cells.

- Transformants are plated, then insert-containing clones are selected by blue-white screening. Unfortunately, not all positive clones prove to be useful for primer design. Thus, some clones may not contain a microsatellite at all (false positive), whereas others contain a microsatellite so close to one of the insert-plasmid boundaries that no flanking primer can be designed. To eliminate such useless positives and minimize unnecessary sequencing, a PCR-based pre-screening

procedure is performed to establish the presence and position of the microsatellites.

- By electophoresis on agarose gel PCR products are selected according to size range (400 to 800 bp) to ensure the sequencing and the developing of specific primers.

- The selected PCR products can be purified by presequencing kit and directly sequenced.

Despite the extraordinary increase of interest in SSRs in the last few years and the technical advances that have been made in recent years to facilitate microsatellite development, the microsatellite library construction is only the first stage in the process of developing a set of working microsatellite primers. The task of developing a working primer set from an enriched library can in itself represent a significant workload (Zane et al. 2002).

As explained previously, the successful isolation of microsatellite markers necessitates several distinct steps to obtain a working set of primers which can amplify polymorphic microsatellite loci. At each stage, there is the potential to "lose" loci and the number of loci which will eventually constitute the working primer set will be a fraction of the original number of sequenced clones. The first "attrition stage", in which losses can occur, consists in the identification of clones containing microsatellite. There are inevitably some sequences which do not contain a microsatellite, and, on those which contain microsatellites, not all will be unique, hence, there is some level of redundancy. On average, approximately 1/3 of the sequenced clones are lost due to the absence of a unique microsatellite.

Ultimately, it is possible to encounter chimeric sequences, in which one of the flanking regions (but not the other) matches that of another clone.

The second "attrition stage" takes into account that not all the microsatellite flanking regions can be suitable for primer design. Microsatellites may be located too close to

the end of an insert to accommodate primer design in the flanking region, and even when there is sufficient length of sequence, the base composition may be unsuitable. The last stage comprises those primer pairs which, seemingly suitable, fail to amplify. Some optimization of reaction conditions can improve success, but this nevertheless represents an additional source of attrition (Figure 2).

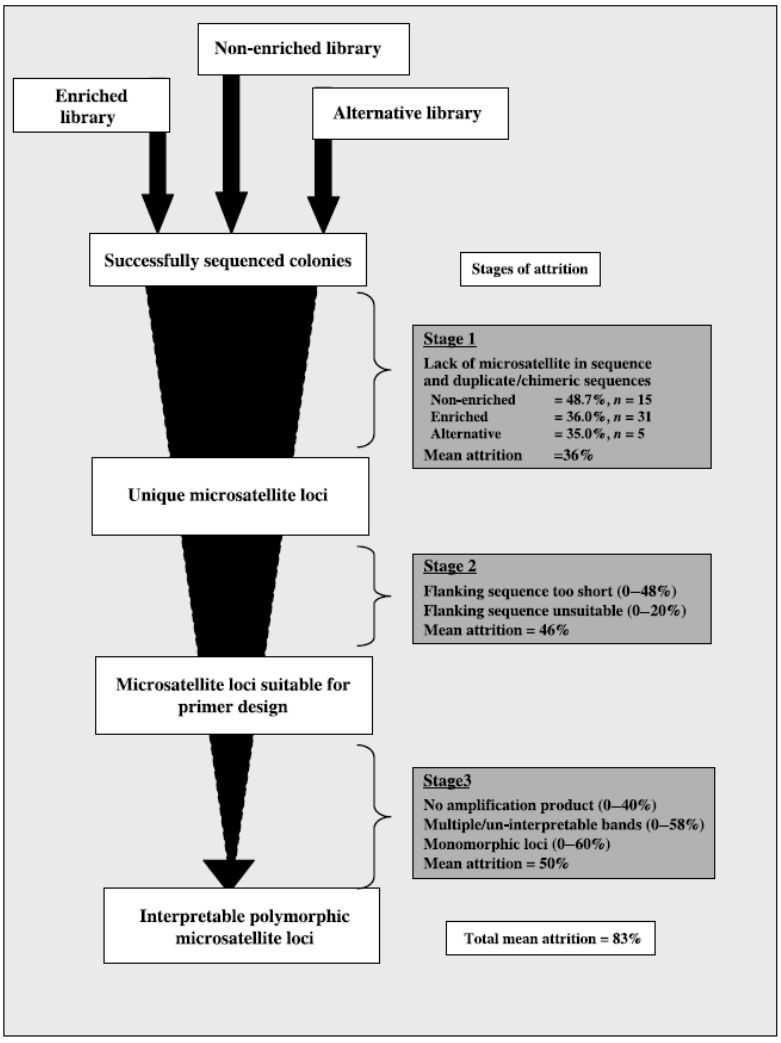


Figure 2: Diagramatical representation of microsatellite attrition (Squirel et al. 2003)

2.2.3.1 DIGESTION AND LIGATION

A total of 200 ng genomic DNA was digested with *MseI* (BioLabs) in a 25-µl volume and ligated to AFLP adaptors using T4 DNA ligase (TaKaRa). The recognition sequence of the *MseI* restriction enzyme is:

5'- T|TAA -3'
3'- AAT|T -5'

and the *MseI* AFLP adaptors are:

- MseAdU (5'- GAC GAT GAG TCC TGA G -3')
- MseAdD: (5'- TAC TCA GGA CTC AT -3')

The reaction mixture was:

- H_2O dd 13,75 µl
- Buffer One Phor All 10X 2,5 µl (GE-Healhcare)
- DTT 100 mM 1,25 µl
- BSA 10 mg/ml 0,125 µl
- Adaptors 50 µM 0,5 µl
- ATP 20 mM 0,25 µl
- T4 Ligase 1 µl (GE-Healhcare)
- *MseI* 0,625 µl
-DNA 200 ng

and was incubated at 37°C for 3 hours. In order to inactivate the *MseI* restriction enzyme and the T4 ligase the reaction mixture was heated at 65°C for 20 minutes.
The digestion was checked with 1,5% agarose gel and a visible product in the form of a smear was considered optimal.

The digestion-ligation mixture was subsequently amplified using AFLP adaptor-specific primers (5'- GAT GAG TCC TGA GTA AN- 3'), i.e. *MseI*-N). In order to reduce, at least partly, the problem of biased amplification (over-amplification was

found to change the average size of amplified fragments), parallel PCR amplifications were progressively performed using two digestion-ligation mixture diluition (1:7, 1:10) and increasing the number of cycles (17-20-23-26).

The PCR reaction mixture was:
- H_2O dd 7,32 µl
- Primer *MseI* 4 µl
- Buffer 10X 2 µl
- $MgCl_2$ 25 mM 1,2 µl
- dNTPmix 2 mM 0,4 µl
- Taq polimerase 0,08 µl
- DNA 5 µl

Then, 1:7 diluition and 23 cycles were chosen, after electrophoresis, as optimal conditions and the polymerase chain reaction (PCR) was performed using a program of 94°C 30s, 53°C 1 min, 72°C 2 min.

To obtain several hundred nanograms of amplified DNA, 10 PCR amplifications under optimal conditions were replicated and quantified by an agarose gel.

The 200 µl of PCR product was precipitated with 20 ml of sodium acetate 3M and 1 ml of ethanol absolute (EtOH 100%), after 15 min on ice and centrifugation of 15 min (14000 rpm), DNA pellet was washed with 500 µl 70% ethanol. After a centrifugation of 15 min (14000 rpm) the supernatant was discarded, dried and dissolved in 50 µl of dH_2O.

2.2.3.2 ENRICHMENT AND HYBRIDIZATION

The enrichment protocol included the hybridization of the DNA fragments with a biotinylated probe $(AC)_{17}$. To isolate the enriched DNA and to remove nonspecific

binding, several washes were done and streptavidin-coated magnetic beads were used to attract the biotin and the fragments binded to it.
The washing solutions TEN 100 and TEN 1000 were prepared as follows:

TEN 100:
- 50 µl Tris 1M pH = 7,5
- 10 µl EDTA 0,5 pH = 8
- 200 µl NaCl 2,5 M
Bring the volume to 5 mL with distilled water.

TEN 1000:
- 50 µl Tris 1M pH=7,5
- 10 µl EDTA 0,5 pH=8
- 2 ml NaCl 2,5 M
Bring the volume to 5 ml with distilled water.

Streptavidin Magnetic Particles (Roche Applied Science) [100 µl particles x n° species x n° probes] were prepared by washing them in 100 µl TEN 100 three times and resuspending them in 40 µl of the same buffer. To minimize nonspecific binding of genomic DNA, 10 µl of an unrelated PCR product (mitochondrial DNA) was mixed with the beads before adding the hybridization mixture.

The hybridation mixture was prepared in 0,5 ml Eppendorf tube as follows:

- saline-sodium citrate (SSC) 20X	21 ml	
- biotinylated $(AC)_{17}$ probe	(75 pM)	5 ml
- sodium dodecyl sulfate SDS 10%	0,7 ml	
-DNA	400 ng	

Bring the volume to 100 µl with distilled water.

DNA was denatured at 95°C (3 min), and annealing was performed at room temperature for 15 min.

The prepared beads were then mixed to the DNA-probe hybrid molecules (diluted with 300 mL of TEN 100) and incubated for 30 min at room temperature with constant gentle agitation to induce the bond between biotinylated probe and magnetic particles.

2.2.3.3 WASHING STEPS

The beads-probe-DNA complex was separated by a magnetic field from the hybridization buffer, which was then discarded. Nonspecific DNA was removed by three nonstringency washes and three stringency washes. Nonstringency washes were performed by adding 400 ml of TEN 1000, while stringency washes were performed by adding 400 ml of SSC 0.2X, 0.1% SDS to the DNA. Each wash was done for 5 min at room temperature and with gentle mixing. DNA was recovered by magnetic field separation each time.

DNA was separated from the beads-probe complex by two denaturation steps. In the first step 50 ml of TLE 1X (Tris-HCl 10 mm, EDTA 1 mm, pH 8) was added to the beads, which were then incubated at 95°C for 5 min. The supernatant, containing target DNA, was separated from magnetic particles with a magnetic field and quickly removed, centrifugated (12000 rpm for 1 min) to eliminate the last magnetic beads and then stored.

The second denaturation step was performed by treating beads with 12 ml of 0.15 M NaOH; in this case the recovered supernatant must be neutralized, before storage, by the addition of an appropriate amount of acetic acid. This was determined in advance by treating the NaOH stock solution with 0.1667 M acetic acid (CH_3COOH). The supernatant was then separated from magnetic particles with a magnetic field and

quickly removed, centrifugated (12000 rpm for 1 min) to eliminate the last magnetic beads and TLE 1X was added to reach a final volume of 50 ml.
To eliminate the residual SDS, DNA recovered from the washing and denaturation steps was precipitated with 500 µl EtOH 100% and put on ice for 15 min. To eliminate the supernant a centrifugation (14000 rpm) of 5 min was carried out. To wash the pellet 250 µl EtOH were added and centrifugated together for 5 min at 14000 rpm removing the supenatant. The pellet was then dried and resuspended in 50 ml of TLE 1X.

Two microliters from each recovered fraction (TLE 1X and NaOH) were amplified by 30 cycles of PCR using the MseI-N primer under the following conditions:

94°C 30 s
53°C 1 min } 30 cycles
72°C 1 min
72°C 10 min

and with this PCR mixture:
-H_20 dd 10,32 µl
-primer *MseI* 4 µl
-buffer 10X 2 µl
-MgCl2 1,2 µl
-$dNTP_{mix}$ 0,4 µl
-*Taq* polimerase 0,08 µl
-DNA 2 µl

Agarose gel visualization of the amplified fragments should display in each of the two PCRs a smear above 200 bp. Ideally the PCR of the last stringency wash should not yield any product, indicating complete removal of nonspecifically bound DNA. The PCR products of the two elution steps were the best candidates for producing a

highly enriched microsatellite library, because they were likely to contain the largest proportion of repeat-containing fragments.

2.2.3.4 CLONING AND TRANSFORMATION

The fragment were ligated into a pGEM-T Easy Vector System I (Promega) as follows (Figure 3):

-2X Rapid Ligation Buffer 5 µl
-T4 DNA Ligase 1 µl
-pGEM-T Easy Vector System I (50 ng) 1 µl
-Insert DNA 2 µl

dH_2O to a final volume of 10 µl
The reactions were mixed by pipetting and incubated overnight at 4°C.

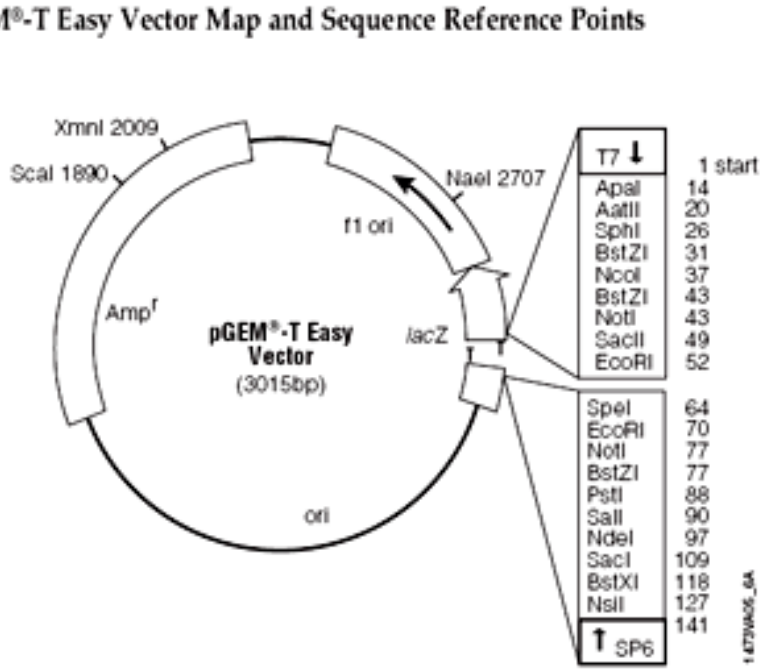


Figure 3: pGEM-T Easy Vector circle map and sequence reference points

Cloning plates were prepared and plasmid vectors were incorporated into competent cells of *Escherichia coli* (JM 109 Competent cells-Promega). These cells are made to take up the plasmid vector P-GEM, which contains two engineered genes, one for ampicillin resistance (ampicillin resistance gene) and the other (LacZ gene that codes for ß-galactosidase) to convert X-gal in nutrient agar to a blue color. A sample of competent cells is made to take up the vector with the two genes. When *E. coli* with this insert is plated on nutrient agar with ampicillin and X-gal, the resulting colonies will be blue. Another sample is made to take up another vector with a DNA insert (recombinant plasmid). The DNA is inserted in a restriction site located within the ß-galactocidase gene. When *E. coli* with the recombinant plasmid are plated out on ampicillin nutrient agar with X-gal, the gene that would normally convert X-gal to a blue color is no longer functioning because the DNA insert disrupted the gene. These colonies will be white (the ampicillin gene is not affected). *E. coli* is sensitive to ampicillin and will not grow on nutrient plates in the presence of this antibiotic. *E. coli* with vector is resistant to ampicillin and will grow on these plates (this shows that DNA equals genes or traits). *E. coli* with plasmid also will be seen as blue colonies. Only the recombinants (those that took up vector with a DNA insert) will be seen as white colonies (changing DNA changes the properties of the plasmid P-Gem) and *E. coli* with the recombinant plasmid, which contains the DNA insert.

Two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction were prepared:
-2,5 g Luria Broth Base
-1,5 g Selected Agar
-Bring the volume to 100 ml with dH_20
-Autoclave for 45 min and allow it to cool until the bottle can be held with bare hands
-Add 0,01 g ampicillin
-Pour enough melted agar into each sterile plastic petri dish to cover the bottom
-Place agar plates on a counter top to cool and set (agar medium will solidify at room temperature)
-Store at 4°C

Place 200 µl of the X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside) in a plastic tube. Add 50 µl of IPTG (Isopropyl β-D-1-thiogalactopyranoside – inducer of β-galactosidase) to the tube and mix the two solutions.
Transfer 40 µl of the X-gal/IPTG mixture on the surface of the ampicillin plates. Dip a glass spreader in 95% ethanol and flame to sterilize and let it cool for a few seconds by touching the agar surface. Use the spreader to distribute the X-gal/IPTG mixture evenly over the surface of the plate. Replace the lid on the plate and invert the plate. Repeat this process for the other plate of ampicillin.

-Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube
-Add 2 µl of each ligation reaction to a sterile 1.5ml microcentrifuge tube on ice.
-Remove tube of frozen JM109 High Efficiency Competent Cells from storage and place in an ice bath until just thawed (about 5 min). Mix the cells by gently flicking the tube.
-Carefully transfer 50 µl of cells into each tube prepared above.
-Gently flick the tubes to mix and place them on ice for 20 min.
-Heat-shock the cells for 30 sec in a water bath at exactly 42°C to induce the transformation.
-Immediately return the tubes to ice for 2 min.
-Add 250 µl room temperature SOC medium (20 g bacto-tryptone, 5 g bacto-yeast extract, 0,5 g NaCl, 10 ml KCl 250mM, 20 mM glucose dissolved in 950 ml dH_2O) to the tubes containing cells transformed with ligation reactions.
-Incubate for 1 hour at 37°C with shaking (200 rpm).
-Plate 150 µl and 100 µl on each of two plates of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates.
-Incubate the plates overnight at 37°C.

Recombinant clones (85) were then identified using blue/white screening on the agar plates and amplified with M13 forward and reverse primers:
-M13 Forward (5'-GTA AAA CGA CGG CCA GT -3')

-M13 Reverse (5'-CAG GAA ACA GCT ATG AC-3')

For each PCR reaction a colony was picked and put on the following PCR mixture:
-dH_20 37 µl
-10X Buffer 5 µl
-dNTPmix 4 µl
-$MgCl_2$ 3 µl
-M13 Forward 0,5 µl
-M13 Reverse 0,5 µl
-*Taq* polimerase 0,25 µl

with the following PCR program:

94°C 2 min
94°C 30s
55°C 30 s } 30 cycles
72°C 1 min
72°C 7 min

Agarose gel visualization of the amplified fragments should display an amplification product between 400 bp and 800 bp in size because it probably contained a microsatellite.

2.2.3.5 PCR PRODUCT PURIFICATION

The PCR product purification was carried out using the Wizard® SV Gel and PCR Clean-Up System (Promega) following this protocol:
-Add equal volume of Membrane Binding Solution to PCR reaction
-Transfer sample to SV Minicolumn
-To induce the binding of the DNA to the membrane leave them at room temperature for 1 min

-Centrifugate (10000 rpm) for 1 min

-Discart the filtrate

-Add 700 µl Membrane Wash Solution

-Centrifugate (10000 rpm) for 1 min

-Add 500 µl Membrane Wash Solution

-Centrifugate (10000 rpm) for 5 min

-Transfer spin column to a 1.5 ml microcentrifuge tube

-Centrifugate (10000 rpm) for 1 min

-Elute with 50 µl Nuclear-Free Water

The DNA concentration was estimated by a 1% agarose gel electroforesis.

2.2.3.6 DNA SEQUENCING

The positive clones were sequenced with ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystem) in a ABI PRISM® 3100 automated sequencer.

The following components were mixed for the sequencing DNA reaction:

- 70 ng purified PCR product
- 2 µl BigDye™ Terminator Cycle Sequencing Kit (Applera)
- 4 µl primer (1 mM)
- dH_20 up to a final volume of 20 µl

using the following program:

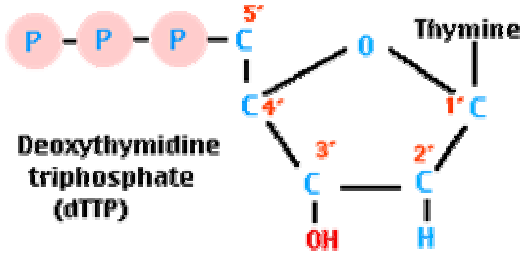
96°C 10 sec
50°C 5 sec
60°C 4 min
} 25 cycles

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA.

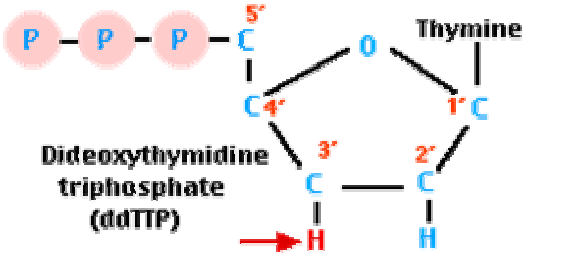
Automated fluorescent DNA sequencing using a capillary DNA sequencing instrument (ABI PRISM® 310 Genetic Analyzer - Applera) is based on the use of a different colored fluorescent dye for each of the four DNA bases. The most popular method for doing this is called the dideoxy method or Sanger method (named after its inventor, Frederick Sanger, who was awarded the 1980 Nobel prize in chemistry for this achievment - Sanger et al. 1977).

DNA is synthesized from four deoxynucleotide triphosphates (fig 4A: deoxythymidine triphosphate (dTTP)). Each new nucleotide is added to the 3′-OH group of the last nucleotide added.

4A



4B



The dideoxy method gets its name from the critical role played by synthetic nucleotides that lack the -OH at the 3′ carbon atom (fig. 4B). A dideoxynucleotide (dideoxythymidine triphosphate-ddTTP) can be added to the growing DNA strand but when it is, chain elongation stops because there is no 3′ -OH for the next nucleotide to be attached to. For this reason, the dideoxy method is also called the chain termination method.

The DNA to be sequenced is prepared as a single strand, denaturing the double stranded DNA.

This template DNA is supplied with:

- a mixture of all four normal (deoxy) nucleotides in ample quantities:

- dATP
- dGTP
- dCTP
- dTTP

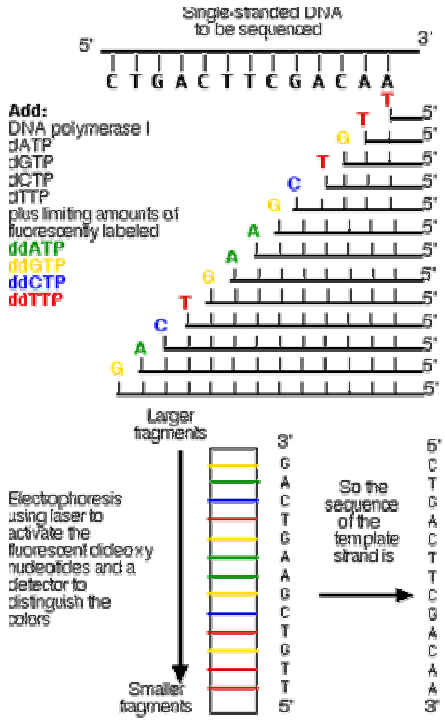
-a mixture of all four dideoxynucleotides, each present in limiting quantities and each labeled with a "tag" that fluoresces a different color:

- **ddATP**
- **ddGTP**
- **ddCTP**
- **ddTTP**

-DNA polymerase I

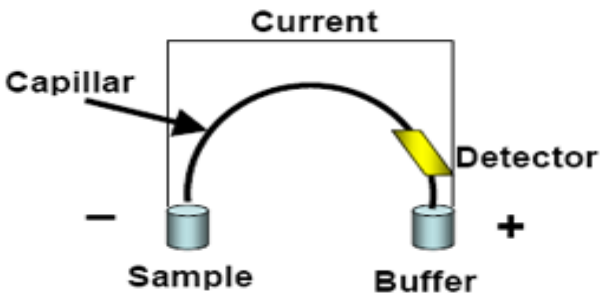
Because all four normal nucleotides are present, chain elongation proceeds normally until, by chance, DNA polymerase inserts a dideoxy nucleotide (shown as colored letters) instead of the normal deoxynucleotide (shown as vertical lines) (Fig. 4C). If the ratio of normal nucleotide to the dideoxy versions is high enough, some DNA strands will succeed in adding several hundred nucleotides before insertion of the dideoxy version halts the process.

4C

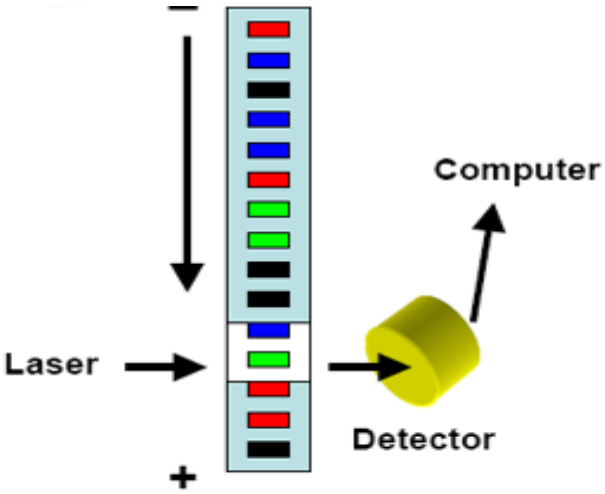


DNA sequencing reactions can be carried out in a single reaction tube and be prepared for loading once the reaction reagents had been filtered out. The capillary system is set up to deliver new polymer to the capillary, load the sequencing reaction into the capillary, apply a constant electrical current through the capillary (fig. 4D), and have the resolved fragments migrate past an optical window where a laser would excite the dye terminator, a detector would collect the fluorescence emission wavelengths (fig. 4E), and software would interpret the emission wavelengths as nucleotides (fig. 4F). At the present time such systems can deliver 500–1000 bases of high quality DNA sequence in a matter of a few hours.

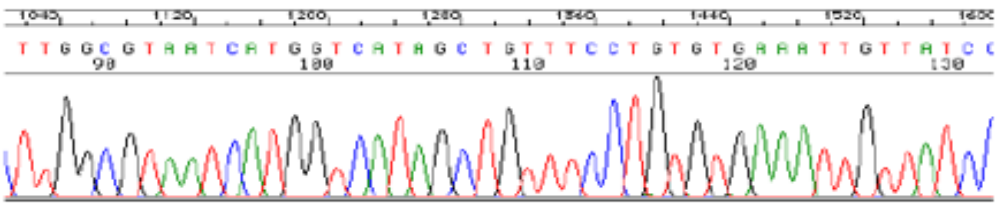
4D



4E



4F



2.2.3.7 PRIMER DESIGN AND PRIMER PAIRS PRESCREENING

The sequences contained microsatellites were aligned using a MEGA version 4.0 (Tamura et al. 2007) and, to discover the location and orientation of the microsatellites into the sequence, the software TRF (Benson 1999) was used.
Primer pairs were designed for the microsatellite inserts using the Primer3 v.0.4.0 computer program (Rozen and Skaletsky 1998) with these conditions:

-primer size: 18-22 bp
-annealing temperature: 54°C-65°C
-self complementarity: 5 – 2

In order to optimize the amplification protocol and to test the primer pairs performance, PCR amplifications were performed with the unlabelled primer pairs in some individuals of *Primula apennina* Widmer collected from Monte Prado. Many parameters which influenced the outcome were changed. These included the $MgCl_2$ concentration, the temperature profile, the number of cycles and the presence of certain additivities (Bovine serum albumine-BSA). The optimal protocol for each primer pair is summarized in the Table 3.

Primer pairs	Annealing temperature	Number of cycles
1	48°	40
2	48°	40
3	54°C	40
4	54°C	40
5	56°C	35
6	58°C	35
7	58°C	35
8	58°C	35
9	60°C	40
10	58°C	35
11	50°C	40
12	52°C	35
13	54°C	35
14	58°C	35
15	56°C	35

Table 3: annealing temperature and number of cycles for each primer pair.

PCR amplifications were performed in a final volume of 10 µl containing:

-1 µl BSA

-1 µl buffer

-1 µl BSA

-0,8 µl DNTp

-0,5 µl each primer

-0,3 µl $MgCl_2$

-0,05 µl Taq polimerase

-20 ng of genomic DNA

The amplification conditions were:

94°C 5 min

94°C 30 sec
…°C 30 sec } … cycles
72°C 30 sec
72°C 7 min

2.2.3.8 FRAGMENT ANALYSIS

After prescreening, forward or reverse primers were labelled with dye-D2, D3 or D4 WELLRED fluorochromes (Sigma-Proligo) and used for the PCR amplifications, following the conditions described above.
To evaluate whether the individual loci were likely to be polymorphic the primer-set was screened against a range of representative samples.

Fluorescently labeled fragments were detected using the automatic sequencer CEQ™ 8000 Genetic Analysis System (Beckman Coulter) and then interpreted using CEQ 8000 analysis software. Four different colored fluorescent dyes can be detected in one sample. One of the dye colors was used for a labelled size standard that was added to each lane to allow comparison of samples from lane to lane. The analysis software uses the size standard to create a standard curve for each lane and then determines the length of each dye-labeled fragment by comparing it with the standard curve for that specific lane.

The differential labelling with three fluorochromes allowed the combination of three primer pairs in a single reaction (multiplexing).

3. RESULTS

3.1 RAPD AND ISSR MARKERS RESULTS

I only used amplification products which were clearly present or absent through all experiments for the data analysis. This approach reduced the influence of non-reproducible, artifactual bands that might bias the analyses. Individuals with a substantial number of missing data were excluded from the analysis.

A total of 172 polymorphic bands were scored using the six RAPD primers. The size of amplification products ranged from 160 to 1084 bp. Each primer produced between 22 and 45 bands corrisponding to an average of 28,67 bands per primer; of these 79,65% (137 in total) were polymorphic among the three species (Table 1). Primer 1 produced three monomorphic bands in *Q. crenata,* seven different monomorphic bands in *Q. suber* and one monomorphic band in *Q. cerris*. Primer 2 gave one monomorphic band in *Q. crenata* and in *Q. cerris* and three monomorphic bands in *Q. suber*. Primer 3 produced five monomorphic bands in *Q. suber*, one monomorphic band in *Q. cerris* and primer 4 only one monomorphic band in *Q. crenata* and two monomorphic bands in *Q. suber;* primer 5 gave only one monomorphic band in *Q. crenata* and primer 6 three monomorphic bands in *Q. crenata,* eleven different monomorphic bands in *Q. suber* and one monomorphic band in *Q. cerris*.

The three ISSR primers generated between 12 and 17 products corresponding to an average of 14 bands per primer; of these 52,38% (22 in total) were polymorphic among the three species. A total of 42 polymorphic bands ranging in size from 174 to 532 bp were scored (Table 1).

Six monomorhic bands were produced by the primer $(AG)_8T$ in *Q. suber*, one in *Q. crenata* and two in *Q. cerris*. The primer $(AG)_8C$ produced 6 monomorphic bands in *Q. suber*, two monomorphic bands in *Q. crenata* and the primer 6 gave monomorphic bands in *Q. suber*, one band in *Q. cerris* and three monomorphic bands in *Q. crenata*.

Primer type	Sequence (5'-3')	No. of recorded fragments	Size range bp
RAPD primers			
1	GGTGCGGGAA	28	160-995
2	GTTTCGCTCC	27	185-946
3	GTAGACCCGT	25	171-741
4	AAGAGCCCGT	22	191-571
5	AACGCGCAAC	25	164-535
6	CCCGTCAGCA	45	169-1084
Total		172	
ISSR primers			
UBC 807	$(AG)_8T$	17	174-499
UBC 808	$(AG)_8C$	12	177-520
UBC 811	$(GA)_8C$	13	197-532
Total		42	

Table 1: genetic data for each primer type

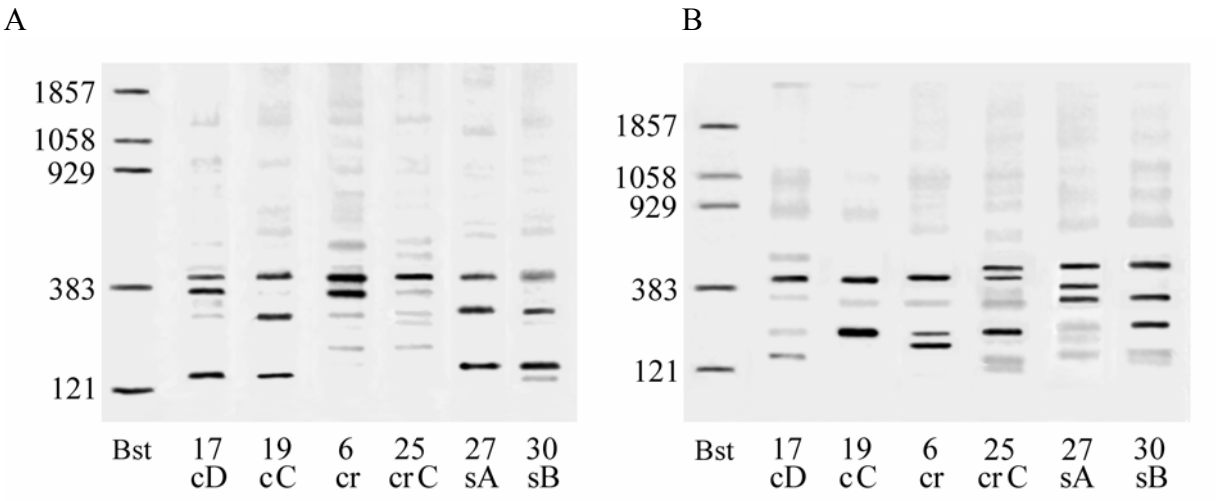


Figure 1: example of the polymorphic banding pattern obtained for six sampled plants with two of the primers used: RAPD primer 1 (A) and ISSR primer $(AG)_8T$ (B).

The percentage of polymorphic bands (PPB) and the estimates of Simpson diversity index (S) for each species based on RAPD and ISSR markers is shown in Table 2, in which *Q. cerris* exhibited the highest level of variability (PPB) according to both RAPD (97,67) and ISSR (92,86) markers, while *Q. suber* showed the lowest for both markers (RAPD: 84,30; ISSR: 57,14). *Q. cerris* had also the highest S value for RAPD (0,82) and ISSR (0,81) primers. The lowest S value was instead obtained for *Q. suber* with RAPD markers (0,63) and for *Q. crenata* with ISSR markers (0,75).

Primer type	**Number of polymorphic bands**	**Percentage of polymorphic bands (PPB)**	**Simpson diversity index (S)**
RAPD primers			
Q. cerris	168	97,67	0,82
Q. suber	145	84,30	0,63
Q. crenata	163	94,77	0,69
ISSR primers			
Q. cerris	39	92,86	0,81
Q. suber	24	57,14	0,77
Q. crenata	36	85,71	0,75

Table 2: Numbers of polymorphic bands, percentage of polymorphic bands (PPB) and Simpson diversity index (S) calculated for the three *Quercus* species.

Molecular data would support a hypothesis of hybrid origin if diagnostic markers (present in all individuals of one species and none of the other) or species-specific markers (unique to one species but not necessarily found in all individuals) found in the putative parents were additive in the putative hybrid. However, in a stabilized hybrid species, strict additivity would not be expected in all individuals due to the often close relationship of the parental species or to hybridization.; rather, combinations of parental markers would be found at the population level (i.e. some

individuals might have both parental markers, whereas others may have markers of one parent or the other; Galez and Gottlieb 1982).

Several studies have therefore used less stringent methods for defining marker bands, calculating the percentage of *private bands* (bands found exclusively in one species), *common bands* (band present in two taxa and absent in the third one), and *shared bands* (bands co-occurring in two taxa, regardless of their presence/absence in the third or recurring in all three species at the same time) (Archibald et al. 2004). Two different private RAPD bands were unique to *Q. cerris* and Q. *crenata*; three different private bands in *Q. cerris*, two in *Q. suber*, and another one in *Q. crenata* were instead detected with ISSR markers. Seven (4,07%) common fragments within the *Q. cerris* and *Q. suber* individuals were scored, 21 (12,21%) within *Q. cerris* and *Q. crenata* individuals, and only one (0,58%) within *Q. suber* and *Q. crenata* individuals. A total of 139 (80,81%) RAPD fragments were shared among the three species, 147 (85,47%) between *Q. cerris* and *Q. suber*, 160 (93,02%) between *Q. cerris* and *Q. crenata*, and 140 (81,40%) between *Q. suber* and *Q. crenata*. Only *Q. cerris* and *Q. crenata* individuals had ten (23,81%) fragments in common, while the species pairs *Q. cerris* - *Q. suber* and *Q. suber* - *Q. crenata* did not show any band in common. A total of 21 (50,00%) ISSR fragments were shared among the three species and between *Q. suber* and *Q. crenata*, 22 (52,38%) between *Q. cerris* and *Q. suber*, 35 (83,33%) between *Q. cerris* and *Q. crenata*. No completely diagnostic or species-specific loci were found (Table 3).

Species	Private bands	Common bands	Shared bands
RAPD			
Q. cerris	2 (1.16)		
Q. suber	0 (0.00) 0/172		
Q. crenata	2 (1.16)		
Q. cerris vs *Q. suber*		7 (4.07)	147 (85.47)
Q. cerris vs *Q. crenata*		21 (12.21)	160 (93.02)
Q. suber vs *Q. crenata*		1 (0.58)	140 (81.40)
Q. cerris vs *Q. suber* vs *Q.*			139 (80.81)
ISSR			
Q. cerris	3 (7.14)		
Q. suber	2 (4.76)		
Q. crenata	1 (2.38)		
Q. cerris vs *Q. suber*		0 (0.00)	22 (52.38)
Q. cerris vs *Q. crenata*		10 (23.81)	35 (83.33)
Q. suber vs *Q. crenata*		0 (0.00)	21 (50.00)
Q. cerris vs *Q. suber* vs *Q.*			21 (50.00)

Table 3: Summary of RAPD and ISSR bands which characterize the three *Quercus* species studied.

DATA ANALYSIS

Similarity matrices computed for each of the two markers with the Nei and Li index were used to cluster the data using the UPGMA strict consensus trees shown in figure 2 as unrooted dendrograms. The RAPD tree scored low consistency indices (CFI = 0,157; MCI = 0,233) and did not appear to discriminate the three species. Individuals from both the putative parental species and the hybrid species were interspersed between three main clusters which hence include a mixture of members of the three species (Figure 2a).

The ISSR tree likewise had low consistency indices (CFI = 0,204; MCI = 0,272), but two main groups with some taxonomic correspondence were defined: in the upper clade individuals from *Q. suber* formed a distinct group consisted of *Q. suber* individuals placed in a separate clade along with few *Q. crenata* individuals; the second group clustered all the *Q. cerris* individuals with most of *Q. crenata* samples (Figure 2b). The genetic relationships among plants, as depicted in the unrooted trees, were largely non-congruent with their morphological classification, nor there was any tendency for geographical proximate plants to cluster together.

The Mantel test between the matrices of cophenetic correlation values gave r = 0,315 (500 random permutations, P = 0,002), showing low correlation between the values of Nei and Li similarity index based on RAPD and ISSR data.

The partitioning of molecular variability for each of the two markers is more sensibly rendered in the plots generated by the PCA (Figure 3). PCA of the RAPD data set accounted for 33.77% of the observed variance with the first 10 components, and as many as 18 components were required to explain 50.75% of the total variance. In the plot of individual component scores along the first three axes, the three species were not identifiable as discrete groups; however, *Q. cerris* and *Q. suber* samples showed a general separation with putative hybrid *Q. crenata* individuals in an intermediate position (Figure 3a). The eigenvalues and the variance by the principal components were less evenly distributed for the ISSR data set: 33.91% of the total variance was extracted by the first 4 components, and 51.98% by the first 8 components. In this case, a plot of individual component scores along the first three axes resulted in two clusters corresponding to the putative parental species with the *Q. crenata* sample

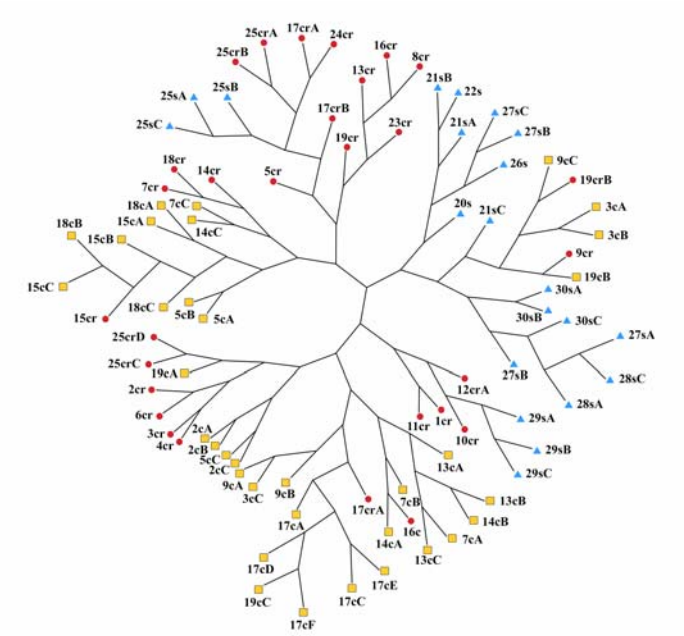
intermediate to them, but mostly overlapping with *Q. cerris* individuals (Figure 3b). Both the dispersal of the total variance in several components and the low measures of consensus trees resolution (CFI, MCI) reflect the high proportion of bands shared by the three species. Moreover, no groupings that correspond to geographical regions could be discerned in these analyses.

The histograms of ML hybrid index scores for *Q. crenata* derived from the two markers were set up so that *Q. cerris* would have high scores and *Q. suber* low scores at the end points on a linear scale between 1 and 0 (Figure 4). Hybrid index scores of *Q. crenata* based on RAPD markers (Figure 4a) ranged from 0,28 to 0,90 (mean 0,59 ± 0,17). The hybrid index values based on ISSR markers (Figure 4b) ranged from 0,14 to 0,97 (mean 0,65 ± 0,19). Although the two methods result in a different arrangement of some individuals, both distributions were not significantly different from normality (W = 0,97, P = 0,633 for RAPD histogram; W = 0,94, P = 0,139 for ISSR histogram) and showed a moderate but significant correlation (ρ = 0,27, P = 0,187). These histograms derived for both markers demonstrate a minimal degree of skewing towards *Q. cerris* indicating a more pronounced molecular similarity of *Q. crenata* to *Q. cerris*.

A

CFI= 0,157

MCI= 0,233



B

CFI=0,204

MCI=0,272

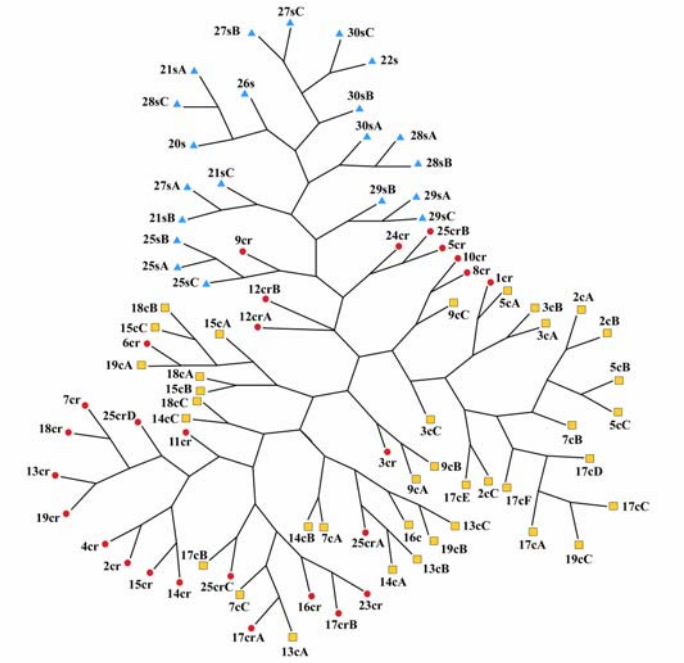


Figure 2: strict consensus unrooted trees from UPGMA analysis depicting molecular relationships among *Q. cerris* (☐) *Q. crenata* (●) and *Q. suber* (▲) individuals. (A) RAPD data. (B) ISSR data. Consensus fork index (CFI) and Mickevich consensus information index (MCI) values are indicated for each tree.

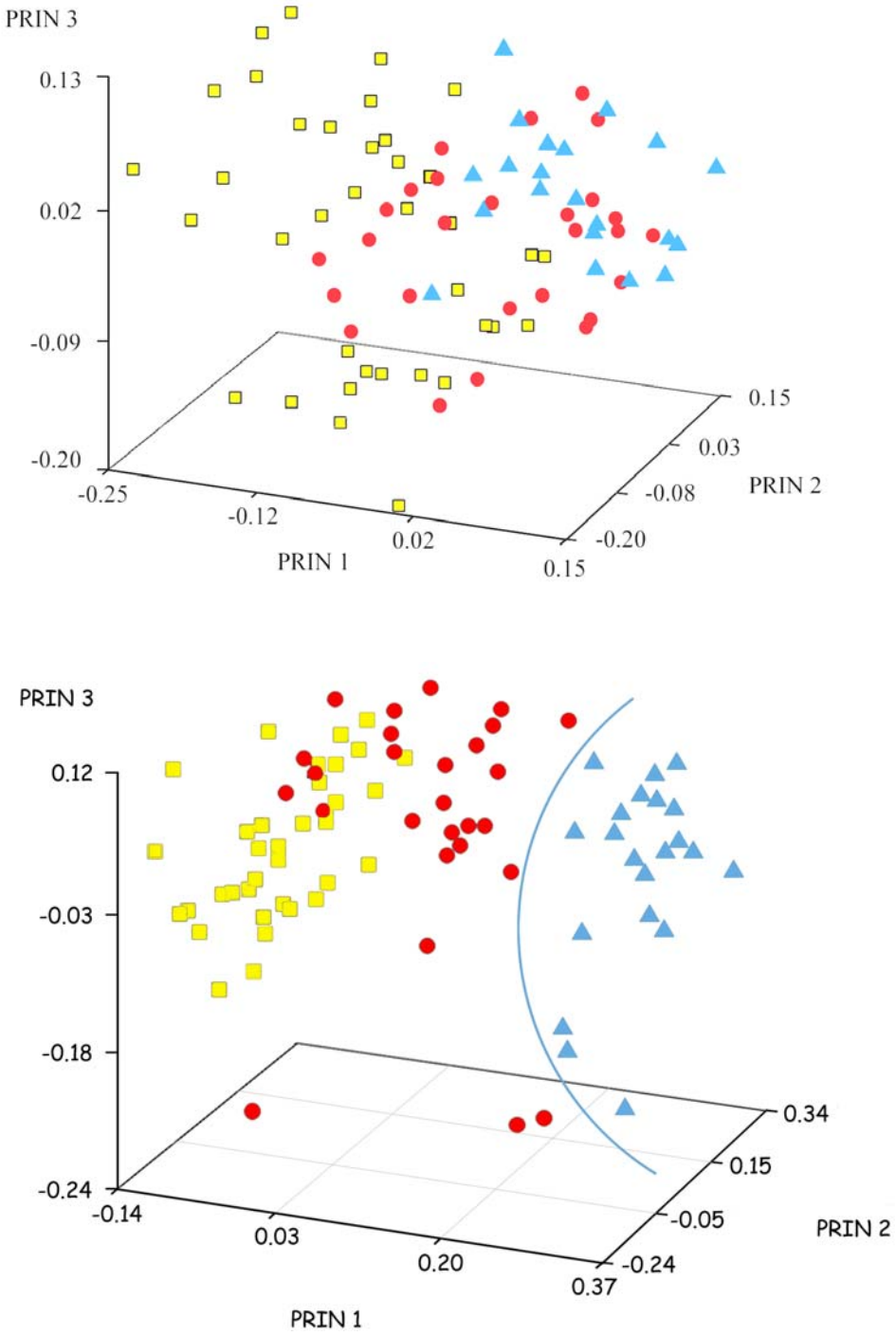


Figure 3: three-dimensional distributions of *Q. cerris* (□), *Q. crenata* (●) and *Q. suber* (▲) individuals obtained by PCA based on the correlation matrices for the presence/absence of RAPD (A) and ISSR (B) fragments. The curved line in the ISSR-based plot indicates the separation of *Q. suber* from *Q. cerris* plus *Q. crenata* individuals.

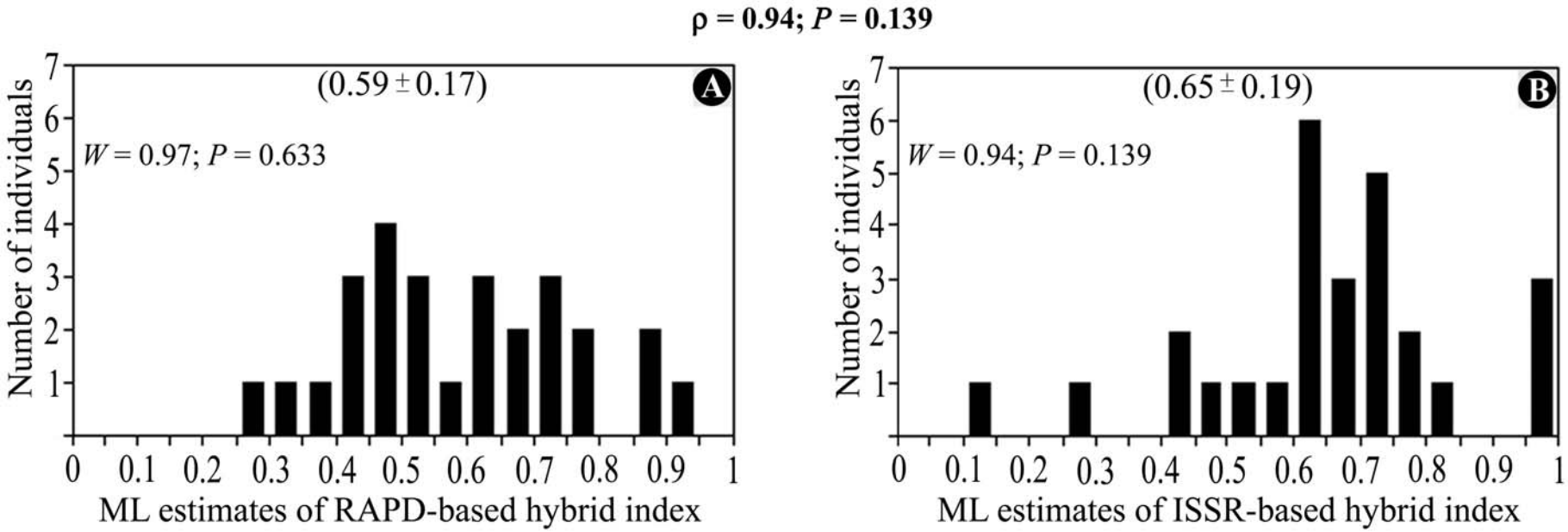


Figure 4: frequency distributions of 27 *Quercus crenata* individuals vs. maximum-likelihood hybrid index based on RAPD (A) and ISSR (B) markers. Low hybrid index scores are indicative of *Q. suber*-like individuals and high scores of *Q. cerris*-like individuals. The mean score is given in the text within each graph along with *W* and *P* values for the Shapiro-Wilk test. Pearson coefficient (ρ) testing the correlation between the two distributions is indicated in bold.

3.2 SSR MARKERS RESULTS

CROSS-SPECIES TRANSFERABILITY WITHIN THE GENUS *PRIMULA*

Though several attempts using a range of reaction and cycling condition were made, 31 primer pairs had no amplification at all and only 5 primer pairs produced a multiple banding patterns. The 5 bands of interest were sequenced but only two of them shared homology with the original species *Primula sieboldii*. Of these two loci, examined for allelic diversity in 32 samples, none were polymorphic. Thus, cross-species transferability can not be used between *Primula apennina* and the three other species.

MICROSATELLITE MARKERS ISOLATION BY FIASCO PROTOCOL

In this study, 63 positive clones were obtained. Of these, 38 sequences were heretofore analyzed and 11 sequenced clones did not contain a microsatellite. Of the 27 remaining sequences, six, two, and three sequenced clones contained identical sequences. The remaining twenty sequences were analysed with the software TRF (Benson 1999). The TRF program discovered 72 microsatellites for the 20 sequences but only the microsatellites above a certain threshold length (a period size of 2 to 9, repeated at least 4 times) and not too close to the insert-plasmid boundaries were selected. The 11 sequences enriched with the selected microsatellites and the 15 correspondent microsatellites are shown in the Table 4.

Sequence name	Microsatellite sequence (5'-3')
2a3	a:TATACATATACATATATATATGTGTGTGTGTGTGTAAGAAAGAGAGG GAAAGAGTGGAACAAAGTGGGAGAAAGCAAGGTAGAGAGAGAGAGA AAGAAGAGATAGTCGTAGTGGGGAGATTGTAGAGACATAAAAGAGGA TAGAGAGAGAGTGCGTGATCCCAGATAGAGAGAGGAGAGAGAGATCG CAGTCATCATCATGAGAGTGCAAAGAGGGAAATAGTGTTTCAAGTTAG GTGTCCACC b:GAGATCGCAGTCATCATCATGAGAGTGCAA
2a4	a:ATCTATATACATATATGTATGTATGTATGTCTATGTAGGTAATCTCTC TATATATATATGTATGTGTGTGTATGTGTATGTGTGTGTGTGTGTATCTC TCCCATAT b: ATAGATAGAGCGGGTACATAGATAGA
2a7	GTGATTACAGGTGTGTGTGTGTGTGTGTGTGAAAAAATAGG
2a11	ACTAGTTTCAATATATATATACATAGATATGTATATGTATGTGTGTTTG A
2a15	CTCCATCCCAACACACACACACAACACAGCATACACCCCATTTTCT
1a17	CAACCCATTCACTTACACACATACATACACGCACAGAGG
2b24	TCGAAAATTTCACATGCACATTGCACACATACATACACACATACACAT ACATACGTACACACATACACACACAAACATATACATACACATACATAC ATATACAAACACTCACATACATATACATATAAACACATACACACATAC ACACATACATACATACATAAACACATAAAAACATAAAAACATAAAAGC ATACACACATACATTCCAACACATACACACATACAAACACACATACAC ATACAAAAAAAAACATGCACACACACACACATA
2c33	CGCTAACCGGTGCTTGCTTTGCTTTGCTTGGTGTCTTCG
2c35	TTTATAATTTTGTGTGTGTGTGTGAGCACACGCG
2d62	GCTATATATATGTGTGTGTGTCATAGTTGGC
1d62	ACAAGAATGCAAAACTATACAAAATAAAAAAAATAGAAAACCCATAA AATAAATGTAATGCGTATA

Table 4: 11 sequences enriched with the selected microsatellites and the 15 correspondent microsatellites.

Primer pairs (Table 5) were designed for the 15 microsatellite inserts using the Primer3 v. 0.4.0 computer program (Rozen and Skaletsky 1998).

Primer pair	Sequence name	Primer sequence (5'-3')
1-L	2a3a-L	TGAAGAGGGTTATGGATATGGAA
1-R	2a3a-R	TGGACTCGGTAATATAGGTGGA
2-L	2a3b-L	TGGGAGAAAGCAAGGTAGAGAG
2-R	2a3b-R	TGGACTCGGTAATATAGGTGGA
3-L	2a4a-L	TGTAGTGTCCGATGTAAAAACGA
3-R	2a4a-R	GGCATCACTCTCCCTATCAATTAC
4-L	2a4b-L	TCGAATGAGTAATTGATAGGGAGA
4-R	2a4b-R	GGTACTTTTCATAATTTCACTTTGT
5-L	2a7-L	CAAGAAATCCAAATAAACCTCCA
5-R	2a7-R	TATATATTATTAGACCCTCATTTTT
6-L	2a11-L	TCTAACTCAAGTCTGGCACGAA
6-R	2a11-R	AGATCCCACACACAAATCACAC
7-L	2a15-L	CCAAGTTGAAGCGCAATTAGA
7-R	2a15-R	ATGAGGTAGGAAGGTACGTGGA
8-L	1a17-L	ATCAAAGCAATGACAGAGGTAACA
8-R	1a17-R	TCAGATAACCTTTCCACCCATC
9-L	2b24-L	TAAGGACGGAGGGAGTAGAAC
9-R	2b24-R	GTGTGTGTCGGTTTGTCTATTTG
10-L	2c33-L	AGGTCGTTGGTTCAAAAAGAAA
10-R	2c33-R	ATTGCCATGACTTCACAAAATG
11-L	2c35-L	TGGGCATGACTTGATAGTTGATAC
11-R	2c35-R	TGGCAATCATATCTTTTTCACATT
12-L	2d62-L	GATGGTTGTCATTTGCTTGTTG
12-R	2d62-R	TTTCCCATATCGCTTTCTGTTT
13-L	1d67a-L	TGCAAATCCATCAAAACCATAA
13-R	1d67a-R	TCAGACCATCAAAATTCAAAACC
14-L	1d67b-L	GGTTTTGAATTTTGATGGTCTG
14-R	1d67b-R	ATTATTCGCGTTTTGGTGATTT
15-L	1d67c-L	GGTTTTGAATTTTGATGGTCTG
15-R	1d67c-R	AAATTATTCGCGTTTTGGTG

Table 5: primer pairs designed for the 15 microsatellites.

In the preescreening the 15 not labelled primer pairs (Invitrogen ^{TM}) were tested in 16 representative individuals of *Primula apennina* collected from Monte Prado (5.1a, 5.8, 5.9a, 10.1b, 10.5a, 10.8, 11.1b, 11.2a, 3.1a, 3.1b, 4.3a, 4.3b, 4.3c, 5.9b, 5.10, 5.11a). The primer pairs 1 (2a3a), 2 (2a3b) failed to amplify.

After prescreening, forward or reverse primers of the 13 loci were labelled with dye-D2, D3 or D4 WELLRED fluorochromes (Sigma-Proligo)(Table 6).

Primer pair	**Sequence (5'-3')**
3L	TGTAGTGTCCGATGTAAAAACGA
4L	TCGAATGAGTAATTGATAGGGAGA
5L	CAAGAAATCCAAATAAACCTCCA
6L	TCTAACTCAAGTCTGGCACGAA
7L	CCAAGTTGAAGCGCAATTAGA
8R	TCAGATAACCTTTCCACCCATC
9L	TAAGGACGGAGGGAGTAGAAC
10R	ATTGCCATGACTTCACAAAATG
11L	TGGGCATGACTTGATAGTTGATAC
12L	GATGGTTGTCATTTGCTTGTTG
13L	TGCAAATCCATCAAAACCATAA
14L	GGTTTTGAATTTTGATGGTCTG
15R	AAATTATTCGCGTTTTGGTG

Table 6: forward or reverse primers labelled with dye-D2, D3 or D4 WELLRED fluorochromes.

Levels of locus polymorfism were assessed in the previous 16 individuals of *P. apennina* from Monte Prado. At this stage 6 (primer n° 3, 4, 7, 10, 14, 15) primer pairs produced monomorphic genotypes in all analysed samples, one primer pairs (primer pair 8) produced three alleles and two primer pairs (primer pairs 6, 9) produced complex multibanded profiles not readily interpretable in term of loci and alleles (Table 7). Primer pairs 5, 11, 12 and 13 were not tested in the Monte Prado

samples because discovered after this stage. To remove the multibanded profiles the annealing temperature was increased; the primer pairs 6 resulted in a interpretable profile while the primer 9 failed to amplify.

Subsequently, to assess polymorphism the screening was extended to Lago Santo and Lago Verde samples, and heretofore, 6 primer pairs tested on 4 individuals randomly selected from the two sites were found to be polymorphic (Table 7).

4. DISCUSSION

4.1 RAPD AND ISSR DISCUSSION

The reproductive biology of species in the genus *Quercus* presents a significant challenge to the use of molecular data to infer organismal relationships. Considering that published accounts of inter- and intraspecific molecular variation indicate that closely related, interfertile oak species are not well differentiated (Whittemore and Schaal 1991) this study examines the hybrid origin of *Q. crenata* from *Q. cerris* and *Q. suber* in northern Italy, where *Q. suber* is currently lacking, and discusses the introgression process to *Q. cerris*, which is the only parent occurring in northern Italy.

By using RAPD and ISSR primers, high levels of molecular polymorphism (S, PPB) in the three oak species were detected. This seems to fit with the general literature on oaks and other long-lived perennials (Bellarosa et al. 1996, Mayes et al. 1998, Coart et al. 2002, Petit et al. 2002, Yakovlev and Kleinschmidt 2002, González-Rodríguez et al. 2005), although comparing results across studies must be done with caution because differences in sampling strategies, geographic scale considered, markers employed, and analytical procedures are customary. The relatively low level of intraspecific diversity in *Q. suber*, stressed by the ISSR markers, might be expected, given that, as reported by Bellarosa (2003), the few relict areas of south-western Italy constitute the far eastern limit of the species and that *Q. suber* is, in these areas, strongly subjected to selection of highly productive varieties. The low level of genetic variation and the range reduction in these marginal populations of *Q. suber* have been documented and explained as a consequence of severe drought periods following the mid-Holocene humid warm phase (Magri 1997, Jiménez et al. 1999). In spite of considerable phenotypic differences existing between the three taxa, both molecular markers show a low degree of neutral genetic differentiation which may be the consequence of high frequency of genetic exchange through hybridization (Bodénès et al. 1997, Bruschi et al. 2000, Tomlison et al. 2000, Williams et al. 2001, Ishida et al. 2003, González-Rodríguez et al. 2005). Indeed, the low frequency of private bands and the distribution of molecular markers in *Q. cerris*, *Q. suber* and *Q. crenata* indicate that most of the molecular diversity found among them is due to

band-frequency differences rather than the fixation of discriminant bands. This study confirms that morphology of oak species hybridizing in nature often does not reflect the degree to which genomes have become contaminated. In that case, interspecific gene flow is masked by strong selection for a limited number of genes controlling striking morphological and physiological features. *Q. cerris*, *Q. suber* and *Q. crenata*, like other hybridizing oak species (Belhabib et al. 2001, Craft et al. 2002, Williams et al. 2001, Tovar-Sánchez and Oyama 2004, Mir et al. 2005), are capable of remaining morphologically or ecologically different in the face of hybridization and possible local introgression. Therefore, according to Hardin (1975), oak species may be appropriately considered as adaptive peaks, in which the tendency of species to merge through semipermeable barriers is balanced by selection for groups of coadapted alleles. In addition, Wu (2001) suggested that if reproductive isolation has once developed between species or populations to some degree, genes responsible for that isolation and submitted to differential selection might not transfer across species even if hybridization occurs. It seems likely that selective factors operate also in this case to maintain coadapted complexes of genomic regions controlling relevant traits, while DNA neutral markers, which are less or not affected by natural selection, may be transferred from species to species through hybridization.

The general inference that genetic distances among oaks tend to be relatively small (Howard et al. 1997) is also evident in cluster and principal components analyses. The relationships among plants depicted in these analyses bolster that *Q. cerris*, *Q. suber* and *Q. crenata* do not represent fully isolated gene pools, although this neutral similarity contrasts rather sharply with their morphological distinctness. RAPD markers provide no resolution of variation among the three species, however the putative hybrid individuals occurred across the range between the clusters of *Q. cerris* and *Q. suber* in the PCA plot. This pattern agrees with the hypothesis of hybridization and indicates that individuals that appear to be typical morphotypes of one species harbour RAPD markers characteristic of another species. ISSR data provided stronger resolution of interspecific variation. Some evidence of taxonomic grouping emerges from the clustering pattern in the UPGMA tree, where *Q. suber* formed a clearly separated subcluster; an additional relationship apparent in the PCA

is that *Q. crenata* group is located among the clusters of individuals belonging to the parental species, but closer to *Q. cerris*.

The low correlation between RAPD and ISSR sets of genetic similarity data has been assessed by the Mantel test and may explain the diverse distribution pattern of polymorphism in the relative UPGMA plots. PCA also supported the result derived from the Mantel test, with different groupings found by the two techniques. Low congruence between the two neutral markers has been reported also in other works (Souframanien and Gopalakrishna 2004, Wu et al. 2004, Hou et al. 2005, Li et al. 2005) and relies on the distinct DNA segments surveyed by the two methods, their distribution throughout the genome, and the extent of the DNA target which is analyzed by each specific assay.

In spite of this, both RAPD and ISSR hybrid index scores computed for *Q. crenata* showed the same general pattern, providing additional indications about the hybrid status of *Q. crenata* individuals in the absence of information about the exact nature of their crossing history. When graphed, the hybrid indices reveal a continuous distribution skewed toward the Turkey oak extreme; shifts of this kind occur as a consequence of asymmetrical backcrossing (Carney et al. 2000, Hardig et al. 2000, González-Rodríguez et al. 2004, Tovar-Sánchez and Oyama 2004, Watano et al. 2004, Burgess et al. 2005). The lack of diagnostic nuclear markers between the two parental species prevents a detailed analysis of introgression, nevertheless the higher number of common and shared RAPD and ISSR bands in *Q. cerris* and *Q. crenata* individuals, the fact that both the species cluster together on the ISSR UPGMA tree and PCA plot, and the greater amount of variation (an assumed consequence of introgression) in the examined samples of *Q. cerris* support the hypothesis of some level of backcrossing in the direction of this parent.

Oak hybrids are often produced in an isolated and sporadic manner and they may introgress with parental species without altering their integrity (Bacon and Spellenberg 1996); asymmetrical introgression is an expected genetic consequence of hybridization when parental taxa differ in abundance (Hill and Buck 1980, Burgess et al. 2005) and hybrid zones with high levels of disturbance may enhance the establishment of backcrossed hybrids (Tovar-Sánchez and Oyama 2004). Both

conditions are satisfied in different parts of the distribution area of *Q. crenata*.

According to palaeobotanical records (Magri 1997), it is possible that cork oak occurred widely in Italy during the Holocene humid warm phase (6000-3000 bp). Subsequent drought periods may have drastically affected the distribution of thermophilous species like *Q. suber* and the decline had to be more severe in the marginal populations exposed to limiting climate conditions. The extinction of *Q. suber* in northern Italy and the low level of genetic variability of the extant Italian stands (Jiménez et al. 1999) may be considered consequences of the past climate history in the easternmost fringe of the species distribution. Moreover, human influence during the last centuries has certainly caused some changes in the genetic diversity and structure of Italian cork oak populations, given that Goiran (1897, 1899) stated that *Q. suber* was cultivated in northern Italy until the beginning of the XVIII century. In his opinion, the specimens of *Q. crenata* surviving at his time were the offspring of hybridization prior to local extinction of *Q. suber*. Under this scenario, introgression to *Q. cerris* has been the unavoidable outcome of backcrossing to the parent favoured by demographic superiority.

Consistent with previous morphological overviews (Goiran 1897-1899, Cristofolini and Crema 2005), the present results support the assumption of a hybrid origin for *Q. crenata* specimens growing in continental Italy, a possible past contact zone of the two parental species, *Q. cerris* and *Q. suber.* On the other hand, the greater affinity between *Q. crenata* and *Q. cerris,* resulting from RAPD and ISSR analyses*,* stands in contrast to patterns of interspecific relationships as recently depicted by Bellarosa et al. (2005); in the reported cladogram, relied upon ITS sequences of nuclear rDNA, *Q. crenata* clustered with *Q. suber* in a sister relation, suggesting greater affinity to this parent than to *Q. cerris*. There are several possible explanations for such discrepancies, the most obvious being the prevailing asymmetrical backcrossing to *Q. suber* along the Tyrrhenian coast, and the exclusive asymmetrical backcrossing to *Q. cerris* in northern Italy.

Though both RAPD and ISSR techniques are equally good at generating fingerprints of individual genotypes, greater resolving power of ISSR markers compared to RAPD markers in unravelling diversity has been previously pointed out (Goulão et al. 2001, Qian et al. 2001, Mattioni et al. 2002, Mort et al. 2003, Tanyolac 2003,

Archibald et al. 2004, Schrader and Graves 2004, Souframanien and Gopalakrishna 2004, Nkongolo et al. 2005) and ascribed to the abundance and hypervariability of repetitive DNA regions targeted by the ISSR primers (Li et al. 2005). In the present survey, ISSR markers produced sharper results with a smaller number of bands, resulting more effective than RAPD in discriminating between the two parental species *Q. cerris* and *Q. suber*, thereby providing information about patterns of hybridization and introgression concerning *Q. crenata*.

Studies about marker transferability often revealed a tendency of microsatellites to be shorter and less polymorphic in species other then that from which they were first isolated (Van Treuren et al. 1997). Mechanistically, this decrease of polymorphism observed in nonfocal species is often caused by the interruption of long, contiguous microsatellite arrays by base substitutions, and/or by shortening of perfect arrays through slippage events (Barrier 2000). However, studies about marker transferability often revealed a tendency of microsatellites to be shorter and less polymorphic in species other then that from which they were first isolated (Van Treuren et al. 1997). Mechanistically, this decrease of polymorphism observed in nonfocal species is often caused by the interruption of long, contiguous microsatellite arrays by base substitutions, and/or by shortening of perfect arrays through slippage events (Barrier 2000).

Moreover, high polymorphism observed in a species does not guarantee that similar polymorphism will be found in related species especially when increasing the evolutionary distance (Morin et al. 1998).

Kijas et al. (1995) tested two primer sets in 10 different *Citrus* species and two related genera and found conservation of the sequences. Cross-species amplification has also been reported between cultivated rice and related wild species (Wu and Tanksley 1993) and between *Vitis* species (Thomas and Scott 1993). Provan et al. (1996) could show successful amplification of two tomato SSR primer pairs tested on potato cultivars. Weising et al. (1997) reported conservation of SSR flanking sites in different species of kiwifruit (*Actinidia chinensis*). Usually, a low percentage of markers also amplifies fragments from species belonging to other genera from the same family. Within the *Poaceae* family, primers worked even across different genera (Röder et al. 1995) but, only 50% of microsatellite loci identified in wheat were also polymorphic in rye and barley cultivars. Whitton et al. (1997) tested 13 SSR loci in 25 representatives of the *Asteraceae*, where it was demonstrated that the regions flanking the repeats are not highly conserved, neither in nucleotide sequence nor in relative position. Indeed, in general, transferability of polymorphic markers in plants is likely to be successful mainly within genera (success rate close to 60% in eudicots and close to 40% in the reviewed monocots) rather than between genera

(transfer rates are approximately 10% for eudicots) within the same family (Barbarà et al. 2007).

17 SSR primer sets developed for *Quercus petraea* were tested on eight different members of the *Fagaceae* family (Steinkellner et al. 1997). In total 66% resulted in interpretable amplification products and most of them were really homologous to the originally cloned SSR fragment from *Q. petraea*. This study demonstrated that, although SSR primers worked even across different genera, with increasing evolutionary distance there was a clear tendency for decreasing ability to successfully amplify loci and a decreasing proportion of polymorphism amongst those markers which could be amplified.

It is yet unclear why microsatellites and their flanking DNA are relatively conserved in some taxa, but not in others. In any case, the chance of a successful cross-species (heterologous) amplification of any DNA sequence by polymerase chain reaction certainly depends on the source and characteristics of the genomic library and on the evolutionary distance of the species sampled (Dayanandan et al. 1997). Given that primer binding sites are expected to be more conserved when the microsatellite flanking sequences are maintained under selective constraints and that microsatellites are surprisingly common in the vicinity of genes (Morgante et al. 2002), microsatellite within genes provide good chances to design primer pairs which are more broadly applicable.

The decline of amplification success with increasing divergence of the species could be the main cause of PCR failure for the primer pairs developed for *Primula sieboldii*, *Primula modesta*, *Primula nutans* and *Primula vulgaris* which were tested for *Primula apennina* Widmer in the present study. All these primrose, belonging to different sections from *Primula apennina*, have sufficiently diverged that they no longer show conserved flanking primer regions, confirming that cross-species transferability depends on the evolutionary relationship of the species sampled and that it can not be applicated between species which are not closely related. Futhermore, the lack of polymorphism could be due to a high conservation of primer binding sites which are present in coding regions, resulting in an absence of variability and rendering impossible the use of this technique.

Consequently, a microsatellite library was developed to isolate microsatellite loci.

The polymorphic microsatellite markers described in this work will be useful tools to evaluate the relative importance of sexual and asexual recruitments in *Primula apennina* Widmer (Sydes and Peakall 1998, Nagamitsu et al. 2004), to identify genets from a large numbers of ramets based on the identity of their genetic composition within each population (Naito et al. 1999, Reusch et al. 1999, Hämmerli and Reusch 2003), and to investigate the process by which the populations were established (Miwa et al. 2001).

Further studies could use the SSR isolated here to investigate the genetic variation and the infraspecific structure of *P. apennina*. In addition, the data obtained could be compared to results from studies with different marker systems in order to evaluate the utility of microsatellite markers in terms of population genetics studies.

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