

HAPLOTYPE RICHNESS IN REFUGIAL AREA OF MARITIME ALPS: PHYLOGEOGRAPHICAL STRUCTURE OF *SAXIFRAGA CALLOSA* AND RELATIOSHIPS WITH RELATED TAXALUIGI MINUTO^A, GABRIELE CASAZZA^A, MASSIMO LABRA^B, FRANCESCO SALA^A, FABRIZIO GRASSI^A^c DIP. TE. RIS, University of Genova, Corso Dogali 1M, I-16136 Genova, Italy.^b Department of Biosciences, University of Milano-Bicocca, Piazza della Scienza 1, 20126 Milan, Italy.^c Botanical Garden, Department of Biology, University of Milan, Via Celoria 26, Milan, Italy.

ABSTRACT

Saxifraga callosa is a species spread in South-Eastern France and Italy with high diffusion in Maritime Alps. This species lives in more or less vertical limestone rocks at an altitude of between 200 and 2,000 m and it ranges from Southwestern Alps (Cottian and Maritime Alps, Provence) through the Apuane Alps and the Apennines to southern Italy, Sardinia and Sicily (Webb & Gornall, 1989). The diffusion of the species occurs exclusively through seeds.

A multiple molecular approach, using ITS, AFLP, cpDNA markers and chloroplast microsatellite (cpSSR), allowed a comprehension of the infraspecific relationships and the construction of haplotype network. By sequencing of ITS regions confirmed that the subsp. *catalaunica* is clearly a separate species. The application of the 'NewHybrids' software to AFLP data-sets recognised *S. lantoscana* as a natural outcross between *S. callosa* subsp. *callosa* and *S. cochlearis*.

Genetic variability within populations was estimated by determining: i), the number of haplotypes per polymorphic population, ii), the haplotype diversity and iii), the Shannon index. Genetic structure was estimated through molecular variance analysis (AMOVA). Total genetic diversity in variance components was calculated in and out the Maritime Alps and in its north and south areal.

The size variant of all tested loci amounts to 11 different haplotypes in *S. callosa*. A haplotype network was constructed by using chloroplast microsatellite (cpSSR). The highest number of haplotypes was found in the Maritime Alps. Analysis of the haplotype distribution showed that population subdivision across all populations was high and the existence of a strong genetic divergence between north and south areals (38%).

The study of haplotypes legitimated conclusions on phylogeographical inferences across the species assortment and on the identification of a centre of diversità in the Ligurian Alps to be interpreted as a peripheral glacial refugia during the Ice Ages.

KEYWORDS

cpSSR, Genetic diversity, Maritime Alps, Periglacial local refugia, Phylogeographic patterns, Pleistocene glaciations, *Saxifraga callosa*, *Saxifraga cochlearis*.

INTRODUCTION

During last decades many botanists were intrigued by the evolution of the flora living in the mountain of South Europe. In the absence of fossils of mountain plants, modern molecular approaches and studies, based on the analysis of the chloroplast genome, offer new tools for studying the phylogeography and the evolution of plant diversity (King & Ferris 1998; Provan et al., 2001) and they can provide data on the biogeographic processes that involved a specific area (Gugerli & Holderegger 2001; Stehlik 2003; Tribsch & Schönswetter 2003). They make possible to test any hypotheses on the glacial refugia survival during Pleistocene glaciations along the Alps (Hewitt, 1996; Taberlet 1998; Widmer & Lexer, C. 2001).

The Mediterranean area was interested the last time during the Oligocene up to 10,000 years ago and the glaciation had a strong influence on the distribution of species. At the LGM large areas of the Alps were covered by a continuous ice-shield. However, in the peripheral calcareous northeastern, southeastern and southwestern sectors, comparatively large areas remained unglaciated. They probably harboured a remarkable number of plants were confined to calcareous bedrocks and thereby revealed their importance as peripheral refugial areas (Schönswetter et al. 2002) and endemic areas were speciation often took place by establishing new taxa. Migration to adjacent lowlands or mountains was a possible strategy to survive for plants. In addition ice-free mountain tops ('nunataks') protruding above the glaciers probably harboured

populations plants belonging to the alpine and nival belts and they later served as sources for the postglacial recolonization.

The flora of the South-Western Alps (Maritime Alps) is recognized for its remarkable plant diversity (Médail & Verlaque, 1997). This area is characterized by high species richness and it includes endemisms (Casazza et al., 2005). From the mid-Tertiary the Maritime Alps were submitted to an extremely disturbed geological and climatic history. Due to the moderate impact of the Quaternary glaciations several zones acted as refugia or nunataker, and by showing more favourable thermal conditions the helped in preserving numerous endemisms (Diadema et al., 2005; Casazza et al., 2005; Minuto et al., 2006) belonging to the tertiary flora and in inducing speciation events for their geographical isolation. Historical and physiogeographical factors played a determinant in creating the biogeographical originality of this region, located at the interface between the Mediterranean and the Alpine biomes.

These mountains are also considered one of the major suture-zones (Taberlet, 1998) but also as hybrid zones in Europe (Hewitt, 2000; Schönswetter et al. 2004; Diadema et al., 2005). The Apennine/Alps postglacial colonization route, originating from South Italy, represents one of major phylogeographical patterns produced by the Quaternary glaciation as recognized for some animal and tree species (Lucchini & Randi, 1998; Taberlet et al., 1998).

Here we present the strict relationship of *Saxifraga callosa* (Figure 1) with some other cogeneric taxa in the Maritime Alps and the range wide fine-scale phylogeographic study of the species. This taxon is endemic to the south-east France and Italy and it belongs to the silver saxifrages (*Saxifraga* sect. *Lingulatae*) polyphyletic group (Conti et al., 1999). Webb & Gornal (1989) distinguished two subentities: subsp. *callosa* and subsp. *catalaunica* (Boiss. & Reut.) D.A. Webb; the first is subdivided in two varieties: subsp. *callosa* var. *callosa* and subsp. *callosa* var. *australis* (Moric.) D.A. Webb (incl. *S. lantoscana* Boiss.). Recent studies pointed out a new intra-taxon subdivision with the Iberian *S. catalaunica* re-assigned to the specific rank (Castroviejo et al., 1997).

S. callosa lives in more or less vertical limestone rocks at an altitude of between 200 and 2,000 m and it ranges from Southwestern Alps (Cottian and Maritime Alps, Provence) through the Apuan Alps and the Apennines to southern Italy, Sardinia and Sicily (Webb & Gornall, 1989). The diffusion of the species occurs exclusively through seeds.



Figure 1. *Saxifraga callosa*.

Taxonomic relationships within *S. callosa* taxon

As reported in details in a previous study of us (Grassi et al., 2006) we performed morphological and molecular analysis on original material belonging to each subspecies of *S. callosa* (subsp. *callosa*, var. *australis* and subsp. *catalaunica*), *S. lantoscana*, (Figure 2) *S. cochlearis* Reichenb. (Figure 3), *S. paniculata* Miller, *S. aizoides* L., *S. hostii* Tausch, *S. valdensis* DC., *S. cotyledon* L., *S. crustata* Vest, *S. florulenta* Moretti, *S. longifolia* Lapeyr. and *S. mutata* L.

A dataset of 32 informative morphological characters, based on 33 diagnostic traits, was produced and the morphological data matrix was run using PAUP 4.0 (Swofford 2001).

ITS1, ITS2 and 5·8S gene amplification and sequences were performed together with the AFLP and CpDNA analyses. The results were transferred in different matrix and performed from the statistical point of view.

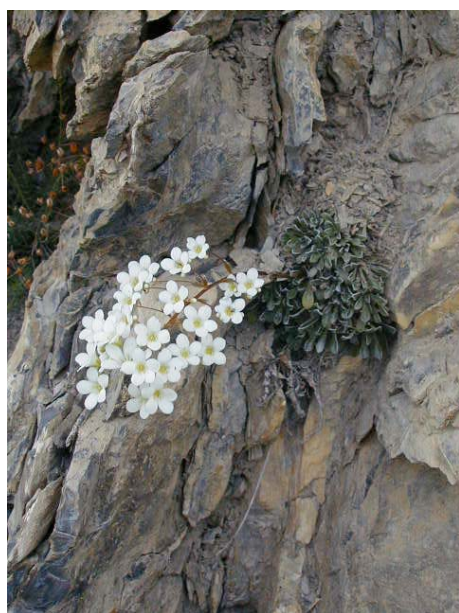


Figure 2. *Saxifraga lantoscana*.



Figure 3. *Saxifraga cochlearis*.

The results of this part of the study let to characterize the genetic structure of *S. callosa* and to identify any possible hybridization event with other related species. We could observe the occurrence of introgression events within the species. Morphological data suggested that *S. lantoscana* is an intermediate taxon resulting from an outcross between *S. callosa* and *S. cochlearis*. Leaf dimension and shape of *S. lantoscana* are a typical example of the gradation in morphologic features. Our molecular results confirm the occurrence of hybridization events between *S. callosa* and *S. cochlearis*. The ITS analysis resolved the *Saxifraga* genus phylogeny (Conti et al., 1999), but was ineffective in resolving relationships within *S. callosa*. Indeed, the ITS marker has already been proven to be a useful source of information for the resolution of phylogenetic relationships at the species level, or above, in many studies (Baldwin, 1992; Alvarez and Wendel 2003). In order to supply this lack of information we were helped by AFLP analysis. In fact, the analysis evidenced large variability within *S. lantoscana* populations. The interspecific transfer of genes through introgressive hybridization is expected to increase levels of genetic variability and to provide new gene combinations on which natural selection can act. The finding of introgression of genomic AFLP markers assigned by 'NewHybrids' software was supported by data on cytoplasmatic marker. Indeed, plastid analysis confirmed the presence within the *S. lantoscana* of different rates of alien alleles belonging to *S. cochlearis*.

Comparison of the results obtained with the two molecular markers (AFLP and cpDNA sequences) confirmed an introgression event and so the confirmation that *S. lantoscana* is to be considered a hybrid taxon.

Another important result of this part of the study was the definition of the phylogenetic position of the *S. callosa* subsp. *catalunica*. The AFLP and the ITS data concur in suggesting a different evolutionary pathway between *S. callosa* and the subtaxon; so, we had the confirmation of its taxonomic transfer to the species range *S. catalunica* Boiss. et Reut. as recently suggested by some Spanish authors (Catroviejo et al., 1997).

Haplotype analysis of the species

A second aim of our study was to determine the phylogeographic structure of *Saxifraga callosa* in order to reconstruct its glacial history, including identification of potential glacial refugia. The haplotype analysis, based on chloroplast microsatellite (cpSSR) variability, was also used to define the hypothetical differentiation centre for the species.

CpDNA analysis was performed on 288 individuals, belonging to 29 different *S. callosa* populations, sampled along the whole distribution area (Grassi et al., in revision).

Further statistical analyses were performed in order to evaluate the number of haplotypes per polymorphic populations, the unbiased gene diversity or haplotype diversity as estimators of genetic variability within population.

The cpSSR analysis in *S. callosa* allowed us to draw phylogeographical inferences across the species assortment. In this species, the genetic variation in cpDNA is high and resulted in 11 haplotypes, geographically separated into the North (above 43° N in latitude) and into the South (Figure 4). This it seems to be an heterogeneity of gene flow due to a potential barrier (Bossart & Prowell, 1998; Lugon-Moulin & Hausser, 2002) which is to be identified between North and Central Apennines. Further indication on the low gene flow between population is also confirmed by the frequent presence of only one haplotype in each population and by the high value for G_{ST} .

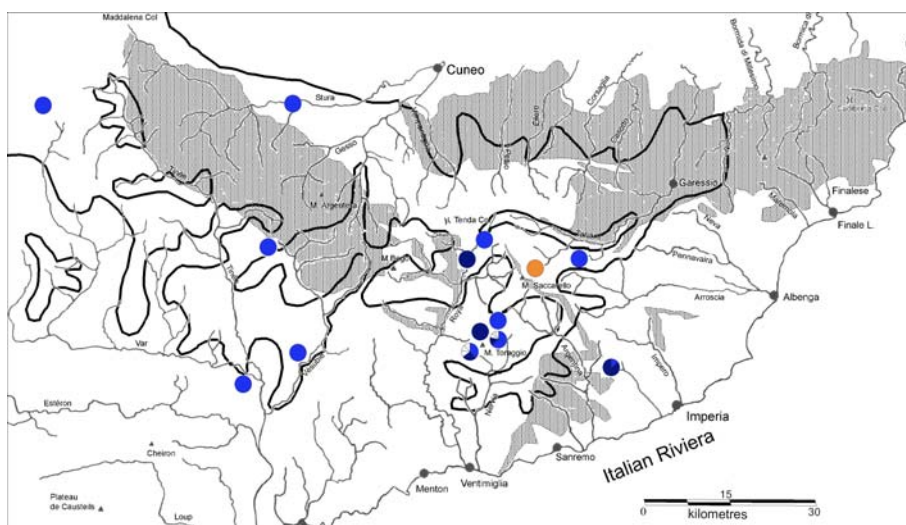


Figure 4. Haplotypes displacement along the area of distribution of the species. With the two colours are distinguished the two main groups of haplotype.

S. callosa probably derived from a common ancestor with those taxa belonging to the *Saxifraga* sect. *Ligulatae* (Conti et al., 1999) that colonized the mountain chains around the Mediterranean during their arisen (Webb & Gornall, 1989), as confirmed by their present. The study species, in particular, was mainly located along the Italian Peninsula reaching also Sicily and Sardinia probably during the Messinian age, when the island was connected to the peninsula (Boquet et al., 1978; Duggen et al., 2003).

The following glacial and interglacial periods moulded the genome of many mountain species (Kropf et al., 2003). The presence of glacial sheets in the Maritime Alps (Malaroda, 2004) and Apennines (Giraudi, 2004) reduced the habitats availability. The combination of the limited gene flow by seed, the patchy distribution of haplotypes, the high haplotype diversity, and the high number of private haplotypes in the mountain chain along the border between France and Italy supports the localization of a glacial survival of *S. callosa* populations in peripheral glacial refugium. Moreover, the high haplotype diversity in Ligurian Alps and the low haplotype diversity in the rest of Maritime Alps is in agreement with the hypothesis that glacial refugia are sanctuaries for allelic richness and that allelic richness decline during colonization events (Comps et al., 2001; Widmer & Lexer, 2001). The survey of these refugia is in agreement with some recent biogeographical studies (Minuto *et al.*, 2006; Casazza et al., 2008) that point out the presence many endemism centres in this sector of the Alps.

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