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Phylogenetic Insights into *Primula* Sect. *Auricula* in the Apennine Peninsula*

Abstract

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The species of *Primula* L. sect. *Auricula* Duby subsect. *Euauricula* Pax are mainly distributed in the southern Alps and Pre-Alps, with a very high rate of endemism: of 16-17 species currently recognized, seven are narrowly endemic and restricted to very small areas, four are endemic to various sectors of the southern Alps, and only five have a wider distribution. The subsection extends southwards to reach peninsular Italy with *P. auricula-P. balbisii*, a species complex distributed across all South European mountain ranges, and with the local endemics *P. apennina* and *P. palinuri*.
We performed a phylogenetic study employing AFLPs (Amplified Fragment Length Polymorphisms) markers of 260 samples collected in the wild from 33 populations, covering all species, with a focus on the three taxa growing in the Apennine peninsula. We also used DNA sequences of six chloroplast markers of all species. Our purpose was to investigate the relationships of the three peninsular species, and hence to understand whether they are the outcome of a single or of multiple independent colonization events.
Our results suggest that: (i) *P. apennina* belongs to the same calcifuge clade as *P. pedemontana* and *P. cottia*, and is the outcome of very recent (late Pleistocene) speciation; (ii) the expansion to peninsular Italy of the problematical *P. auricula-P. balbisii* species complex represents an independent event; (iii) *P. palinuri*, the only species of Mediterranean coastal habitats, is not related to *P. auricula-P. balbisii* in spite of their geographical contiguity and of some morphological similarity; (iv) *P. palinuri* appears to be most closely related to *P. allionii, P. latifolia* and *P. marginata* implying either long-distance dispersal from the west Alps or, more likely, the existence of a now extinct ancestor which was widespread in peninsular Italy. However, the placement of *P. palinuri* in a phylogenetically derived position is incongruent with an accumulation of plesiomorphic characters in this species which may support its interpretation as a palaeoendemic. (v) our results provide convincing evidence in favour of a "multiple-origin" of the peninsular Auriculas.

Key words: AFLP, chloroplast DNA, Endemism, Italy, Phylogeny, *Primula apennina*, *Primula auricula*, *Primula balbisii*, *Primula palinuri*.

Introduction

The genus *Primula* L. encompasses more than 400 species of perennial herbs, mainly distributed in the mountain areas of the Holarctic Floristic Kingdom. The main diversity

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centre is in the Sino-Himalayan Region, harbouring about 225 species (Richards 2003). Europe, with more than 30 species currently recognized, is a secondary diversity centre, and species here probably diversified during the Pleistocene (Richards 2003). *Primula* Sect. *Auricula* Duby is endemic to the mountain ranges of Central and southern Europe and includes 25 species, with the highest specific diversity (22 species) in the Alps (Zhang & Kadereit 2004). The group has been subject to a phylogenetic and biogeographical analysis (Zhang & al. 2004) which showed that the section can be divided into two groups, a 'western clade' (subsect. *Euauricula* Pax) mainly distributed in the western part of the Alps, the Apennines, Pyrenees and Cordillera Cantabrica, and an 'eastern clade' (subsect. *Cyanopsis* (Schott) Pax) mainly distributed in the eastern part of the Alps, the Dinaric Alps, the Balkans, Carpathians and Sudetan Mountains (Zhang & Kadereit 2004, 2005). The split of sect. *Auricula* into these two groups was dated, using a molecular clock approach, to approximately 2.4 million years ago, and it was hypothesized that this initial split of the section may have been caused by an early glaciation of the Alps forcing the last common ancestor of the two clades into western and eastern refugia, respectively (Zhang & al. 2004).

Primulas of subsect. *Euauricula* are evergreen, dwarf plants mostly growing in alpine or mountain habitats; leaves are fleshy, not rugose, and have involute vernation; floral bracts are shorter than the pedicels; calyx teeth are rounded; corollas are mostly pink to red or violet, and only two species have yellow corollas (Crema 2009). Chromosome numbers are $2n = 62$ or 64, rarely $2n = 66$ or $2n = 126$ (Kress 1963, 1989), presumably hexaploid, hypohexaploid or hexaploid- or hypohexaploid-derived, based on $x = 11$. Typical habitats are stony or rocky soils, either basic or acidic. Sixteen species are currently recognized in subsect. *Euauricula* (Crema & Cristofolini 2013): five of them have a more or less wide distribution across the mountain ranges of southern Europe, four are endemic to one or more sectors of the Alps, with some disjunctions to the Pyrenees, while as many as seven species are narrowly endemic.

Only three species extend into the Apennine peninsula, i.e. *P. auricula* L. *s. l.*, *P. apennina* Widmer and *P. palinuri* Petagna. *Primula auricula* L. *s. l.* grows on dolomitic or calcareous rocks and debris at subalpine and alpine elevations in all south European mountain ranges (Aeschiman & al. 2004). It is one of two yellow-flowered species in this group. Leaves are almost entire or crenate, glabrous or with glandular hairs on the margin; flowering time is from May to July, and fruiting time from June to August. Chromosome counts determined $2n = 62$ (Chiarugi 1956, Giordani & al. 1980) or $2n = 64$ (Kovačević 1980, sub *P. balbisii*). *Primula auricula* is the morphologically most variable taxon in the section, and also the most widely distributed. Its taxonomy has always been controversial and never was resolved satisfactorily. In their phylogeny based on ITS, Zhang & Kadereit (2004) identified two distinct taxa: *P. auricula* L., confined to the northern part of the range (northern Alps, Tatra Mountains, west Austria, France, Germany, Poland, Slovakia and Switzerland), and *P. balbisii* Lehm., distributed in the southern part of the range (southwest Alps, Apennines, Balkans, Tatra Mountains and southwest Carpathians). On the other hand, Crema (2009), in a phylogeny based on AFLPs, found that populations of *P. auricula* were embedded in *P. balbisii*, making the latter paraphyletic. Although the taxonomy of this species remains to be resolved, we here will provisionally distinguish two taxa.

Primula apennina Widmer is endemic to the northern Apennine. Its distribution range is a narrow corridor of approximately 45 km x 5 km, where it forms disjunct populations on the higher mountain tops. Its habitat are the north facing ledges and sandstone crevices (acid soils) at 1400–2000 m (Crema & al. 2009). Leaves are efarinose, wedge-shaped, rather square-ended and usually with entire margins or occasionally very shallowly wavy-toothed. The scape exceeds the leaves and has 2-18 flowers with a hint of blue in the pink to red perianth. All flowers in a population develop fairly synchronously from May to June, and flowering lasts for 2-3 weeks. The fruits reach maturity at the end of August. *Primula apennina* is an obligate outcrosser (Fisogni & al. 2011). Its chromosome number is $2n = 62$ (Kress 1963, 1989).

Primula palinuri Petagna is endemic to a narrow area between Palinuro Gulf and Scalea (Campania, southern Italy) where it grows on limestone and sandstone of north facing rocky slopes of the coasts; the substrate is typically neutral (pH = 6.90-7.20) because of the presence of limestone powder mixed with the sand (Pizzolongo 1963). It is a robust plant up to 20 cm tall and summer-dormant; the leaves are efarinose and irregularly toothed. The long inflorescence stalk carries a cluster of 10-20 funnel-shaped flowers with foliose bracts and long calyces; the corolla is yellow, as in *P. auricula*. Flowering time is from February to April, fruiting time from April to May. Most chromosome counts gave $2n = 66$ (Chiarugi 1941; Honsell 1961; Kress 1963, 1989), but also $2n = 44$ (Garbari 1974), which is the only record of tetraploidy in the subsection. *Primula palinuri* shares several characters with subsect. *Cyanopsis*, e.g. foliose bracts, long calyces, and a smooth seed coat. Consequently, Zhang (2002) regarded it as the most primitive species of the entire section, and Chiarugi (1952, 1956) even proposed accommodating it in its own section, *Palaeoauricula*, within subgen. *Auricula*.

Our purpose was to investigate the phylogenetic relationships of these three species from the Apennine, and hence to understand whether they are the outcome of a single colonization event of peninsular Italy, or of multiple independent events.

Materials and Methods

Sampling.- For the AFLP analysis, we sampled all species of sect. *Auricula* subsect. *Euauricula* (Tab. 1). *Primula glaucescens* Moretti, *P. spectabilis* Tratt. and *P. wulfeniana* Schott (subsect. *Cyanopsis*) were used as outgroups. In order to represent infraspecific variation, more than one population per species was sampled for species with a broad distribution area (see electronic supplementary file ESF1). We used only fresh material collected in natural populations. Each population was represented by nine plants, but in two cases where populations were very small, sampling was reduced to two or three individuals. Altogether we sampled 33 populations for a total of 260 individual plants. Plants were sampled at a distance of at least ca. 0.5 m to avoid sampling of clones. We collected one leaf from the basal rosette of each sampled plant; the leaf was put immediately in a vial with silica gel, carried to the laboratory, and used for DNA extraction as soon as possible. The origin of plant material for the cpDNA analysis is shown in the Appendix.

Molecular analysis and data processing of AFLPs.- AFLPs (*Amplified Fragment Length Polymorphisms*) are polymerase chain reaction (PCR)-based, dominant multilocus

Table 1. The species of *Primula* sect. *Auricula* subsect. *Euauricula*: accepted names and relevant synonyms (col. 1), geographic distribution (col. 2), edaphic requirements (col. 3: Ca = calciphilous, Si = silicicolous), number of populations sampled (col. 4), species code (col. 5). Each population was represented by 5 to 9 individuals. Details of sampling are given in the Appendix (ESF1).

1	*2*	*3*	*4*	*5*
Species	***Geographic distribution***	***Substr.***	***No. of pops***	***Code***
South-European Orophytes				
P. auricula L.	northern Alps, Tatra	Ca	2	AUR
P. balbisii Lehm. = *P. auricula* subsp. *balbisii* (Lehm) Nyman ≡ *P. auricula* subsp. *ciliata* (Moretti) Lüdi	southern Alps, Apennines, Balkans	Ca	3	BAL
P. carniolica Jacq.	eastern Alps, Dinaric and Illyrian Mountains	Ca	1	CAR
P. hirsuta All.	central Alps and Pyrenees	Si	4	HIR
P. latifolia Lapeyr.	central Alps and eastern Pyrenees	Si	3	LAT
P. villosa Wulfen	western Alps, eastern Alps, Karawanken	Si	2	VIL
Endemics to the Alps				
P. allionii Lois.	Cottian and Maritime Alps	Ca	1	ALL
P. daonensis (Leyb.) Leyb.	central Alps	Si(Ca)	2	DAO
P. pedemontana Thomas ex Gaudin	western Alps (also recorded for Cantabrian Mts)	Si	1	PED
P. marginata Curtis	western Alps	Ca	3	MAR
Narrow endemics, Alps				
P. albenensis Banfi & Ferlinghetti	western Pre-Alps	Ca	1	ALB
P. cottia Widmer	Cottian Alps	Si	2	COT
P. grignensis Moser	western Pre-Alps	Ca	1	HIRg
P. recubariensis Prosser & Scortecagna	eastern Pre-Alps	Ca	1	REC
P. valcuvianensis (S. Jess. & L. Lehm) Cristof. & Crema = *P. hirsuta* subsp. *valcuvianensis* S. Jess & L. Lehm	western Pre-Alps	Ca	1	HIRv
Narrow endemics, Italian peninsula				
P. apennina Widmer	northern Apennine	Si	1	APP
P. palinuri Petagna	Cilento	Si/Ca	2	PAL

molecular markers. This technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA using PCR. The AFLP procedure was that described by Vos & al. (1995) with minor modifications. All reactions were performed simultaneously for all species to ensure comparability. Amplified fragments were scored for presence (1) or absence (0) of co-migrating bands. Details of the AFLP procedure are described in Crema (2009).

Popgene 3.2 was used to calculate the number of polymorphic loci of each population, and these values were used to calculate the percentage of polymorphic loci per species and for the subsection. Nei & Li (1979) genetic distances were calculated from the AFLP data and analysed with Neighbor Joining (NJ; Saitou & Nei 1987) using PAUP* v4.0b10 (Swofford 2000) with the three species of subsect. *Cyanopsis* used as outgroup. Nei–Li distances are particularly suitable for restriction fragment data because they emphasize the presence of shared fragments, assumed to be the result of common descent, rather than the absence of fragments, which may result from various causes. Statistical bootstrap support (Felsenstein 1985) was evaluated in the same program based on 1,000 bootstrap re-samples. Parsimony analysis of the AFLP data was conducted in PAUP v4.0b10 using a heuristic search starting from random trees with 10 replicates of random taxon addition and TBR branch swapping. One hundred bootstrap replicates were carried out with the same settings as for the parsimony analysis. To further clarify relationships within the subsection, principal co-ordinates (PCOs) were extracted from the Jaccard distance matrix among all AFLP phenotypes, and plotted using the package Past 1.89 (Hammer & al. 2001).

Finally, following the AFLP molecular clock developed by Kropf & al. (2009), Nei's original measures of genetic identity and genetic distance were calculated using Popgene v. 3.2. The same software was used to obtain a dendrogram based on Nei's genetic distances among populations. The AFLP divergence rate of $D_{N72} = 0.037$ (SD = 0.046) per 10,000 years was used to roughly estimate the temporal course of diversification in *P.* subsect. *Euauricula*.

Molecular analysis and data processing of cpDNA sequences.- Six different cpDNA markers were analysed. These were: psbA-trnH spacer, rps16-trnK spacer, ndhF-rpl32 spacer, rpl16 intron, trnL intron and rps16 intron. The standard 25µl PCR reaction mix consisted of 0.5mM $MgCl_2$, 0.2mM dNTPs, 0.8mM primers, 0.04 U/µl *Taq* polymerase, 0.4 mg/ml BSA, and 1-2 µl DNA extract in water and reaction buffer provided by the manufacturer of the polymerase. Primers psbAF (5'-GTT ATG CAT GAA CGT AAT GCT C-3') and trnHR (5'-CGC GCA TGG TGG ATT CAC AAA TC-3' [Sang et al., 1997]) were used for amplification of the psbA-trnH spacer, and primers rps16x2F2 (5'-AAA GTG GGT TTT TAT GAT CC-3') and trnK(UUU)x1 (5'-TTA AAA GCC GAG TAC TCT ACC-3' [Shaw et al., 2007]) for amplification of the rps16-trnK spacer. For amplification of the ndhF-rpl32 spacer we used primers ndhF (5'-GAA AGG TAT KAT CCA YGM ATA TT-3') and rpL32-R (5'-CCA ATA TCC CTT YYT TTT CCA A-3' [Shaw et al., 2007]), and for the rpl16 intron primers F71 (5'-GCT ATG CTT AGT GTG TGA CTC GTT G-3') and R1516 (5'-CCC TTC ATT CTT CCT CTA TGT TG-3' [Watts et al., 2008]). The trnL intron was amplified with primers ucp-c (5'-CGA AAT CGG TAG ACG CTA CG-3') and usp-f (5'-ATT TGA ACT GGT GAC ACG AG-3' [Taberlet & al. 1991]), and the rps16 intron with primers rps16F (5'- GTG GTA GAA AGC AAC GTG CGA CTT-3') and rpsR2 (5'-

TCG GGA TCG AAC ATC AAT TGC AAC-3' [Oxelman et al., 1997]). PCR reactions were carried out using the program: 60sec at 94°C, followed by 35 cycles with 20sec at 94°C, 30sec at 56°C and 60sec at 72°C, and a post-treatment with 80sec at 56°C and 8min at 72°C. Cycle sequencing was carried out with BigDye Terminator 3.1 (Applied Biosystems, Foster City, California, USA) using the same primers as for the PCR amplifications. All markers were sequenced in both directions. The cycle-sequenced samples were run on an ABI 3130xl Genetic Analyzer at Johannes Gutenberg-Universität Mainz (Germany) for sequencing. For each marker forward and reverse sequences were edited and merged to consensus sequences, which then were aligned using Sequencher 4.1.4 (Gene Codes Corporation). All alignments were checked and corrected manually. Marker matrices were then concatenated to a combined data set. For the combined data set of the six cpDNA markers Maximum Likelihood phylogenetic analyses were performed using RaxML (Stamatakis 2006; Stamatakis & al. 2008) using the GTR+G subsitution model and including bootstrapping that was halted automatically following the majority-rule 'autoMRE' criterion. The sequences will be deposited in genbank.

Results

Percentage of Polymorphic Loci.- The value computed for the entire subsection was 99.84% (639 fragments), ranging from 48% to 76.47%, with a mean of 63.74% per population (two populations with fewer than five individuals analysed were excluded from the calculations). *Primula palinuri* had a value of 59.75%, only slightly lower than the average per species in the subsection, while in *P. apennina* the value was 48%, the lowest within the subsection, and much lower than in *P. cottia* (61.8%) and *P. pedemontana* (71.79%). In *P. auricula-P. balbisii* the value was 50 to 66.5*%,* i.e. around the mean of the subsection.

Neighbor Joining and Parsimony analysis of AFLPs.- The trees resulting from the Neighbor Joining (electronic supplementary file ESF2) and Parsimony analyses (electronic supplementary file ESF3) were largely congruent. In both cases the species of subsection *Euauricula* form a strongly supported cluster or clade (NJ: 100% bootstrap, parsimony: 96% bootstrap). Also, in both trees the populations of each species form monophyletic and strongly supported groups. All acidophilous species form a cluster (NJ: 91% bootstrap, parsimony: 67% bootstrap). *Primula apennina* forms a cluster or clade with *P. cottia* (NJ: 79% bootstrap, parsimony: 60% bootstrap), and the two together are most closely related to *P. pedemontana* (100% bootstrap in both analyses). The group *apennina-cottia-pedemontana* is part of the larger group including all acidophilous species. *Primula palinuri* is part of a large basal polytomy in the subsection comprising seven clades in the parsimony analysis, while in the Neighbor Joining analysis it forms a weakly supported (57%) cluster with *P. allionii*, and this cluster in turn is part of a large basal polytomy in the subsection comprising six clusters The species complex *P. auricula-P. balbisii* forms a highly supported group (100% bootstrap in both analyses); within this group, the two populations of *P. auricula sensu stricto* are monophyletic (99% - 100% bootstrap). They are nested among the three populations of *P. balbisii* in the parsimony analysis, and part of a trichotomy with two clusters of *P. balbisii* in the NJ analysis.

Principal Coordinate Analysis.- An ordination of the whole subsection based on the matrix of interspecific Jaccard's similarities is given in Fig. 1a. The first two coordinates, accounting for 11.91% and 9.97% of the total variance, respectively, produced four distinct clusters: (1) a group of acidophilous species (upper-left corner) including *P. apennina*; (2) a group of basiphilous species excl. *P. auricula s. l.* (right hand side of the plot); (3) *P. palinuri*, isolated on the lower right hand side of the plot but most similar to the preceding group; (4) *P. auricula* and *P. balbisii* (lower left hand corner). In a more detailed

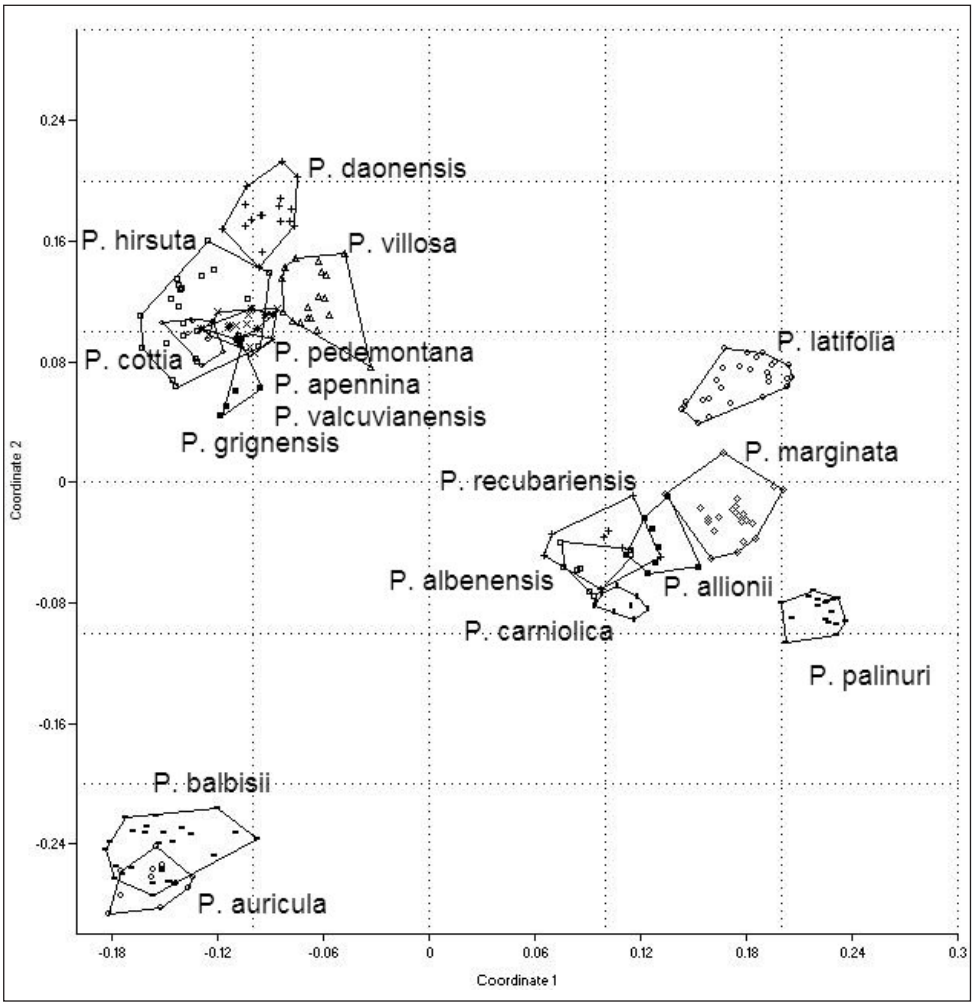

Fig. 1. Ordination of the species of *Primula* sect. *Auricula* subsect. *Euauricula* based on the Principal Coordinates extracted from the matrix of Nei's genetic similarities (see ESF 4).
a) Ordination of the whole subsection on the first two coordinates, accounting for 11.91% and 9.97% of the total variance respectively.

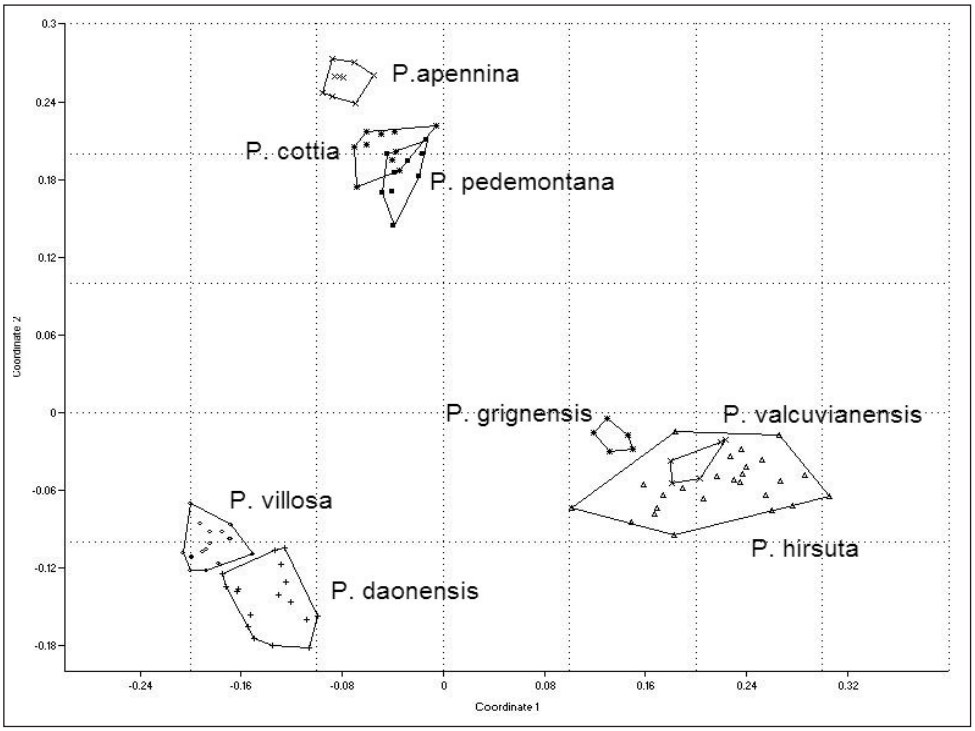

Fig. 1. b) Ordination of the acidophilous group on the first two coordinates, accounting for 20.08% and 15.93% of the total variance respectively.

view focused on the acidophilous group (Fig. 1b), the first two coordinates, explaining respectively 20.08% and 15.93% of the total variance, showed *P. pedemontana-P. cottia-P. apennina* forming a group in the upper left hand corner of the plot.

Nei genetic identity and molecular clock.- Nei genetic identities and distances were calculated (electronic supplement ESF4) in order to investigate diversification through time of *P.* subsect. *Euauricula*. The highest values of genetic diversity were recorded for *P. palinuri,* with an average genetic distance to the other taxa of 0.175, a maximum distance (0.191) to *P. auricula-P. balbisii* and a minimum (0.150) to *P. allionii*. The average genetic distance of *P. apennina* to the other taxa was 0.146, with minimum distances to *P. cottia* (0.060) and *P. pedemontana* (0.063). The average genetic distance among accessions of the complex *P. auricula- P. balbisii* was 0.066 (0.033 within *P. auricula s. str.* and 0.067 among the three populations of *P. balbisii*).

In the unrooted dendrogram based on Nei's genetic distances (Fig. 2), *P. palinuri* forms the first branch; the cluster *P. auricula-P. balbisii* is sister to the remaining species, and the cluster *P. apennina-P. cottia-P. pedemontana* is nested within the major clade of acidophilous species. The divergence times calculated using the AFLP clock calibrated by Kropf & al. (2009) indicate that diversification of *P.* subsect. *Euauricula* took place dur-

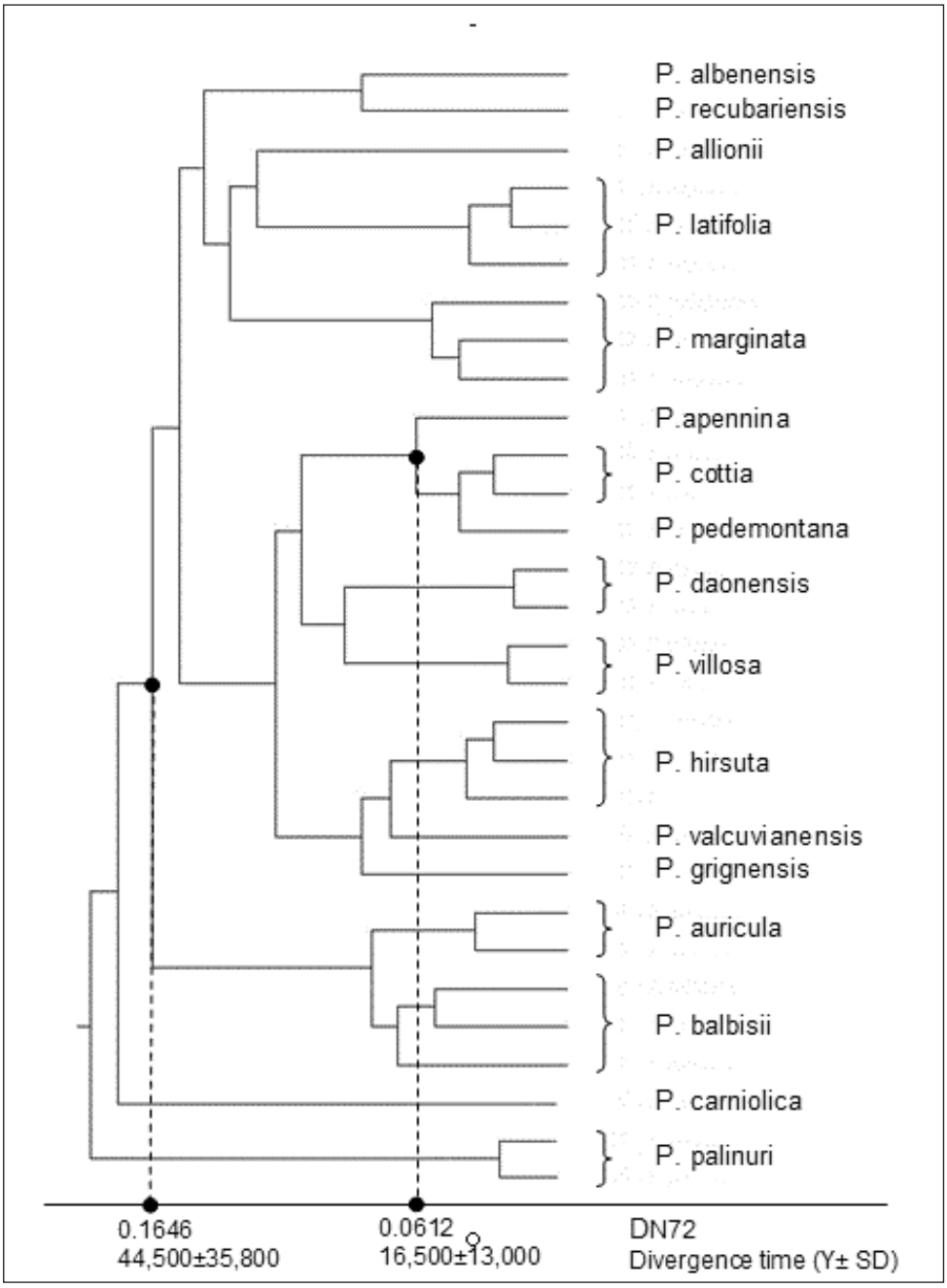

Fig. 2. UPGMA dendrogram of the species of *Primula* sect. *Auricula* subsect. *Euauricula* based on the matrix of Nei's genetic distance (see electronic supplementary file ESF 4). The AFLP divergence rate of DN72 = 0.037 (SD = 0.046) per 10,000 years was used to estimate the temporal course of diversification (Kropf & al. 2009).

ing the last 50,000± 40,000 years, the split of *P. palinuri* being oldest, and the diversification of the clade *P. apennina-P. cottia-P. pedemontana* starting 16,500±13,000 yrs b.p.

Molecular analysis of cpDNA sequences.- The complete cp marker alignment comprises 4874 nucleotides. In the ML analysis (Fig. 3), *P. palinuri* was part of a well-supported clade (86%) with *P. allionii*, *P. latifolia* and *P. marginata*. Within this clade, *P. palinuri* and *P. marginata* shared a 77bp deletion and a 6bp insertion. *P. appenina* was weakly supported (57%) sister to *P. cottia*, and these two were part of a well-supported (100%) clade with *P. pedemontana*. *P. auricula* and *P. balbisii* were sister to each other (99%) and part of a larger clade with *P. villosa*, *P. recubariensis*, *P. daonensis* and *P. hirsuta*.

Discussion

The information obtained from nuclear and cp DNA shed some light on the evolutionary history of Primulas in the Italian peninsula. Phylogenetic and phenetic analyses based both on AFLPs and on six plastid markers strongly supported the monophyly of *P.* subsect. *Euauricula* as circumscribed by Zhang & Kadereit (2004, 2005). However, there is a divergence between phylogenies obtained from the nuclear and chloroplast markers. Whereas the AFLPs indicate that the acidophilous species form a partly well supported clade, acidophilous and basiphilous species are intermixed in the tree based on plastid DNA. Such incongruences are not new, and may be the result of either hybridization or incomplete lineage sorting (Dillenberger & Kadereit 2013 and references therein).

Both AFLPs and plastid markers were consistent in indicating that *P. apennina*, *P. palinuri* and the complex *P. auricula* - *P. balbisii* are not closest relative to each other, implying that the presence of Primulas of subsect. *Euauricula* in the Italian peninsula is due to three separate and independent events.

The complex P. auricula-P. balbisii.- There is no general consensus regarding the taxonomy of this group. Recent floristic inventories (e.g. Conti & al. 2005) recognized only one species, while Zhang & Kadereit (2004) distinguished two species, based both on morphological and molecular evidence. Our analysis provided strong evidence for the monophyly for *P. auricula s. l.* (100% bootstrap support in all cladograms). Inside this clade, the two populations of *P. auricula s. str.* are similar to each other, contain little genetic diversity, and form a well-supported group, while the three populations of *P. balbisii* contain higher diversity, and relationships among populations remain unclear because branch support is low. Within this complex there is a remarkable contrast between morphological uniformity and molecular distinctiveness among populations, which possibly is the result of ancient allopatric differentiation combined with uniform selective pressures. In conclusion, relationships within *P. auricula s. l.* require further research, including sampling across the full distribution range, and in particular along the Apennine, in order to better understand variation patterns and to clarify its taxonomy.

P. apennina.- This species is represented by few disjunct populations distributed on the higher mountain tops along a narrow corridor in the northern Apennine. The percentage of polymorphic loci revealed by AFLPs (48%) is the lowest within the subsection, in accor-

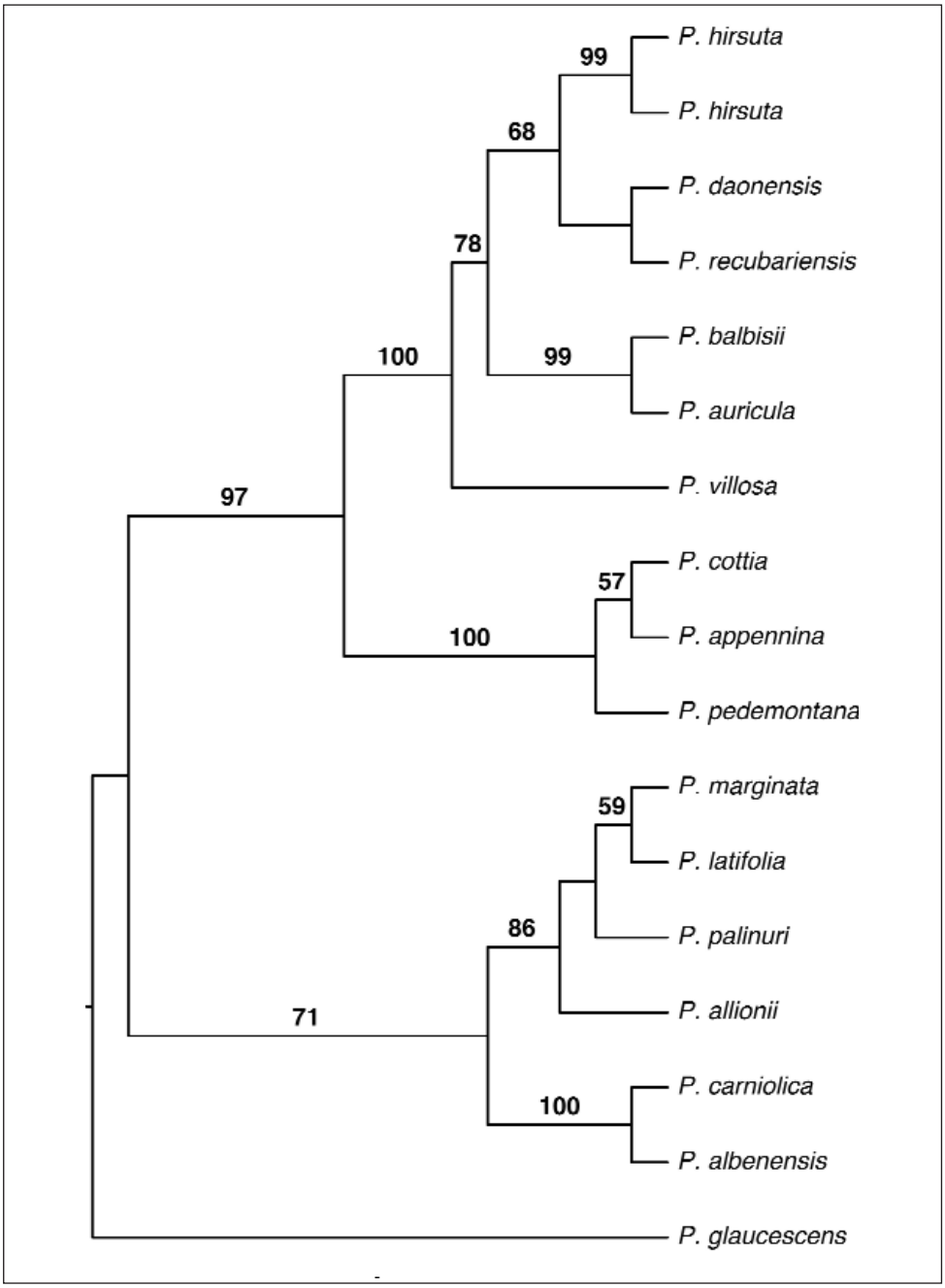

Fig. 3. Phylogeny of *Primula* sect. *Auricula* subsect. *Euauricula*. Maximum Likelihood tree obtained from the analysis of six cpDNA markers (psbA-trnH spacer, rps16-trnK spacer, ndhF-rpl32 spacer, rpl16 intron, trnL intron, rps16 intron). Bootstrap values >50% are indicated above branches. The tree was rooted with *P. glaucescens* (subsect. *Cyanopsis*).

dance with its isolation and small population size. *Primula apennina* is genetically most similar to *P. cottia* and *P. pedemontana* (genetic distance 0.060), and its genetic distance to all other species is twofold or more (≥ 0.110). In all our trees *P. apennina*, *P. cottia* and *P. pedemontana* form a well-supported group (100% bootstrap), in accordance with the phylogeny based on ITS (Zhang & al. 2004). The mountain complex in which *P. apennina* grows is a part of the Tusco-Emilian Apennine whose summits exceed 2000 m a.s.l., surrounded on both sides by mountains of lower elevation; towards W-NW a gap of about 200 km separates it from the Maritime Alps where the two most closely related species grow, causing ecological isolation by altitude. A number of species of alpine habitats share the same distribution pattern as the *P. pedemontana-P. cottia- P. apennina* group, with a main range extending to the Maritime Alps, and a disjunct distribution, with scattered small populations, on the higher tops of the Tusco-Emilian Apennine. Examples include *Antennaria carpatica* (Wahlenb.) Bluff. & Fingerh., *Artemisia umbelliformis* Lam., *Carex foetida* All., *Cystopteris montana* (Lam.) Desv., *Empetrum hermaphroditum* Hagerup, *Eriophorum scheuchzeri* Hoppe, *Ranunculus küpferi* Greuter, *Rhododendron ferrugineum* L. and *Salix breviserrata* Flod (Ferrarini 1974; Alessandrini & Branchetti 1997; Bonafede & al. 2001; Alessandrini & al. 2003 and references therein). Through very small population sizes and low genetic diversity, genetic drift may have resulted in rapid genetic divergence, as in *P. apennina*.

The cladogenesis of this group seems to date back to the Würmian late glacial. A possible scenario is that the ancestor of the three species spread from the western Alps to the northern Apennine in this period, when the species could grow at lower elevations. The highest peaks of the Tusco-Emilian Apennine were covered by ice during the last glacial period (Giraudi 2003), but the lower peaks on which the species grows today (Mt. Sterpara, Mt. Orsaro, Mt. La Nuda etc) could have served as refugia and/or as a colonization route. The Holocene caused the species to move from low elevations towards the mountain tops, resulting in their present isolation. A similar scenario had recently been postulated for the northern Apennine endemic *Minuartia laricifolia* subsp. *ophiolitica* Pignatti by Moore & al. (2013). However, more information about Quaternary glacials in the Apennine, palaeobotanic, palaeoclimatic data, and species distribution models would be necessary to better understand the evolutionary history of *P. apennina*.

With regard to taxonomy, our evidence does not justify merging *P. apennina* in *P. pedemontana* (Richards 2003) and *P. cottia* in *P. villosa* (Aeschiman & al. 2004; Richards 2003).

Primula palinuri.- An ecological and geographical outlier in sect. *Auricula*, *P. palinuri* is the only species growing on maritime cliffs on neutral to acidic substrate, the only species from a Mediterranean habitat, and the most southerly in the whole section. With *P. auricula* s.l. it shares a yellow corolla, a feature unique in the subsection but frequent in related sections and likely a plesiomorphic characters.

Evidence concerning the relationships of the species, which in our analyses was clearly part of subsect. *Euauricula* as it was in the ITS based phylogenetic reconstructions by Zhang & Kadereit (2004), is somewhat contradictory.

On the one hand, molecular evidence mostly favours a close relationship between *P. palinuri* to a group of alpine species. In the analysis of cpDNA sequences, *P. palinuri* is most closely related to *P. allionii*, *P. latifolia* and *P. marginata*. Such placement is not con-

tradicted by the parsimony analysis of the AFLP data, where these four species are part of a large polytomy, and in the NJ analysis of the AFLP data *P. palinuri* grouped with *P. allionii*, although with low support. In the PCO analysis, *P. palinuri* is distinct, but most similar to the above three species (plus *P. albenensis*, *P. carniolica* and *P. recubariensis*). Its genetic distance from all other species of the subsection also explains its position as first branch in the UPGMA analysis of Nei's genetic distances (Figure 3).

On the other hand, *P. palinuri* has some non-molecular characters which conflict with its rather derived molecular position. First, a Calabrian population of the species was found to have a sporophytic chromosome number of $2n = 44$ (Garbari 1974), interpreted as tetraploid on the base x = 11. Tetraploid chromosome numbers are otherwise unknown in sect. *Auricula* and have only been found in North American representatives of the related sections *Cuneifolia* and *Parryi* (Richards 1993, Zhang & al 2004). Second, *P. palinuri* shares several characters with subsect. *Cyanopsis*, e.g. foliose bracts, long calyces, and a smooth seed coat. Therefore, Chiarugi (1941, 1952) and Zhang (2002) regarded it as the most primitive species of the entire section, and Chiarugi (1952, 1956) even proposed accommodating this species in its own section *Palaeoauricula* within subgen. *Auricula*. Considering that genetic distances are highest between *P. palinuri* and *P. auricula s. l.*, and that these two are not closely related to each other, the treatment by Pax (1905) revived by Richards (2003), who included *P. auricula* (incl. *P. balbisii*) and *P. palinuri* as the only members of subsect. *Euauricula*, is clearly refuted.

When relying on the molecular data, the geographical distribution of *P. palinuri* can be explained in two different ways. First, the ancestor of the species could have reached southern Italy by long-distance dispersal from the western Alps. The possibility of long-distance dispersal of alpine plants into southern Italy (and Sicily) has recently been shown for *Adenostyles* Cass. by Dillenberger & Kadereit (2013). Second, it is conceivable that the ancestor of *P. palinuri*, a relative of the western Alps species, once was more widespread in peninsular Italy - probably in a cold period of the Quaternary - but became extinct in this area upon climatic warming resulting in the geographical isolation of *P. palinuri*. Considering 1) that the seeds of *Primula* sect. *Auricula* are not easily dispersed over long distances, 2) that genetic diversity in extant *P. palinuri* is as high as the average in the subsection, which excludes at least recent dispersal which, as a genetic bottleneck, should be recognizable as substantial reduction of genetic diversity, and 3) that *P. balbisii* (which is not a close relative of *P. palinuri*!) is widespread in peninsular Italy and thus illustrates the general potential of subsect. *Euauricula* for such distribution, we clearly prefer the second of our explanations.

However, even if the phylogenetically derived position of *P. palinuri* and its origin from the western Alps appear likely, the accumulation of plesiomorphic characters in this species, supporting its interpretation as a palaeoendemic, still requires explanation.

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