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Plastid DNA fingerprinting of the rare *Fritillaria moggridgei* (Liliaceae) reveals population differentiation and genetic isolation within the *Fritillaria tubiformis* complex.

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Abstract

A phylogenetic analysis based on combined DNA sequences of the partial *matK* gene and the *rpl16* intron showed that the rare alpine endemic *Fritillaria tubiformis* subsp. *moggridgei* (Liliaceae) and the more widespread *F. tubiformis* var. *burnatii* are exclusively related. A genetic study used plastid DNA markers, due to limits imposed by nuclear DNA fingerprinting in species with large genomes, to study variation within and between populations. Five length-variable homopolymer repeats (polyA and polyT) and four regions with one or two insertion/deletions (indels) of different lengths were identified. Of the total of 56 plastid haplotypes obtained, 32 were fixed in the seven populations of subsp. *moggridgei* and the rest were variable in var. *burnatii*. The analysis of molecular variance (AMOVA) showed higher genetic variation among rather than within subsp. *moggridgei* populations. Indel mutations, on the other hand, were fundamental in distinguishing the two taxa.

Key words: endemism, genetic diversity, Ligurian Alps, population differentiation, species range

Introduction

More than 11% of the world flora is endemic to the Alps, and 81% of the narrow endemics are restricted to the Maritime and Ligurian Alps in the Italian provinces of Cuneo, Imperia and Savona (Aeschimann *et al.* 2011). Owing to the overall richness of the flora and incredibly high number of endemic species, this portion of the mountains has long been recognised as one of the biodiversity hotspots of the Mediterranean basin (Médail & Verlaque 1997, Casazza *et al.* 2005, 2008, Aeschimann *et al.* 2011).

The peculiar co-occurrence in this region of geological, edaphic and climatic discontinuities has largely contributed to the current high level of diversity (Minuto *et al.* 2006, Grassi *et al.* 2009, Szövényi *et al.* 2009). Geographical and climatic gradients acting as natural barriers among species and populations can eventually lead to the differentiation of new endemic taxa (Thompson *et al.* 2005, Ægisdottir *et al.* 2009, Schwienbacher *et al.* 2010 and references therein). Peripheral or locally rare populations can develop at boundaries of a species range and, in the presence of atypical habitats, diverge from central populations both morphologically and genetically (Jones *et al.* 2001, Hardie & Hutchings 2010).

Fritillaria tubiformis Grenier & Godron (1855: 13) subsp. *moggridgei* (Boissier & Reuter ex Planchon, 1873: 116) Rix in Heywood (1978: 356) (Liliaceae) provides a good example of a rare taxon sporadically distributed across the southwestern Alps at a few localities in Italian Piedmont and Liguria (Charpin & Salanon, 1985). In contrast, *Fritillaria tubiformis* subsp. *tubiformis* with dark purple flowers is not present in Liguria but has a much more widespread range in the Central Alps, the Piedmont to Valle d'Aosta and provinces of Bergamo and Brescia (Conti *et al.* 2005). However, in a recent paper reporting typification of *Fritillaria tubiformis*, Bartolucci and Peruzzi (2012) questioned the above-mentioned distribution and stated that the species has long been confused and often fully synonymized by Italian botanists with *F. meleagris* Linnaeus (1753: 304) subsp. *burnatii* (Planchon, 1873: 115) Rix in Heywood (1978: 356) (= *F. delphinensis* Grenier &

Godron (1855: 180) var. *burnatii* Planch.). In this regard, a biosystematic study is in progress aimed at clarifying the systematic status and distribution of the two taxa; however, irrespective of its closeness to *F. meleagris* or *F. tubiformis*, all Italian populations with dark purple flowers have been referred to *F. tubiformis* var. *burnatii* (Planch.) Rouy (1910: 403) (L. Peruzzi, Pisa, pers. comm.).

Understanding the details of population biology of endemics of this part of the Alps is a prerequisite for their conservation and predicting the impact of the global change on these rare species. Small, isolated populations often have low genetic variability and in changing environments are more prone to genetic erosion than larger populations (Frankham 1995, Booy *et al.* 2000, Ouborg *et al.* 2006, Jones *et al.* 2011 and references therein). Peripheral and small populations, nevertheless, may maintain a high degree of local differentiation as a consequence of scattered or geographically oriented distributions as documented in some rare plants (Booy *et al.* 2000, Ægisdottir *et al.* 2009, Jones *et al.* 2011). Marginal populations indeed may contain unique genetic variation and be of particular value for conservation of a species.

Here, we have measured genetic variability of *F. tubiformis* subsp. *moggridgei*, including all seven presently known Italian populations. Genome size can limit the range of molecular markers available for nuclear DNA fingerprinting because these techniques perform suboptimally, and levels of polymorphism identified are typically an underestimate in taxa with large genomes (Fay *et al.* 2005). For example, multilocus fingerprinting methods such as AFLP (amplified fragment length polymorphism) have proved to be an efficient way of detecting polymorphisms in endangered and highly fragmented populations (Kitner *et al.* 2012), but this approach was ineffective (Fay *et al.* 2009) in species with large genomes as found in *Fritillaria* Linnaeus (1753: 303) (1C = 87.3 pg in *F. japonica* Miquel, 1867: 158; Ambrožová *et al.* 2011).

For characterization of population diversity and genetic structure in *F. tubiformis* subsp. *moggridgei*, we designed ten plastid DNA markers. As demonstrated by Fay *et al.* (2009) for

British plants of *Cypripedium calceolus* Linnaeus (1753: 951), plastid microsatellites represent an effective alternative to nuclear DNA markers to reveal genetic variability in species with large nuclear genomes.

Investigations that take into consideration divergence of infraspecific variants can provide information about how speciation happens and which factors are most important to promote divergence (McGlaughlin & Friar 2011). This analysis has therefore been implemented with four populations of *F. tubiformis* var. *burnatii* sampled in the Piedmont at the boundary of the range of *F. tubiformis* subsp. *moggridgei.*

Given the possibility as hypothesised by Bartolucci & Peruzzi (2012) that var. *burnatii* could be closer to *F. meleagris* than to *F. tubiformis*, before proceeding with the plastid DNA fingerprinting we conducted a maximum parsimony analysis on a combined DNA sequence matrix of the plastid *matK* gene and *rpl16* intron to evaluate placement of *F. tubiformis* subsp. *moggridgei* relative to *F. tubiformis* var. *burnatii*, *F. meleagris* sensu stricto and other Eurasian *Fritillaria* species, as sampled in Rønsted *et al.* (2005)

The main foci of this research were: i) an analysis of genetic diversity and differentiation among and within populations of *F. tubiformis* subsp. *moggridgei* and potential ii) gene flow between this species and neighbouring populations of *F. tubiformis* var. *burnatii*. We will refer to the former taxon as subsp. *moggridgei* and the latter as var. *burnatii* throughout the remainder of this paper. In addition, we hope that our results would provide guidance for future conservation programs of this rare microendemic.

Materials and methods

Plant material

The plant material used in this study was collected from 11 populations of *F. tubiformis s.l.* distributed across the Maritime and Ligurian Alps. Italian populations of subsp. *moggridgei* are known to be distributed along the southernmost boundaries of the species range and were all

sampled (Fig. 1, Table 1). Plant material was collected from plants in full bloom in May and June, 2009 and 2010. DNA was extracted from 40 mg silica gel-dried leaf tissues using the E.Z.N.A.® SP Plant DNA Kit (Omega Bio-Tek, Norcross, GA) and purified on columns following the manufacturer's protocol.

Phylogenetic analysis

Sequences of plastid *matK* and *rpl16* intron were obtained from subsp. *moggridgei* populations (MAR, CRA and FRO; Table 1) and var. *burnatii* (SER population; Table 1) and from one DNA sample of *F. tubiformis* deposited in the DNA Bank at the Royal Botanic Gardens, Kew (Table 2; http://apps.kew.org/dnabank/search). A further 26 sequences corresponding to most of the Eurasian *Fritillaria* species (*F. tubiformis* included) in Rønsted *et al.* (2005) were downloaded from GenBank (Table 2).

The *matK* region was amplified using a combination of -19F and trnK-2R primers with the internal primers 390F and 1326R, which allowed for amplification and sequencing of the whole gene (approx. 1800 bp). The *rpl16* intron was amplified using primers 158F and 1661R (Rønsted *et al.* 2005).

PCR amplifications were run in a Gene Amp 9700 PCR system (ABI, Applied Biosystems, Warrington, Cheshire, UK) using 5X Green GoTaq® Reaction Buffer (Promega BioScience, LLC. San Luis Obispo, CA, USA), EuroTaq (BioCat GmbH, Heildelberg, Germany) with 2.5 mM $MgCl_2$ (ABGene, Epsom, Surrey, UK) and starting with 40-60 ng of genomic DNA in final reaction volumes of 20 μl. PCR profiles consisted of 15 s of initial denaturation at 90°C, followed by 30 cycles of 94°C for 60 s, 50°C for 60 s and 65°C for 4 min, and a final elongation step at 65°C for 5 min for the *rpl16* gene and of 150 s initial denaturation at 94°C, followed by 29 cycles of 94°C for 60 s, 52°C for 45 s and 72°C for 150 s with 8 s increments of the ramp at the completion of each cycle and a final elongation step at 72°C for 7 min for the *matK* gene. PCR products were cleaned as detailed in the following section.

Sequences were edited and assembled using Sequencher version 4.1 (Gene Codes, Ann Arbor, Michigan, USA). They were aligned manually with those retrieved from GenBank in PAUP* 4.0b4 for Macintosh (Swofford 2002). Matrices can be obtained from the first author (marco.mucciarelli@unito.it); DNA sequences produced for this study were deposited in GenBank (accession numbers KC409110, KC409111, KC409112, KC409113, KC409114, KC409115, KC409116, KC409117, KC409118). Cladistic analysis were conducted using PAUP* 4.0b4 (Swofford 2002). Sequences of *Cardiocrinum giganteum* (Wallich, 1824:21) Makino (1913:125) and *Notholirion thomsonianum* (Royle, 1839: 92) Stapf (1934: 95) were designated as outgroups in accordance with Rønsted *et al.* (2005). Phylogenetic analysis was conducted as described by Rønsted *et al.* (2005) using the heuristic search option. In brief, we performed 1000 replicates of random taxon addition using subtree-pruning-regrafting (SPR) branch swapping algorithm with 25 trees held at each step. Trees collectively found in these 1000 replicates were used as starting trees for a second search with no tree limit until all trees at this length were found. Robustness was assessed with bootstrap using 1000 replicates of simple addition and TBR swapping. Here we report only the strict consensus tree of 451 shortest trees (Fig. 2).

Identification of length-variable loci

We searched for length-variable loci flanked by conserved regions in non-coding spacers and introns of plastid DNA of *F. tubiformis* using primers given in literature (Taberlet *et al.* 1991, Shaw *et al.* 2007, Ebert & Peakall, 2009) and starting with three representative samples of subsp. *moggridgei* and one sample of var. *burnatii*. Ten out of 35 loci (Table 3) proved to be variable. PCR amplifications were run in a Gene Amp 9700 PCR system (ABI, Applied Biosystems, Warrington, Cheshire, UK) using ReddyMix PCR Mastermix with 2.5 mM $MgCl_2$ (ABGene, Epsom, Surrey, UK) and starting with 20–40 ng of genomic DNA in final reaction volumes of 25 µl. PCR profiles consisted of 3 min of initial denaturation at 94°C, followed by 28 cycles of 94°C for 60 s, 48°C for 60 s and 72°C for 60 s, and a final elongation step at 72°C for 7 min. PCR products were cleaned

using QIAquick™ PCR purification columns (QIAGEN Ltd., Crawling, West Sussex, UK) following the manufacturer's protocols. Samples were sequenced on an ABI 3730 capillary DNA genetic analyser using ABI Big Dye® Terminator v3.1 chemistry, following the manufacturer's protocols. For purifying products, we used ethanol precipitation. Raw sequences were edited and assembled using Sequencher version 4.1. They were aligned manually in PAUP* 4.0b4 (Swofford 2002), and variable regions were identified. Primers to amplify the length-variable regions were designed in conserved flanking regions. For each region, one primer was labelled with a fluorescent dye to enable amplification products to be visualized using an ABI 3100 genetic analyser. Sizes (bp) were determined using ABI GeneScan 3.1 and Genotyper 2.0 using the internal size standard ABI GeneScan™-500ROX™ for all primer pairs. Fragments containing unique or rare indels were re-sequenced from new PCR products to test for reproducibility and exclude possible Taq-induced artefacts. Ten variable sites were scored for all individuals. A matrix was prepared, using different numbers for alleles of different lengths for each microsatellite and 1 vs. 2 for the simple indels and repeats (Table 4). If two different indel events occurred at the same position in different samples, as for the *psbD-trnT* spacer (single indels of different lengths), alleles were numbered (Table 4).

Definition of haplotypes

As defined by a combination of alleles for the 10 loci, 56 were identified. Each haplotype was defined by the exact number of repeats, which allows these to be analyzed according to the stepwise mutation model (SMM) (measuring genetic differentiation among populations according to variance in allele size; Slatkin 1995). A table with haplotype definitions and frequencies is available upon request from the first author.

Statistical analysis

Haplotype frequencies in the sampled populations were estimated using ARLEQUIN software (Excoffier *et al.* 2005; data not shown). Overall gene diversity of *F. tubiformis s.l.* was estimated

for each microsatellite locus as the total (A_{tot}) and average number of alleles per population (A_{pop}), allelic richness ($r_{(21)}$, i.e. expected number of alleles observed in n genets of each population corrected for differences in sample size by rarefaction as in Kalinowski, 2004, with n = 21, the smallest population minus one, and the overall H_t, Nei 1987, average within-population, H_s, Nei 1987 and average among populations gene diversity, D_{st}; Table 5) using FSTAT (Goudet 2011).

Genetic diversity at the population level was estimated as the number of polymorphic loci (*N*), proportion of polymorphic loci (polymorphic/usable loci) (*P*), average number of alleles per locus (*A*), average number of alleles over polymorphic loci (allele range) (A_{poly}) and average (across loci) gene diversity (*H*; Edh *et al.* 2007; Table 6) using ARLEQUIN. *H* is the probability that two randomly chosen sites are different, which is equivalent to gene diversity at the nucleotide level for DNA data assuming no recombination and selective neutrality (Excoffier *et al.* 2005). The proportion of private alleles ($f_{private}$), (i.e. alleles unique to a particular population; Table 6) was calculated in CONTRIB (Rémy Petit, Petit@pierroton.inra.fr, INRA-Bordeaux). Total gene diversity (H_t), average gene diversity across loci (H_s) and proportion of private alleles ($Allf_{private}$) were also calculated for all populations taken together (see below).

Genetic diversity was also estimated by calculating allelic richness ($r_{(21)}$) per population using CONTRIB (Table 6). Although there were no differences in sample size of *F. tubiformis s.l.* populations except one, allelic richness was corrected using the rarefaction method (Kalinowski 2004). To provide robust estimates, the sample size of the smallest population sample minus one (n = 21; Table 6) was used for rarefaction (Fay *et al.* 2009).

Garza-Williamson statistics (*G-W* index; Garza & Williamson 2001) as modified by Excoffier *et al.* (2005) were calculated with ARLEQUIN (Table 6) in to test whether populations have suffered bottlenecks. This parameter takes into account the number of alleles at a given locus in a population and allelic range (for microsatellite data this is the difference between maximum and minimum

number of repeats at all loci). Finally, number of haplotypes distributed within the 11 populations of *F. tubiformis s.l.* and their absolute frequencies were estimated using ARLEQUIN.

For estimating population differentiation, genetic divergence (G_{st}) of Nei's statistics (Nei 1973) (relative differentiation in Pons & Petit 1996) was also calculated in CONTRIB on unordered haplotypes (Table 6) and for the two subspecies also (G_{st}; Table 7; Fay *et al.* 2009). Because standardized measurement of G'_{st} allows more appropriate measures of genetic differentiation than G_{st} (or F_{st}) (it is independent of number of samples and rate of mutation; Slatkin 1995, Ægisdóttir *et al.* 2009 and references therein), G_{st} and G'_{st} were also calculated locus by locus in FSTAT.

To evaluate differences in resolution of genetic diversity based on the two types of repeat motifs (i.e. indels appear to evolve more rapidly than mononucleotide repeats and have a different frequency in the plastid DNA; Graham *et al.* 2000; Shaw *et al.* 2007), Nei's estimators of diversity (H_s, H_t, G_{st}, G'_{st}) were also calculated in FSTAT for the indels and the five mononucleotide repeats separately.

For each taxon, total number of haplotypes, percentage of exclusive haplotypes, average number of polymorphic loci (*N*), average number of alleles per locus (*A)*, allelic range (*R*), proportion of private alleles ($f_{private}$), total (H_t) and average within-population gene diversity (*Hs*) (Nei 1987) were calculated in CONTRIB (Table 7). Average gene diversity across loci (*H*) and Garza–Williamson statistics (*G-W* index) were calculated with ARLEQUIN (Table 7).

Genetic structure in subsp. *moggridgei* populations was studied by analysis of the overall molecular variance (AMOVA) estimated among and within populations with ARLEQUIN (Table 8; Excoffier *et al.* 1992). To differentiate the contribution to variation derived from indels and single mononucleotide substitutions, degree of population differentiation and relative fixation indices were estimated by two statistical methods. The F_{ST} statistic following Weir & Cockerham (1984) allows estimates of genetic structure indices based on the number of alleles and their frequencies in haplotypes. The R_{ST} statistic (Slatkin 1995) takes into account differences between allelic sizes

(sum of square size differences). Both F_{ST} and R_{ST} statistics with the corresponding fixation indices were therefore calculated on overall variance (Table 8) and each locus (Table 9).

The AMOVA analysis was also performed by creating a genetic structure with two groups of populations corresponding to the two taxa (Table 8, lower part). To test significance of AMOVA covariance components and fixation indices with and without this genetic structure, a non-parametric permutational approach (Excoffier *et al.* 1992) was used by permuting haplotypes among populations among groups (F_{ST}), haplotypes among populations within groups (F_{SC}) and populations among groups (F_{CT}) for a total of 10100 iterations. By implementing a hierarchical analysis of variance, total variance was partitioned into covariance components due to inter– and intrapopulation differences as reported (Table 8).

Haplotype network

Two median-joining (MJ) networks (Bandelt *et al.* 1999) containing all shortest least complex trees (MP trees) were constructed using NETWORK 4.6.0.0. (www.fluxus–engineering.com). This method arranges haplotypes into a network where number of mutations along branches is a measure of divergence between haplotypes. In addition, the software uses parsimony criteria to identify median vectors, i.e. consensus of mutually similar sequences of markers, possibly equivalent to unsampled/extinct haplotypes. In order to evaluate the contribution of the five indels alone, and by implementing the data matrix with the remaining hypervariable regions (loci 1, 2, 3, 5, 6), two MJ networks were obtained (Fig. 3A-B). To reduce complexity of the resulting MJ network, rapidly changing and less discriminating characters were down-weighted (Fig. 3B) according to criteria in the program guidelines. The MP option, which deletes all non-MP links from the network, was in effect.

Multidimensional scaling analysis (MSD)

To clarify genetic differentiation of *F. tubiformis s.l.* populations, a multivariate analysis was carried out by subjecting population pairwise estimates of F_{ST} (Weir & Cockerham 1984; Fig. 5) and R_{ST} (Slatkin 1995; data not shown; see comments in Results) to a multidimensional scaling (MDS) in two dimensions with PCO 1.0 (principal coordinate analysis; Iwata 2004). This method allows for an interpretation of population association in a spatial model and is preferable to PCA (principal component analysis) methods since differences between near groups of individuals are resolved better by MDS, especially when number of individuals is large.

Results

Analysis of *matK* and *rpl16*

The combined plastid matrix contained 31 taxa including *F. tubiformis s.l.* (Table 1, Fig. 1) and other *Fritillaria* species (Table 2). The strict consensus tree (Fig. 2) showed that *Fritillaria* species fell into a well-supported group (99 bootstrap percentage, BP). *Fritillaria tubiformis s.l.* samples fell in a single clade (81 BP) sister to the clade containing most species of subgenus *Fritillaria sensu* Rix (2001). The four Piedmont samples of var. *burnatii* (SER) and subsp. *moggridgei* (MAR. CRA, FRO) formed a further subclade (61 BP) sister to *F. tubiformis*. *Fritillaria meleagris* was positioned far from this clade (Fig. 2).

Identification of length-variable regions

A total of 264 individuals representing seven populations of subsp. *moggridgei* and four populations of var. *burnatii* (Table 1) from the southwestern Piedmont and Liguria (Fig. 1) were analysed for ten variable plastid DNA regions (Table 3). Five mononucleotide repeats (A or T homopolymers) were found in the *trnL-trnF*, *psbK-trnS*(GCU), *trnE*(UUC)-*trnT*(GGU), *trnP*(UGG)-*petE/G* and *clpPexon1-psbB* intergenic spacers of the LSC region of *F. tubiformis s.l.* These homopolymers were highly variable in the samples studied and ranged in size from 6 to 26

bp (Table 4). Two species-specific simple indels (insertion/deletion) events and two simple plus one double species-specific indels of tandem repeats (repeats of an adjacent sequence) were observed. One indel consisting of a 5 bp tandem repeat (ACTTT) was found at position 288 of the aligned matrix in the intron of the *rpoC1*gene (*rpoC1*exon2-*rpoC1*exon1). Two single indels (tandem repeats of 30 and 17 bp) were found at position 316 of the aligned matrix in the *psbD-trnT*(GGU) intergenic spacer of the LSC. An indel due to a tandem repeat of 9 bp (TTTTAGAAA) was detected at position 274 of the aligned matrix in the *rpl33-rps18* intergenic spacer (Table 4). Two indels of unique sequence at positions 228 and 388 of the aligned matrix, consisting of 7 (ATTCTTT) and 5 bp (CCGAA), respectively, were found in the *petA-psbJ* intergenic spacer. Among the six indels, four were deletion events (5, 9, 7 and 5 bp) shared between all members of *F. tubiformis burnatii* (Table 4; Fig. 3), and locus 7 (*psbD–trnT*) showed a 30-bp deletion shared between all the members of subsp. *moggridgei* (211 bp fragment length; Table 4; Fig. 3), except for a few members of the Vallone di Cravina (CRA) population, in which the deletion at locus 7 was 13 bp (228 bp fragment length; Table 4; Fig. 3). In all cases where sequences were redone from new PCR products, the same indels were found.

Haplotypes recovered and their distribution

All 10 length-variable regions were successfully scored; the final matrix contained information for 260 out of 264 individuals. All loci were highly variable, corresponding to a total number of 51 alleles (Table 4). Fifty-six haplotypes were obtained from the combined alleles for the ten scored regions (data not shown); nine haplotypes had at least one missing value and were therefore deleted from the data matrix. We found 24 haplotypes in var. *burnatii* and 32 in subsp. *moggridgei*, none of which was shared between the two species. Three haplotypes with the highest absolute and relative within-population frequencies, 45.5, 66.7 and 40.7%, were found in populations GAL, MAR and SAC, respectively. Population CRA with nine was the richest population in haplotypes. Haplotypes in var. *burnatii* ranged from 5–8 per population, and only those belonging to PRA and DRO were

population-exclusive. One haplotype from population PRA and one from ROS were the most frequent in var. *burnatii* (relative frequency 46.1 and 34.6%, respectively).

Microsatellite diversity locus by locus

All loci were polymorphic, with the total number of alleles per locus ranging from 2–13, with a total of 51 different alleles at the ten loci (Tables 4, 5). The average number of alleles per population (A_{pop}) ranged from 1.09–3.55 within a single locus, and almost all loci (four) containing indels were fixed within populations ($A_{pop} = 1$). Values for allelic richness ($r_{(21)}$) ranged from 2.00–10.34, and overall gene diversity (H_t) varied considerably from 0.463–0.847 for the most polymorphic locus (*psbK-trnS*; Table 5). According to locus, average within-population gene diversity (H_s) ranged from 0.031–0.444 for mononucleotide repeats (average 0.179 ± 0.042) and was zero for indels. Estimates of gene diversity among populations (D_{st}) defined by Nei (1987) as the absolute differentiation ($H_t - H_s$) ranged from 0.329–0.506, corresponding to H_t at loci with indels.

Distribution of genetic diversity in *F. tubiformis s.l.* populations

Degree of population differentiation was high for all loci (Tables 6, 9). With a few exceptions, the 11 populations of *F. tubiformis s.l.* were variable in terms of number of polymorphic loci (*N*) and proportion of polymorphic loci (*P*). These were low in MAR (two loci; *P* = 20%), SAM and CRA populations (three loci; *P* = 30–33%) and high in ROS, FRO and SAC (five loci; *P* = 50%; Table 6). Average number of alleles per locus (*A*) varied even more, especially in subsp. *moggridgei*, ranging from 1.0 to 5.3 in MAR and SAM populations, respectively (Table 6). However, when considering only polymorphic loci (A_{poly}), differences among populations decreased considerably (Table 6). The proportion of private alleles ($f_{private}$), unique to a single population, ranged from 8.3–15.4% in var. *burnatii* and from 6.7–10.0% in subsp. *moggridgei.* Populations SER and GAL had the highest proportions of private alleles in the two taxa; five populations had no

unique alleles. Gene diversity (*H*) varied considerably, ranging from 0.055–0.272 in populations MAR and PLU (Table 6).

Total gene diversity across all populations was $H_t = 0.988 \pm 0.002$ and the average gene diversity across loci $H_s = 0.829$. Total percentage of private alleles ($Allf_{private}$) for *F. tubiformis s.l.* was 5.11%. Allele richness was high for all populations except population MAR ($r_{(21)} = 1.88$) and ranged from 7.14–11.15 in var. *burnatii* and from 5.82–13.18 in subsp. *moggridgei* (Table 6). *G-W* indexes varied between 0.817–1.000 (Table 6).

Genetic differentiation (Nei 1973) of *F. tubiformis s.l.* populations ranged from $G_{st} = 0.111$ in CRA population to 0.323 of MAR (Table 6), the most divergent population. When relative contribution to the genetic differentiation was evaluated per locus, indels showed the highest G_{st} values ($G_{st} = 0.936–1.000$ for locus 4 and loci 7-10). Estimated over all populations, G_{st} was 0.714 when calculated over all variable loci and $G_{st} = 0.528$ when only mononucleotide repeats were considered and similarly with G'_{st} values ($G'_{st} = 0.733–0.552$, respectively).

Distribution of genetic diversity in the two taxa

Among the 56 haplotypes, the proportion of subspecific-specific haplotypes was distributed as follows: 32 exclusive haplotypes belonged to subsp. *moggridgei* and 24 were exclusive to var. *burnatii* (Table 7). Genetic diversity was similar for the two subspecific taxa. There were six polymorphic loci in subsp. *moggridgei* and five in var. *burnatii* (as for other loci with indels, locus *psbD-trnT* was monomorphic in the four populations sampled for the latter taxon). The average number of alleles per locus was 2.9 for var. *burnatii* and 3.4 for subsp. *moggridgei*, probably as a consequence of the high allelic variation in the mononucleotide repeat loci in individuals of population SAM (Tables 4-7). The allelic range was $R = 4.35$ for the species (4.35 is the average difference between the maximum and minimum number of repeats at all loci) and not statistically different in subsp. *moggridgei* ($R = 4.5$) or var. *burnatii* ($R = 4.2$). The proportion of private alleles was high in subsp. *moggridgei* ($f_{private}$ = 4.5% unique alleles) and considerably higher in var.

burnatii ($f_{private}$ = 5.9%) for the contribution of populations SER and DRO (Table 7; see also Table 6). The remaining genetic indices were similar between the subspecies; values for total (H_t), across loci (H) and within population (H_s) gene diversities were 0.998–0.988, 0.325–0.329, 0.805–0.870 in subsp. *moggridgei* and var. *burnatii*, respectively (Table 7). *G–W* indices were high for both subspecies (0.961–0.942), supporting estimates at the population level of Table 6. As expected from the similar values of H_t and H_s, population differentiation (G_{st} values) was low (0.193–0.128, subsp. *moggridgei* and var. *burnatii*, respectively).

Distribution of genetic variation

AMOVA showed that overall divergence among populations of subsp. *moggridgei* was high and that molecular variation was almost equally distributed among and within populations based on the infinite alleles and stepwise-mutation models: among populations F_{ST} = 54.06% and R_{ST} = 56.57%; within populations F_{ST} = 34.20% and R_{ST} = 22.23% (Table 8).

When the relative contribution of each locus to the total variation encountered in *F. tubiformis s.l.* was considered, we found that loci 4, 7, 8, 9 and 10 with only indels made the largest contribution to divergence among populations with no appreciable differences according to the statistical algorithm employed (96.27–100% and 99.04–100% for F_{ST} and R_{ST}, respectively). In addition, all populations were fixed for these monomorphic loci (F_{ST} = 0.963–1) (Table 9). On the other hand, microsatellite loci 1, 2, 3, 5 and 6 (and to some extent locus 7) made different contributions to the two components of total variation in accordance with the statistical algorithm employed. It is clear (Table 9) that microsatellite loci made the greatest contribution to the "among populations" component when evaluated according to the stepwise mutation model (R_{st} = 61.89–94.57%) and to the "within populations" component when in the infinite alleles model (F_{ST} = 53.38–83.07%; Table 9). Overall F_{ST} for the plastid loci in *F. tubiformis s.l.* was 0.66.

Table 8 (lower part) also shows results for the distribution of genetic variation between the two subspecific taxa. The between−group component of total molecular variance was high (F_{CT} = 0.625; P < 0.01; Table 8) and the proportion due to differences among population within subspecies (F_{SC} = 0.510, P < 0.0001; Table 8) and due to differences within populations (F_{ST} = 0.816, P < 0.0001; Table 8) was similarly high and statistically significant. Similar results were obtained using the *R*-statistics (F_{ST} = 19.11/18.36% and R_{st} = 14.06 /10.66%; Table 8). Existence of two main gene pools within *F. tubiformis s.l.* was therefore evident. Genetic variance among haplotypes of the two taxa represented the greatest portion of total variance and ranged from 62.53% with the infinite allele model (F_{ST}) and 75.28% with the stepwise mutation model (R_{st}) (Table 8).

Haplotype network

Haplotype distribution and composition of *F. tubiformis s.l.* produced an MJ network (Fig. 3A) with no torso and only three nodes (Ha, Hb1 and Hb2), which form two main clusters (A and B) clearly partitioning the two taxa; clusters were separated from each other by five positions corresponding to the five indels at loci 4, 7, 8, 9, 10 (Fig. 3; Table 4). Haplotypes of var. *burnatii* clustered at the Ha node (37.8% of the total number of individuals) and were characterized by a 241-bp fragment at locus 7 (region *psbD-trnT*) and by four deletions (5-9 bp) at loci 4, 8, 9 and 10 (Table 4). Populations of subsp. *moggridgei* accounted for the remaining 62.2% of the total number of individuals, partitioned between haplotypes Hb1 and Hb2. Haplotype Hb1 is directly connected to the var. *burnatii* cluster and consists of the subsp. *moggridgei* individuals characterized by a 228-bp fragment at locus 7 (Fig. 3A; Table 4). These are all individuals exclusive to the Cravina population (frequency 1.8%) of the Valle Pesio (site # 1 in Fig. 1). The second and larger cluster Hb2 (frequency 60.4%) is composed of all remaining subsp. *moggridgei* individuals characterized by a 211-bp fragment at locus 7 (*psbD-trnT*; Fig. 3A).

By weighting phylogenetic distances within the network (ε = 10) a high-resolution full median MJ network of all ten microsatellites was obtained (Fig. 3B). It consisted of a torso connecting two

main clusters with maximum parsimony, each one of closely related haplotypes (just one change between nodes). Cluster A comprised seven haplotypes for var. *burnatii* (total frequency 42.6%), and cluster B was made up of 11 haplotypes of subsp. *moggridgei* (frequencies 57.4%) (Fig. 3B).

The phylogenetic distance between the two clusters was due to six changes clearly separating the two taxa (Fig. 3B) and corresponding to the short indels at loci 4, 8, 9 and 10, the 13-bp indel at locus 7 (*psbD-trnT*) and the hypervariable locus 5 in the *trnP-petE-G* region. The median vector mv1 (Fig. 3B) is positioned between the two subspecific taxa in order to accommodate a hypothesised ancestral or missing sequence bearing a further 17bp indel in the *psbD–trnT* region (locus 7; Fig. 3B; Table 4). The haplotype distribution within the two taxa (cluster A and B) gave two complex networks. Two median vectors (mv3 and mv4) were positioned within cluster B in order to accommodate three peripheral haplotypes (Hp, Hq and Hr) belonging to the southern populations of subsp. *moggridgei* from the Valle Tanaro (FRO and GAL; sites # 3, 4, Fig. 1). On the other hand, the most common subsp. *moggridgei* haplotypes (SAC, CRA, MAR and SAM; site # 9, 1, 5, 10, Fig. 1) occupied a central position in cluster B (Fig. 3B). Cluster A grouped all accessions of var. *burnatii* and showed a poorly resolved network with the four sampled populations largely intermixed.

Multidimensional scaling analysis (MDS)

According to multidimensional scaling, two main genetic groups were detected among the 11 populations of subsp. *tubiformis s.l.* (Fig. 4). The first two dimensions (PCO1 and PCO2) of the MDS plot accounted for 71.3% of total variance. The first axis (PCO1) corresponded to the greatest variation and broadly separated the two subspecific taxa in accordance with network results. This component is likely to represent the contribution of the five indel mutations to the overall differentiation between the two subspecies, and this was confirmed by populations of var. *burnatii* showing the same kind of indels here grouped closely (cluster B; Fig. 4). MSD was also useful for detecting populations possessing some intermediacy between the two genetic groups. This was

reliable in the case of subsp. *moggridgei* populations, which were well resolved by the second axis (PCO2) accounting for 20.9% of variation. In accordance with NETWORK (Fig. 3, cluster B), the Val Tanaro populations (GAL and FRO) clustered together in the PCO plot, well separated from the remaining northernmost populations. Populations CRA SAC and SAM (Val Stura) grouped together irrespective of their geographic position (Fig. 4). Pian del Lupo, on the other hand, was separated from all other populations, in accordance with NETWORK results.

The MDS analysis stressed genetic differentiation between the taxa and supported a higher level of genetic complexity within the subsp. *moggridgei* populations. Similarly, when using R_{ST} estimates, which relies on the sum of square size differences, the PCO plot split all populations in two narrow clusters, with PCO1 accounting for almost all total variance (88.79%; data not shown).

Discussion

Genetic variation in large genomes

As demonstrated by Fay *et al*. (2009) for British plants of *Cypripedium calceolus*, plastid DNA fingerprinting represent an alternative to nuclear DNA markers for revealing genetic variability in species with large nuclear genomes. Similarly, this problem has been addressed for *Fritillaria*, among which some of the largest genome sizes in the plant kingdom have been recorded (Ambrožová *et al.* 2011 and references therein). Li and colleagues (2011) found low genetic diversity in landraces of *Fritillaria thunbergii* Miquel (1867: 157), employing only nine inter-simple sequence repeat (ISSR) bands out of 100 total markers scored. A total of 31 polymorphic (out of 54) RAPD markers were scored in the triploid *F. camschatcensis* Ker Gawler (1809: 1216) (Yamagishi *et al.* 2010) one of the smallest genomes in *Fritillaria* (1C = 38.4 pg; Ambrožová *et al.* 2011). Our study has shown that plastid DNA fingerprinting is able to reveal genetic polymorphisms in natural alpine populations of *F. tubiformis* subsp. *moggridgei*, providing units of conservation.

Indel-based DNA polymorphism and infraspecific diversification in *Fritillaria tubiformis s.l.*

Many studies so far have scored plastid regions and found substantial intraspecific indel polymorphisms, thus providing a useful tool for plant DNA fingerprinting (reviewed by Provan *et al.* 2001, Hamilton *et al.* 2003 and references therein, Fay *et al.* 2006, Micheneau *et al.* 2010, Schroeder *et al.* 2012). Inversions and insertions/deletions (indels) are major structural mutations of plastid DNA (reviewed by Kelchner 2000). Both may include phylogenetically relevant information, but indels are regarded as more reliable phylogenetic markers given the low levels of homoplasy of these microstructural characters (Graham *et al.* 2000). In contrast, several authors have acknowledged the possible highly homoplasious nature of small inversions, especially when employed at higher taxonomic level (Lehtonen *et al.* 2009). In this regard, large indels have been efficiently employed to infer phylogenetic relationships at the species and family level as demonstrated in Caryophyllaceae (*Silene* L.; Ingvarsson *et al.* 2003) and in Solanaceae (*Capsicum annuum* L.; Jo *et al.* 2011). In *F. tubiformis* five regions of the plastid genome contained indels that were subspecies-specific (Figs. 3,4) and allowed for a clear assignment of all the populations with a total of 24 var. *burnatii* and 32 subsp. *moggridgei* exclusive haplotypes (Table 7; Fig. 3).

Through a combined matrix of *matK* and the *rpl16* intron, we tested overall relatedness of the two subspecific groups of *F. tubiformis* populations in order to provide a basis for including both taxa in an analysis of genetic variation. The phylogenetic analysis was congruent with results obtained by Rønsted *et al.* (2005), positioning *F. tubiformis s. l.* with *F. tenella* Marschall von Bieberstein (1808: 269), *F. lusitanica* Wickström (1821: 352) and *F. meleagris* in the core clade of subgenus *Fritillaria sensu* Rix (2001). Our phylogenetic analysis identified a distinct *F. tubiformis s. l.* clade (B5) containing populations belonging to both flower morphotypes, taxonomically ascribed to var. *burnatii* and subsp. *moggridgei*. The former was clearly more related to the populations of subspecies *moggridgei* than to any other taxa in the analysis, including *F. tubiformis sensu stricto* employed as reference species (RBG DNA Bank samples and GenBank sequence

accessions). This result was meaningful in testing the level of resolution of plastid markers in distinguishing among closely related populations of the two taxa. Analysis of molecular variance at each locus confirmed that plastid DNA indels were essential in indicating separation between the two taxa and that two genetic pools are recognizable in *F. tubiformis* based on the number of different alleles (100% percentage variation, F_{ST} = 1.000 and F_{ST} = 0.963 for the *psbD-trnT* region; Table 9) and on differences at the polymorphic loci (100% percentage variation, R_{ST} = 1.000 and R_{ST} = 0.990 for the *psbD-trnT* region; Table 9). When the MJ network resulting from the five indels was incorporated with variation in the *trnP-petE/G* spacer, which at the population level had the most informative of the mononucleotide repeats (D_{ST} = 0.506, G'_{ST} = 0.704, the highest values for all scored loci), it was clear that the two taxa were separated by at least six steps (Fig. 3B).

Although one must be careful in employing polymorphic haplotypes to make evolutionary inferences (Hamilton *et al.* 2003), the level of plastid DNA divergence recovered here was striking (99.8% of the total gene diversity found in both taxa; Table 7), thus confirming utility of indels in measuring genetic variation between taxa. They differed in the proportion of private alleles and genetic divergence, which was greater in subsp. *moggridgei* (G_{st} = 0.193 vs. 0.128; Table 7). The latter found support in the PCO analysis (Fig. 4) based on allele frequencies, in which subsp. *moggridgei* populations were dispersed along the PCO2 component due to their greater genetic divergence (see, for example, populations MAR, CRA and PLU from Valle Pesio).

The *psbD-trnT* spacer of the plastid LSC (large single copy) DNA has been found to be highly variable in Solanaceae (Daniell *et al.* 2006) and contains large indels (57-94 bp) in *Minuartia* L. (Caryophyllaceae; Shaw *et al.* 2007). The *psbD-trnT* spacer of *F. tubiformis* subsp. *moggridgei* revealed a 30-bp indel (211 bp fragment) in all but a few individuals, and these individuals possessed a larger fragment (228 bp) at the same position. The MJ network of all *Fritillaria* indels showed that individuals bearing the 228 bp fragment are ancestral to the whole subsp. *moggridgei* gene pool (Fig. 3A). Considering that this sub-population is located in the uppermost part of the

Vallone di Cravina (CRA), we speculate that they may represent a founder population for all others in Valle Pesio.

Gene diversity and its distribution in populations of subsp. *moggridgei* and *F. tubiformis s. l.*

Average genetic diversity within populations of *F. tubiformis s. l.* was low (H_s = 0.179; Table 5), especially when compared with values obtained by Fay *et al.* (2009) for *Cypripedium calceolus* (H_s = 0.675), Ægisdottír *et al.* (2009) in alpine *Campanula thyrsoides* L. (H_s = 0.760) and Hodgins & Barrett (2007) in *Narcissus triandrus* L. Otherwise, in *Fritillaria* H_s was comparable with the low values obtained by Edh *et al.* (2009) for nuclear microsatellites in the endemic *Brassica cretica* Lam. *(*H_s = 0.211). Here, unlike nuclear DNA, no variation in plastid DNA was found, similar to the consistent indels in *F. tubiformis*.

When gene diversity was considered across all loci, however, it varied significantly between populations ranging from 0.055 in population Vallone del Marguareis (MAR) to 0.272 for population Pian del Lupo (PLU; Table 6). Low genetic diversity within endangered, rare or small populations is common and normally interpreted as a result of stochastic events like genetic drift, founder effects or population isolation (Edh *et al.* 2007). Furthermore, compared to widespread species, lower levels of gene diversity are normally expected within populations of endemic species regardless of their size (Edh *et al.* 2007 and references therein). However, the overall gene diversity of *F. tubiformis* was high at the species level (H_t = 0.623, Table 5), falling in the range found in other species studied so far with plastid microsatellites (H_t = 0.245-0.408; 0.907; 0.420-0.786 in Edh *et al.* 2009, Fay *et al.* 2009, and Honjo *et al.* 2009, respectively) and largely dependent on the overall gene diversity existing among populations (D_{ST} = 0.445; Table 5).

AMOVA supported these observations and, consistent with genetic indices, showed that greater genetic variation is to be expected "among" rather than "within" subsp. *moggridgei* populations (FST 54.06 vs. 45.94%, Table 8 upper part). Percentage variation of the "within populations" component, however, ranged between 45.9–43.4%, proving that despite a consistent degree of

genetic isolation, gene flow still creates variability among individuals of subsp. *moggridgei*. When AMOVA was repeated on both taxa (Table 8, lower part), percentage variation was equally distributed "among" and "within" populations (FST 19.11 vs. 18.36%; RST 14 vs. 10%, Tab 8 lower part), but in this case most variation was due to genetic differences between the two taxa.

With regard to the possible causes of the observed low gene diversity within subsp. *moggridgei* populations, we can exclude bottleneck effects. Most populations exhibited high numbers of polymorphic loci and alleles per locus (Table 6) and a high proportion of private alleles (on average 5.11%) compared, for example, with the results of Fay *et al.* (2009) for *C. calceolus*. Allelic richness was particularly high, higher than those recorded by Andrianoelina *et al.* (2009) in the endangered tropical tree *Dalbergia monticola* Bosser & Rabevohitra (H_{cp} = 0.26–0.66) and *Narcissus triandrus* by Hodgins & Barrett (2007). In addition, *G-W* values were close to 1 in all populations sampled (Table 6). This statistic is sensitive to population bottlenecks and is expected to be close to one in stationary populations (Garza & Williamson 2011).

Genetic drift and population isolation along narrow alpine valleys is probably the cause of low gene diversity observed within most of subsp. *moggridgei* populations. Allelic richness is a gene diversity parameter suggested to be useful for identifying populations that deserve special management because of reduced diversity, i.e. population bottlenecks reduce allelic richness more quickly than heterozygosity (Kalinowski 2004). In this context, population MAR actually showed gene diversity indices notably lower (A = 1 and allelic richness $r_{(21)}$ = 1.88) than any other population. We feel, therefore, that this population should be regarded as a priority target in future conservation of *F. moggridgei* in Valle Pesio. In consideration of its lower elevation with respect to the other two populations in the same valley (PLU and CRA) population MAR is likely to have originated from a founder/s brought down during transhumance of cattle.

As argued by Ægisdottír *et al.* (2009) for *C. thyrsoides*, we believe that considerable genetic differentiation is to be expected in *F. tubiformis* considering that, except for occasional long-

distance transport of seeds by grazing animals, this species has limited seed dispersal capacities. As observed in the literature, geological and climatic discontinuities typical of the Alps acting as natural barriers among species and populations (Ægisdottir *et al.* 2009, Schwienbacher *et al.* 2010 and references therein) may lead to fragmentation of a widespread ancestral taxon (Hughes & Hollingsworth 2008, Xu *et al.* 2011, Takahashi *et al.* 2011). Limitation of seed dispersal, clonality through bulb dispersion and habitat fragmentation may have resulted in genetically and morphologically distinct populations that diverged from the more widespread species towards development of localized endemics. *Fritillaria tubiformis* subsp. *moggridgei* is one such endemic from the Ligurian Alps.

Conclusions

Based on plastid DNA variation and haplotype distribution, we can claim that subsp. *moggridgei* and Italian populations of var. *burnatii* clearly belong to different genetic pools. This finding is consistent with our phylogenetic analysis and with: i) the high proportion of species-specific private alleles that fixed plastid haplotypes within the two taxa (Table 7; Fig. 3), ii) similar levels of gene diversity, iii) the fact that, when a genetic structure is introduced in the analysis, most genetic variation among populations is due to differences between subspecies *moggridgei* and var. *burnatii* (AMOVA, Tables 8). Based on the inferred steps among plastid DNA haplotypes, the two taxa are separated by a minimum of six with no observed intermediate haplotypes (Fig. 3).

Based on all these results, we feel that subsp. *moggridgei* may merit species rank. Further studies aimed at clarifying the systematic status and actual distribution of this taxon are already in progress. Our study indicates the hypothesis of a long and independent history with little or restricted gene flow of local populations of *F. moggridgei*. Special attention, therefore, should be devoted to all these populations because they may contain specific genetic variation e.g. adaptive germination and dormancy traits (Carasso *et al.* 2011, 2012) of high value for maintenance of the species in a long-term evolutionary perspective.

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References

Ægisdóttir, H.H., Kuss, P. & Stöcklin, J. (2009) Isolated populations of a rare alpine plant show high genetic diversity and considerable population differentiation. *Annals of Botany* 104: 1313–1322.

Aeschimann, D., Rasolofo, N. & Theurillat, J.P. (2011) Analyse de la flore des Alpes. 1: historique et biodiversité. *Candollea* 66: 27–55.

Ambrožová, K., Mandáková, T., Bureš, P., Neumann, P., Leitch, I.J., Koblizkova, A., Masac, J. & Lysak, M.A. (2011) Diverse retrotransposon families and an AT-rich satellite DNA revealed in giant genomes of *Fritillaria* lilies. *Annals of Botany* 107: 255–268.

Andrianoelina, O., Favreau, B., Ramamonjisoa, L. & Bouvet, J.M. (2009) Small effect of fragmentation on the genetic diversity of *Dalbergia monticola*, an endangered tree species of the eastern forest of Madagascar, detected by chloroplast and nuclear microsatellites. *Annals of Botany* 104: 1231–1242.

Bandelt, H. J., Forster, P. & Roehl, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37–48.

Bartolucci, F. & Peruzzi, L. (2012) Typification of *Fritillaria tubiformis* Gren. & Godr., *Fritillaria delphinensis* f. *moggridgei* Planch. and *Fritillaria delphinensis* var. *burnatii* Planch. (Liliaceae) from SW Europe. *Candollea* 67: 23–29.

Booy, G., Hendriks, R.J.J., Smulders, M.J.M., Van Groenendael, J.M. & Vosman, B. (2000) Genetic diversity and the survival of populations. *Plant Biology* 2: 379–395.

Carasso, V., Fusconi, A., Hay, F.R., Dho, S., Gallino, B. & Mucciarelli, M. (2012) A threatened alpine species, *Fritillaria tubiformis* subsp. *moggridgei*: seed morphology and temperature regulation of embryo growth. *Plant Biosystems* 146: 74–83.

Carasso, V., Hay, R.F., Probert, J.R. & Mucciarelli, M. (2011) Temperature control of seed germination in *Fritillaria tubiformis* subspecies *moggridgei (Liliaceae)* a rare endemic of the south-west Alps. *Seed Science Research* 21: 33–38.

Casazza, G., Barberis, G. & Minuto, L. (2005) Ecological characteristics and rarity of endemic plants of the Italian Maritime Alps: the relationship between habitats and endemic/endangered taxa. *Biological Conservation* 123: 361–371.

Casazza, G., Zappa, E., Mariotti, M.G., Médail, F. & Minuto, L. (2008) Ecological and historical factors affecting distribution pattern and richness of endemic plant species: the case of the Maritime and Ligurian Alps hotspot. *Diversity and Distributions* 14: 47–58.

Charpin, A. & Salanon, R. (1985) *Materiaux pour la flore des Alpes maritimes: catalogue de l'herbier d'Emile Burnat déposé au Conservatoire botanique de la ville de Genève. 1: Lycopodiaceae-Lentibulariaceae*. Editions des Conservatoire et Jardin botaniques de la Ville de Genève, Genève, 258 pp.

Conti, F., Abbate, G., Alessandrin, A. & Blasi, C. (2005) *An annotated checklist of the Italian vascular flora.* Palombi, Roma, 420 pp.

Daniell, H., Lee, S., Grevich, J., Saski, C., Quesada-Vargas, T., Guda, C., Tomkins, J. & Jansen, R.K. (2006) Complete chloroplast genome sequences of *Solanum bulbocastanum, Solanum lycopersicum* and comparative analyses with other Solanaceae genomes. *Theoretical and Applied Genetics* 112: 1503–1518.

Ebert, D. & Peakall, R. (2009) A new set of universal *de novo* sequencing primers for extensive coverage of noncoding chloroplast DNA: new opportunities for phylogenetic studies and cpSSR discovery. *Molecular Ecology Resources* 9: 777–1075.

Edh, K., Widén, B. & Ceplitis, A. (2007) Nuclear and chloroplast microsatellites reveal extreme population differentiation and limited gene flow in the Aegean endemic *Brassica cretica* (Brassicaceae). *Molecular Ecology* 16: 4972–4983.

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47-50.

Excoffier, L., Smouse, P.E. & Quattro, J.M. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.

Fay, M.F., Bone, R., Cook, P., Kahandawala, I., Greensmith, J., Harris, S., Pedersen, H.A.E., Ingrouille, M.J. & Lexer, C. (2009) Genetic diversity in *Cypripedium calceolus* (Orchidaceae) with a focus on north-western Europe, as revealed by plastid DNA length polymorphisms. *Annals of Botany* 104: 517–525.

Fay, M.F., Chase, M.W., Rønsted, N., Devey, D.S., Pillon, Y., Pires, J.C., Petersen, G., Seberg, O. & Davis, J. (2006) Phylogenetics of *Liliales*: summarized evidence from combined analyses of five plastid and one mitochondrial loci. *Aliso* 22: 559–565.

Fay, M.F., Cowan, R.S. & Leitch, I.J. (2005) The effects of genome size on the quality and utility of AFLP fingerprints. *Annals of Botany* 95: 237–246.

Fischer ex J.E., Alexander (1829) Notice regarding the Salt Lake Inder, in Asiatic Russia. Communicated by Lieutenant J. E. Alexander, 16th Lancers, K. L. S. M. R. A. S. Corres. Mem. S. A. E., & c. *Edinburgh New Philos. J.* 8: 18–20.

Frankham, R. (1995) Conservation genetics. *Annual Review of Genetics* 29: 305–327.

Garza, J.C. & Williamson, E.G. (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology* 10: 305–318.

Goldstein, D.B., Ruiz Linares, A., Cavalli-Sforza, L.L. & Feldman, M.W. (1995) Genetic absolute dating based on microsatellites and the origin of modern humans. *Proceedings of the National Academy of Sciences of the United States of America* 92: 6723–6727.

Goudet, J. (2011): FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www.unil.ch/izea/softwares/fstat.htlm (accessed: 10 October 2009)

Graham, S.W., Reeves, P.A., Burns, A.C.E. & Olmstead, R.G. (2000) Microstructural changes in noncoding chloroplast DNA: interpretation, evolution, and utility of indels and inversions in basal angiosperm phylogenetic inference. *International Journal of Plant Sciences* 161: S83–S96.

Grassi, F., Minuto, L., Casazza, G., Labra, M. & Sala, F. (2009) Haplotype richness in refugial areas: phylogeographical structure of *Saxifraga callosa*. *Journal of Plant Research* 122: 377–387.

Grenier, J.C.M. & Godron, D.A. (1855) Notices botaniques sur quelques plates des genres *Fritillaria*, *Asphodelus* et *Melica*. *Mémoires de la Société d'Emulation du Département du Doubs* 6: 12-15 pp.

Grenier, M. & Godron, M. (1855) Flore de France ou description des plantes qui croissant naturellement en France et en Corse, tome troisième, premièr partie. Dodivers et C^{e}, Besançon, 779 pp.

Hamilton, M.B., Braverman, J.M. & Soria-Hernanz, D.F. (2003) Patterns and relative rates of nucleotide and insertion/deletion evolution at six chloroplast intergenic regions in new world species of the Lecythidaceae. *Molecular Biology and Evolution* 20: 1710–1721.

Hardie, D.C. & Hutchings, J.A. (2010) Evolutionary ecology at the extremes of species' ranges. *Environmental Reviews* 18: 1–20.

Heywood, V.H. (1978) Flora Europaea, notulae systematicae ad floram Europaeam spectantes. *Botanical Journal of the Linnean Society* 76: 297–384.

Hodgins, K.A. & Barrett, S.C.H. (2007) Population structure and genetic diversity in tristylous *Narcissus triandrus*: insights from microsatellite and chloroplast DNA variation. *Molecular Ecology* 16: 317–332.

Honjo, M., Kitamoto, N., Ueno, S., Tsumura, Y., Washitani, I. & Ohsawa, R. (2009) Management units of the endangered herb *Primula sieboldii* based on microsatellite variation among and within populations throughout Japan. *Conservation Genetics* 10: 257–267.

Hughes, M. & Hollingsworth, P.M. (2008) Population genetic divergence corresponds with species-level biodiversity patterns in the large genus *Begonia*. *Molecular Ecology* 17: 2643-2651.

Ingvarsson, P.K., Ribstein, S. & Taylor, D.R. (2003) Molecular evolution of insertions and deletion in the chloroplast genome of *Silene*. *Molecular Biology and Evolution* 20: 1737–1740.

Iwata, H. (2004) *PCO ver.1.0.* Available from *URL:* http://cse.naro.affrc.go.jp/iwatah/others/pco/index.html (accessed: 20 October 2010)

Jo, Y.D., Park, Y., Kim, J., Song, W., Hur, C.G., Lee, Y.H. & Kang, B.C. (2011) Complete sequencing and comparative analyses of the pepper (*Capsicum annuum* L.) plastome revealed high frequency of tandem repeats and large insertion/deletions on pepper plastome. *Plant Cell Reports* 30: 217–229.

Jones, B., Gliddon, C. & Good, J.E.G. (2001) The conservation of variation in geographically peripheral populations: *Lloydia serotina* (Liliaceae) in Britain. *Biological Conservation* 101: 147–156.

Kalinowski, S.T. (2004) Counting alleles with rarefaction: Private alleles and hierarchical sampling designs. *Conservation Genetics* 5: 539–543.

Kelchner, S.A. (2000) The evolution of non-coding chloroplast DNA and its application in plant systematics. *Annals of the Missouri Botanical Garden* 87: 482–498.

Ker Gawler, J.B. (1809) *Fritillaria racemosa*, (α) oriental fritillary. *Curti's Botanical Magazine or, Flower Garden Displayed* 30: pl. 1216.

Kitner, M., Majeský, L., Gillová, L., Vymyslický, T. & Nagler, M. (2012) Genetic structure of *Artemisia pancicii* populations inferred from AFLP and cpDNA data. *Preslia* 84: 97–120.

Lehtonen, S., Myllys, L. & Huttunen, S. (2009) Phylogenetic analysis of non-coding plastid DNA in the presence of short inversions. *Phytotaxa* 1: 3–20.

Li, S., Hu, K., Guo, J., Yang, X., Zhu, Y. & Cheng, Z. (2011) Genetic diversity and relationship of *Fritillaria thunbergii* Miq. landraces and related taxa. *Biochemical Systematics and Ecology* 39: 725–731.

Linnaeus, C. (1753) *Species plantarum*. Salvius, Stockholm, 560 pp.

Makino, T. (1913) Observations on the flora of Japan. *Botanical Magazine Tokyo* 27: 124–127.

Marschall von Bieberstein, F.A. (1808) *Flora Taurico-Caucasica exhibens stirpes phaenogamas, in Chersoneso Taurica et regionibus caucasicis sponte crescentes.* Charkouiae, Typis Academicis, 478 pp.

McGlaughlin, M.E. & Friar, E.A. (2011) Evolutionary diversification and geographical isolation in *Dubautia laxa* (Asteraceae), a widespread member of the Hawaiian silversword alliance. *Annals of Botany* 107: 357–370.

Médail, F. & Verlaque, P. (1997) Ecological characteristics and rarity of endemic plants from southeast France and Corsica: implications for biodiversity conservation. *Biological Conservation* 80: 269–281.

Micheneau, C., Duffy, K.J., Smith, R.J., Stevens, L.J., Stout, J.C., Civeyrel, L., Cowan, R.S. & Fay, M.F. (2010) Plastid microsatellites for the study of genetic variability in the widespread *Cephalanthera longifolia*, *C. damasonium* and *C. rubra* (Neottieae, Orchidaceae), and cross-amplification in other *Cephalanthera* species. *Botanical Journal of the Linnean Society* 163: 181–193.

Minuto, L., Grassi, F. & Casazza, G. (2006) Ecogeographic and genetic evaluation of endemic species in the Maritime Alps: the case of *Moehringia lebrunii* and *M. sedoides* (Caryophyllaceae). *Plant Biosystems* 140: 146–155.

Miquel, F.A.W. (1867) *Fritillaria*, in Prolusio florae Japonicae. *Annales Musei Botanici Lugduno-Batavi* 3: 157–158.

Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the United States of America* 70: 3321–3323.

Nei, M. (1987) *Molecular evolutionary genetics*. Columbia University Press, New York, 512 pp.

Nei, M., Tajima, F. & Tateno, Y. (1983) Accuracy of estimated phylogenetic trees from molecular data. 2. Gene-frequency data. *Journal of Molecular Evolution* 19: 153–170.

Ouborg N.J., Vergeer, P. & Mix, C. (2006) The rough edges of the conservation genetics paradigm for plants. *Journal of Ecology* 94: 1233–1248.

Planchon, J.E. (1873) Sur les espèces de fritillaries de France, a propos des icons et d'un manuscrit inédit de Pierre de Belleval. *Bulletin de la Société Botanique de France* 20: 96–124

Pons, O. & Petit, R.J. (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. *Genetics* 144: 1237–1245.

Provan, J., Powell, W. & Hollingsworth, P. (2001) Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends in Ecology & Evolution* 16**:** 42–147.

Rix, M. (2001) *Fritillaria: a revised classification, together with an updated list of species*. Alpine Garden Society, Worcestershire, 14 pp.

Rønsted, N., Law, S., Thornton, H., Fay, M.F., Chase, M.W. (2005) Molecular phylogenetic evidence for the monophyly of *Fritillaria* and *Lilium* (Liliaceae; Liliales) and the infrageneric classification of *Fritillaria*. *Molecular Phylogenetics and Evolution* 35: 509-527.

Rouy, G. (1910) *Flore de France ou description des plantes qui croissant spontanément en France, en Corse et en Alsace-Lorraine*, tome douzième. Société de sciences naturelles de la Charente Inférioire, Paris, 505 pp.

Royle, J.F. (1839) Illustrations of the botany and other branches of the natural history of the Himalayan Mountains and the flora of Cashmere, V. 2 plates. Allen, London, 97 pp.

Schrenk, A. G. von (1841) *Enumeratio plantarum novarum a cl. Schrenk lectarum* / [F.E.L. Fischer, C.A. Meyer]. Fischer, Petrópolis, 113 pp.

Schroeder, H., Hoeltken, A.M. & Fladung, M. (2012) Differentiation of *Populus* species using chloroplast single nucleotide polymorphism (SNP) markers – essential for comprehensible and reliable poplar breeding. *Plant Biology* 14: 374–381.

Schwienbacher, E., Marcante, S. & Erschbamer, B. (2010) Alpine species seed longevity in the soil in relation to seed size and shape – A 5-year burial experiment in the central Alps. *Flora* 205: 19–25.

Shaw, J., Lickey, E.B., Schilling, E.E. & Small, R.E. (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany* 94: 275–288.

Slatkin, M. (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139: 457–462.

Stapf, O. (1934) *Lilium*, *Notholirion* and *Fritillaria*. *Bulletin of Miscellaneous Information* (Royal Gardens, Kew) 1934: 94–96

Sweet, R. (1826) *Sweet's hortus Britannicus: or a catalogue of plants cultivated in the gardens of Great Britain, arranged in natural orders*. Ridgway, London, 524 pp.

Szövényi, P., Arroyo, K., Guggisberg, A. & Conti, E. (2009) Effects of Pleistocene glaciations on the genetic structure of *Saxifraga florulenta* (Saxifragaceae), a rare endemic of the Maritime Alps. *Taxon* 58: 532–543.

Taberlet, P., Gielly, L., Pautou G. & Bouvet, J. (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

Takahashi, Y., Takahashi, H. & Maki, M. (2011) Comparison of genetic variation and differentiation using microsatellite markers among three rare threatened and one widespread toad lily species of *Tricyrtis* section *Flavae* (Convallariaceae) in Japan. *Plant Species Biology* 26: 13–23.

Thompson, J.D., Lavergne, S., Affre, L., Gaudel, M. & Debussche, M. (2005) Ecological differentiation of Mediterranean endemic plants. *Taxon* 54: 967–976.

Wallich, N. (1824) *Tentamen florae Nepalensis illustratae*, fasc. I, tav. 12–13. The Asiatic Lithographic Press, Calcutta, 117 pp.

Weir, B.S. & Cockerham, C.C. (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution* 38: 1358–1370.

Wickström, J.E. (1821) Beskrifning af tvenne nya arter af växtslägtet *Fritillaria*, jemte anmärkningar om åtskilliga arter af samma slägte. *Kungliga Vetenskaps Academiens Handlingar* 1821: 350-359.

Xu, T.T., Chen, J.A., Zhu, L.L. & Li, Z.H. (2011) Development of microsatellite loci for *Aconitum gymnandrum* (Ranunculaceae), a species endemic to the Qinghai-Tibetan plateau. *American Journal of Botany* 98: e7–e9.

Yamagishi, M., Nishioka, M. & Kondo, T. (2010) Phenetic diversity in the *Fritillaria camschatcensis* population grown on the Sapporo campus of Hokkaido University. *Landscape and Ecological Engineering* 6: 75–79.

TABLE 1. Populations studied for *Fritillaria tubiformis* s.l.

Acronym	#	Subspecies	Locality*	Latitude (N)	Longitude (E)	Elevation (m)	Sample size§
CRA	1	*F. t. moggridgei*	Vallone di Cravina (CN)	44°14′	007°38′	1420-1540	24
DRO	2	*F. t. burnatii*	Droneretto (CN)	44°31′	007°12′	1700-1800	24
FRO	3	*F. t. moggridgei*	Monte Frontè (IM)	44°03′	007°45′	1980-1890	24
GAL	4	*F. t. moggridgei*	Monte Galero (CN)	44°09′	008°00′	1690	22
MAR	5	*F. t. moggridgei*	Vallone del Marguareis (CN)	44°11′	007°40′	1430	24
PLU	6	*F. t. moggridgei*	Pian del Lupo (CN)	44°11′	007°41′	1990	23
PRA	7	*F. t. burnatii*	S. Michele di Prazzo (CN)	44°31′	007°02′	1960-2020	24
ROS	8	*F. t. burnatii*	Alpe Pian Rosso (CN)	44°09′	007°46′	1800-1910	24
SAC	9	*F. t. moggridgei*	Monte Saccarello (IM)	44°04′	007°43′	1900	24
SAM	10	*F. t. moggridgei*	Sambuco (CN)	44°20′	007°05′	1432	24
SER	11	*F. t. burnatii*	Vallone di Serpentera (CN)	44°13′	007°41′	1890-2000	23

*The two-letter code after localities indicates the administrative province (CN = Cuneo, Piedmont; IM = Imperia, Liguria). Numbers (#) correspond to the sampled sites of Fig.1. §Sample size refers to the number of individuals for which haplotype data were collected.

TABLE 2. Details of the plant and GenBank accessions used in the phylogenetic study.

	Taxa	Voucher information	*matK*	*rpl16*
	Subgenus *Fritillaria*			
	Section *Olostylea*			
1	*F. caucasica* Adam	Chase 3488 (K), Kew 1989-1112	AY624432	AY624378
2	*F. alburyana* Rix	Chase 3470 (K), Kew1994-3139	AY624429	AY624375
	Section *Fritillaria*			
3	*F. aurea* Schott	Chase 3487 (K), Kew 1973-21448	AY624430	AY624376
4	*F. acmopetala* Boissier	Chase 2565 (K), Kew 1959-59401	AY624426	AY624372
5	*F. hermontis* Fenzl subsp. *amana* Rix	Chase 2563 (K), Kew 1974-2043	AY624440	AY624387
6	*F. crassifolia* Boissier & Huet du Pavillon. subsp. *kurdica* (Boissier & Noë) Rix	Chase 2559 (K), Kew1985-926	AY624432	AY624378
7	*F. michailowskyi* Fomin	Chase 2583 (K), Kew1981-3060	AY624434	AY624380
8	*F. minuta* Boissier & Noë	Chase 2562 (K), Kew1978-3492	AY624448	AY624396
9	*F. olivieri* Baker	Chase 2569 (K), Kew1963-51207	AY624449	AY624397
10	*F. reuteri* Boissier	Chase 2568 (K), Kew1969-6106	AY624456	AY624404
11	*F. tubiformis* Grenier & Godron	Chase 2558 (K), Kew	AY624459	AY624408
12	*F. tubiformis* Grenier & Godron	Chase 2558 (K), Kew	This paper	This paper
13	*F. t. burnatii*	population SER; see Table 1	This paper	This paper
14	*F. tubiformis* Grenier & Godron subsp. *moggridgei* (Boissier & Reuter ex Planchon) Rix	population MAR; see Table 1	This paper	This paper
15	*F. tubiformis* Grenier & Godron subsp. *moggridgei* (Boissier & Reuter ex Planchon) Rix	population CRA; see Table 1	This paper	This paper
16	*F. tubiformis* Grenier & Godron subsp. *moggridgei* (Boissier & Reuter ex Planchon) Rix	population FRO; see Table 1.	This paper	This paper
17	*F. lusitanica* Wickström	Chase 2603 (K), Kew1980-3020	AY624443	AY624391
18	*F. meleagris* Linnaeus	Chase 2566 (K), Kew1990-3088	AY624445	AY624393
19	*F. tenella* Marschall von Bieberstein.	Chase 2561 (K), Kew1955-12701	AY624458	AY624407
20	*F. pallidiflora* Schrenk	Chase 2567 (K), Kew1959-1103	AY624450	AY624398

TABLE 2. (continued)

	Taxa	Voucher information	*matK*	*rpl16*
	Subgenus *Japonica* Rix			
21	*F. japonica* Miquel *var. koidzumiana* (Ohwi) Hara & Kanai	AY624442	AY624389	AY624442
	Subgenus *Rhinopetalum* Fisch			
22	*F. gibbosa* Boissier	Photo M. W. Chase	AY624438	AY624385
23	*F. karelinii* (Fischer ex. D. Don) Baker	Chase 3461 (K), Kew1994-218	–	AY624390
	Subgenus *Petilium* Ludwig			
24	*F. raddeana* Regel	Chase 745 (K), Kew1973-54	AY624454	AY624402
25	*F. imperialis* Linneus	Chase 2557 (K), Kew1970-3943	AY624441	AY624388
26	*F. chitralensis* B. Mathew	Chase 3472 (K), Kew1970-4109	AY624433	AY624379
	Subgenus *Karolkowia* Rix			
27	*F. serwerzowii* Regel	Chase 743 (K), Kew1933-Hoog	AY624457	AY624405
	Subgenus *Theresia* Koch			
28	*F. persica* Linneus	Chase 3496 (K), Kew1923-41201	AY624451	AY624399
	Subgenus *Davidii* Rix			
29	*F. davidii* Franchet	B. Mathew 2000. Bulblets.	AY624435	AY624381
	Outgroups			
30	*Notholirion thomsonianum* (Royle) Stapf	Chase 448 (K), Kew 1974-025	AB024393	AY624424
31	*Cardiocrinum giganteum* (Wall.) Makino	Chase 3689 (K), Kew1988-4907	AY624469	AY624425

Classification of *Fritillaria* according to Rix (2001). Sample numbers (#) in first column. For other information on species and vouchers see Rønsted *et al.* (2005). GenBank accession numbers as in http://www.ncbi.nlm.nih.gov.

TABLE 3. Primer sequences used to amplify ten variable regions of plastid DNA in *Fritillaria tubiformis s.l.*

Region	Internal primers	Primer sequences 5'-3'	References	Original primer references
trnL-trnF	trnL-F Frit F trnL F	F: GCCCATTTTAAGTACTAAC R:AATTGAACTGGTGACACGAG*	This paper -	*primer of the region in Taberlet *et al.* 1991
psbK-trnS(GCU)	psbK F psbK R	F: TGTTTGGCAAGCTGCTGTAA* R: GAAGAGTTTGAGAGTAAGC	- This paper	*primer of the region in Ebert & Peakall 2009
trnE(UUC)-trnT(GGU)	AB1 F AB1 R	F: CAGATGAGATATGCCTATC R: GAGCTCATTATCAACATAGA	This paper "	Ebert & Peakall 2009
rpoC1exon2-rpoC1exon1	BC2 F BC2 R	F: CCCGGTAAGAACCGATTTTT R: ATCATGACATAACCATCGGG	This paper "	Ebert & Peakall 2009
trnP(UGG)-petE/G	HI5 F HI5 R	F: AACTGAACTAAGAGCGCTT R: GCATACTATACTAGGTGTTTG	This paper "	Ebert & Peakall 2009
clpPexon1-psbB	LM6 2F LM6 2R	F: CAATACGCAATGGGGGTTGG R: ACGCACGGGTCGATCATTTG	This paper "	Ebert & Peakall 2009
psbD-trnT(GGU)	SI7 F psbD Frit R	F: GCCGCAAAACGACCTAACC R:AGGGGTTTTCATTTTCAGCC	This paper "	Shaw *et al.* 2007
rpl33-rps18	DE3 F DE3 R	F: CCACTTTGGCCGKATCTTAA R: AGAGAGCTTGGATTTACGAA	This paper "	Ebert & Peakall 2009
petA-psbJ	FG 4 1F FG4 1R	F: GAAATAACAAAAGGGGGATC R: TGAGCAAATGGAACTTCTTC	This paper "	Ebert & Peakall 2009
petA-psbJ	FG 4 2F FG4 2R	F: GAAGAAGTTCCATTTGCTCA R: CTATCTTTTTCGTGTCTATCG	This paper "	Ebert & Peakall 2009

TABLE 4. Characterization of the five microsatellites and five indels of *Fritillaria tubiformis s.l.*

Locus	Gene	Type of repeat motif	Repeat motif	Size range (bp)	Fragment length* (bp)	Alleles
1	*trnL-trnF*	polyT	T	16-25	235-245	1,2,3,4,5,6,7,8,9,10
2	*psbK-trnS*	polyA	A	13-26	181-194	1,2,3,4,5,6,7,8,9,10,11,12,13
3	*trnE-trnT*	polyT	T	13-18	153-158	1,2,3,4,5,6
4	*rpoC1ex2-ex1*	repeat indel	$(ACTTT)_2$	5/10	257/262	1,2
5	*trnP-petE/G*	polyA	A	6-12	97-103	1,2,3,4,5,6,7
6	*clpPex1-psbB*	polyT	T	8-11	86-89	1,2,3,4
7	*psbD-trnT*	repeat indel	~	0/17/30	211/228/241	1,2,3
8	*rpl33-rps18*	repeat indel	$(TTTTAGAAA)_2$	9/18	101/110	1,2
9	*petA-psbJ*	indel I (from 5′ end)	ATTCTTT	0/7	140/147	1,2
10	*petA-psbJ*	indel II (from 5′ end)	CCGAA	0/5	115/120	1,2

Name of the gene, type of repeat, motif and size in base pairs, total fragment length (bp) and alleles found are given for each locus. * For indels and repeats the total length of the corresponding sequenced fragment is given; otherwise, it refers to the size range (bp) of the microsatellites.

TABLE 5. Overall gene diversity analysed locus by locus.

Locus	A_{tot}	A_{pop}	$r_{(21)}$	H_t	H_s	D_{st}
1	10	2 .82	7 .796	0 .838	0 .395	0 .444
2	13	3 .55	10.337	0 .847	0 .436	0 .411
3	6	2 .27	5 .938	0 .773	0 .444	0 .329
4	2	1 .00	2 .000	0 .463	0 .000	0 .463
5	7	2 .18	6 .124	0 .741	0 .234	0 .506
6	4	1 .64	3 .983	0 .694	0 .244	0 .450
7	3	1 .09	2 .575	0 .486	0 .031	0 .455
8	2	1 .00	2 .000	0 .463	0 .000	0 .463
9	2	1 .00	2 .000	0 .463	0 .000	0 .463
10	2	1 .00	2 .000	0 .463	0 .000	0 .463
Average ± s.d.	-	-	-	0 .623 (0 .040)	0 .179 (0 .042)	0 .445 (0 .021)

* For locus definition see Table 2. A_{tot} = total number of alleles; A_{pop} = average number of alleles per population; $r_{(21)}$ = allelic richness; H_t = total gene diversity; H_s = average within population gene diversity; D_{st} = gene diversity among populations.

TABLE 6. The overall gene diversity of *F. tubiformis s.l.* populations as estimated by a series of standard diversity indices.

	Populations										
Gene diversity indices	ROS	SER	DRO	PRA	GAL	FRO	SAC	SAM	PLU	MAR	CRA
No. of haplotypes	7 .0	7 .0	8 .0	5 .0	3 .0	4 .0	6 .0	8 .0	6 .0	3 .0	9 .0
No. of polymorphic loci (*N*)	5 .0	4 .0	4 .0	4 .0	4 .0	5 .0	5 .0	3 .0	4 .0	2 .0	3 .0
Allele proportion (*P*)	0 .50	0 .40	0 .40	0 .40	0 .40	0 .50	0 .50	0 .30	0 .44	0 .20	0 .33
Average no. of alleles per locus (*A*)	3 .00	2 .50	1 .75	2 .75	1 .25	2 .00	2 .60	5 .33	2 .80	1 .00	2 .25
Allele range (A_{poly})	3 .20	3 .00	2 .75	3 .00	2 .25	2 .60	2 .20	4 .33	2 .80	2 .00	3 .25
Proportion of private alleles ($f_{private}$)	0 .000	0 .154	0 .083	0 .000	0 .100	0 .077	0 .000	0 .067	0 .071	0 .000	0 .000
Gene diversity across loci (*H*)	0 .251	0 .169	0 .223	0 .157	0 .105	0 .140	0 .121	0 .209	0 .272	0 .055	0 .228
Allelic richness ($r_{(21)}$)	11 .15	9 .35	9 .34	7 .14	5 .82	6 .34	6 .34	13 .18	10 .47	1 .88	12 .84
G-W index	0 .927	0 .900	1 .000	0 .917	1 .000	0 .900	0 .860	0 .833	0 .817	1 .000	1 .000
Genetic divergence (G_{st})	0 .149	0 .136	0 .126	0 .198	0 .213	0 .181	0 .196	0 .116	0 .124	0 .323	0 .111

*For explanation of the populations acronyms see Table 1. *P* is the proportion of polymorphic loci (polymorphic over usable loci); A_{poly} is the average number of alleles over polymorphic loci; $f_{private}$ is the proportion of alleles unique to that particular population; $r_{(21)}$ is the allelic richness. For explanations of the remaining genetic parameters, see Materials and Methods.

TABLE 7. Overall gene diversity within the two subspecific taxa of *F. tubiformis s.l.* as estimated by a series of standard diversity indices.

Gene diversity indices	subsp. *moggridgei*	var. *burnatii*
No. of haplotypes	32.00	24.00
Proportion of exclusive haplotypes (%)	100.0	100.0
No. of polymorphic loci (*N*)	6.000	5.000
Average no. of alleles per locus (*A*)	3.400	2.900
Allelic range (*R*)	4.500	4.200
Proportion of private alleles ($f_{private}$)	0.045	0.059
Gene diversity (H_t)	0.998	0.998
Gene diversity across loci (*H*)	0.325	0.329
Average within population gene diversity (H_s)	0.805	0.870
G-W index	0.961	0.942
Genetic divergence (G_{st})	0.193	0.128

**R* is the difference between the maximum and minimum number of repeats at all loci; $f_{private}$ is the proportion of alleles unique to each subspecies. For explanations of the remaining genetic parameters, see Materials and Methods.

TABLE 8. Distribution of plastid DNA variation among and within the seven populations of subsp. *moggridgei* (upper part) and when the analysis was implemented with the addition of four var. *burnatii* populations (lower part) as estimated by analysis of molecular variance (AMOVA).

	Sum of squares		Variation components			Variation (%)		Fixation indices	
Source of variation	F_{ST}	R_{ST}	F_{ST}	R_{ST}	d.f.	F_{ST}	R_{ST}	F_{ST}	R_{ST}
Among populations	137.44	676.20	0.938	4.631	6	54.06	56.57	F_{ST} = 0.541***	R_{ST} = 0.566***
Within populations	125.95	561.73	0.797	3.555	158	45.94	43.43		
Total	263.39	1237.93	1.735	8.186	64	100.00	100.00		
Source of variation	F_{ST}	R_{ST}	F_{ST}	R_{ST}	d.f.	F_{ST}	R_{ST}	F_{ST}	R_{ST}
Between subspecies	383.30	3545.10	2.992	28.328		62.53	75.28	F_{CT} = 0.625**	R_{CT} = 0.753**
Among populations within subspecies	202.31	1161.37	0.914	5.291		19.11	14.06	F_{SC} = 0.510***	R_{SC} = 0.569***
Within populations	218.75	998.60	0.879	4.010	ϑ	18.36	10.66	F_{ST} = 0.816***	R_{ST} = 0.893***
Total	804.36	5705.08	4.785	37.630	ϑ	100.00	100.00		

** 0.001 < P < 0.01; ***P < 0.0001

TABLE 9. Comparison of the distribution of plastid DNA variation among and within populations of *F. tubiformis s.l.* estimated with locus by locus analysis of molecular variance (AMOVA) employing the number of different alleles (F_{ST}) and the sum of square size differences (R_{ST}) as a distance method.

	Among populations		Within populations				
Source of variation	Variation (%)		Variation (%)		Fixation indices		
Locus	F_{ST}	R_{ST}	F_{ST}	R_{ST}	F_{ST}	R_{ST}	P^*
1	19.32	61.89	80.68	38.11	0.193	0.619	<0.0001
2	18.98	72.43	81.02	27.57	0.190	0.724	<0.0001
3	16.93	69.45	83.07	30.55	0.169	0.695	<0.0001
4	100.00	100.00	0.00	0.00	1.000	1.000	<0.0001
5	46.62	94.57	53.38	5.43	0.466	0.946	<0.0001
6	44.53	78.48	55.47	21.52	0.445	0.785	<0.0001
7	96.27	99.04	3.73	3.73	0.963	0.990	<0.0001
8	100.00	100.00	0.00	0.00	1.000	1.000	<0.0001
9	100.00	100.00	0.00	0.00	1.000	1.000	<0.0001
10	100.00	100.00	0.00	0.00	1.000	1.000	<0.0001
Average over loci	65.98	77.80	34.02	22.20	0.660	0.778	<0.0001

For locus definition see Table 4. * The significance threshold was determined by 10100 permutations.

Figure legends.

FIGURE 1. Map of the Italian populations of *F. tubiformis s.l.* Populations belonging to var. *burnatii* are labelled with a star symbol and those belonging to subsp. *moggridgei* with a solid circle. Most of the sampled populations are located at the boundary between Piedmont (P) and Liguria (L) (Italy). Insets show, top left, sampling sites numbered as for populations (see Table 1) and, top right, the position of the sampled area within the Alps.

FIGURE 2. Strict consensus tree of more than 2600 most parsimonious trees from analysis of the combined plastid *matK* and *rpl16* intron sequences. Tree length = 451 steps, CI = 0.89 and RI = 0.85. Bootstrap percentages (> 50%) are indicated above branches. *Cardiocrinum giganteum* and *Notholirion thomsonianum* are chosen as the outgroups. See also Table 2.

FIGURE 3. Median-joining (MJ) network for the combined plastid DNA data set of *Fritillaria tubiformis s.l.* A. MJ network based on indel repeats at locus 4, 7, 8, 9, 10. Haplotypes (Ha, Hb1 and Hb2) are indicated by the circles, the size of each circle being proportional to the observed frequency of each haplotype. B. MJ network for the combined plastid DNA data set of *Fritillaria tubiformis s.l.* based on all ten microsatellites. Median vectors are labelled mv1, mv2, mv3 and mv4. Number of changes required to explain transitions among haplotypes is indicated along the lines of the networks, except for connections that required only a single change. Information on the type and number of repeat motifs contributing to the network is provided for the two main clusters (A and B) corresponding to var. *burnatii* and subsp. *moggridgei*, respectively.

FIGURE 4. Principal coordinate analysis (PCO) of eleven populations of *Fritillaria tubiformis s.l.* analyzed for ten microsatellite loci. The first (PCO1) and the second (PCO2) axes explain 50.4% and 20.9% of total variation, respectively. Acronyms correspond to populations (see Table 1). The two genetic groups corresponding to the two subspecific taxa are circled.

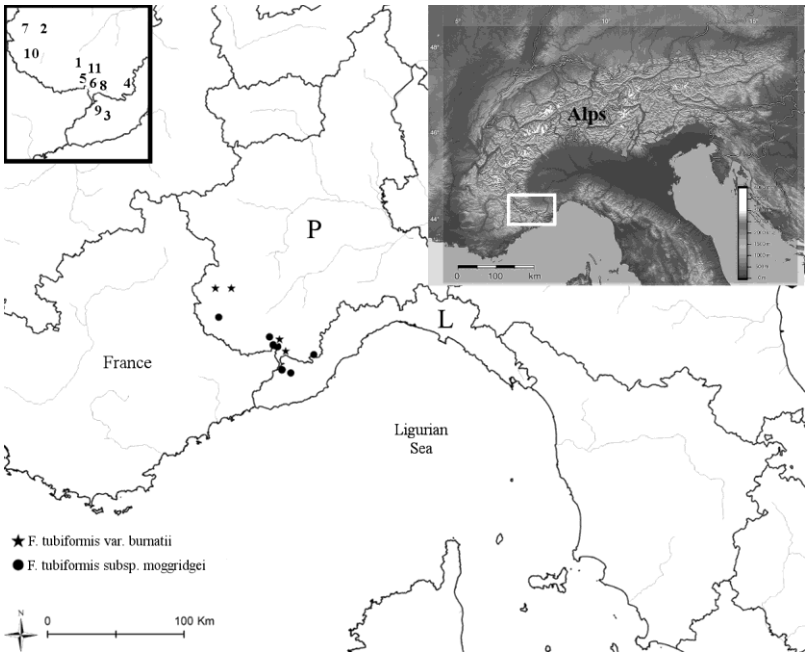
7 2
10
1 11
5 6 8 4
9 3
Alps
P
L
France
Ligurian
Sea
F. tubiformis var. burnatii
F. tubiformis subsp. moggridgei
0
100 Km

Fig. 1

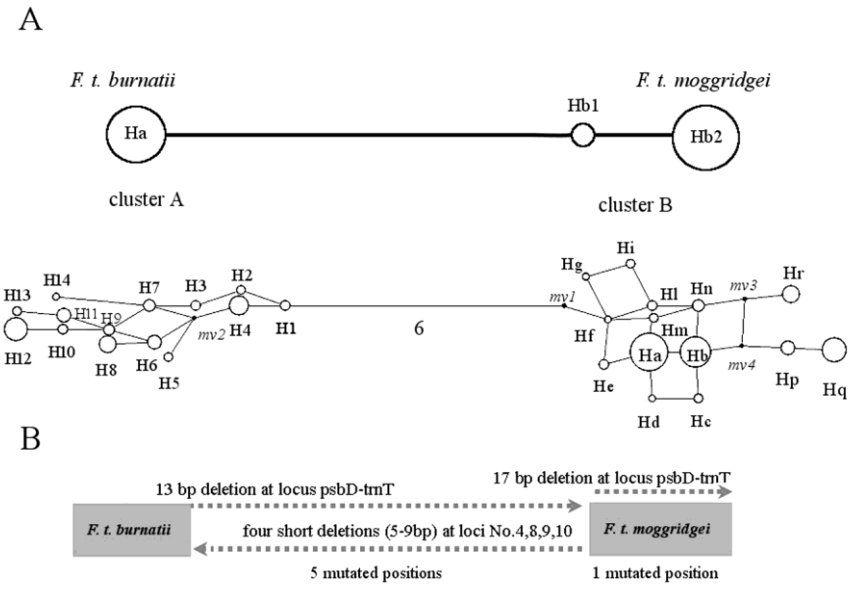
A
F. t. burnatii
F. t. moggridgei
Ha
Hb1
Hb2
cluster A
cluster B
Hi
Hg
Hr
Hl4
H7
H3
H2
Hl3
Hl1
Hl5
mv1
Hl
Hn
mv3
mv2
H4
H1
6
Hf
Hm
Ha
Hb
H12
Hl0
H8
H6
mv4
Hp
Hq
H5
He
Hd
Hc
B
13 bp deletion at locus psbD-trnT
17 bp deletion at locus psbD-trnT
F. t. burnatii
four short deletions (5-9bp) at loci No.4,8,9,10
F. t. moggridgei
5 mutated positions
1 mutated position

Fig. 2

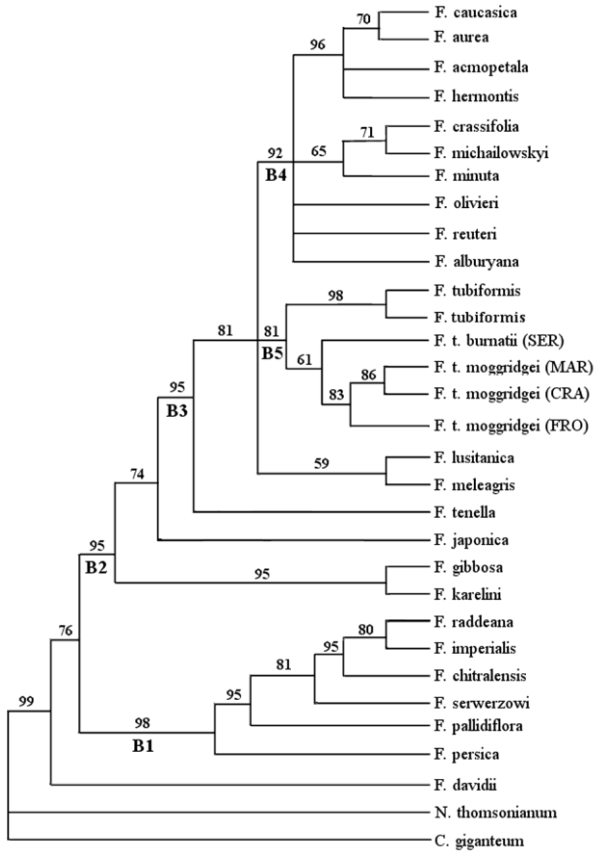
F. caucasica
F. aurea
F. acmopetala
F. hermontis
F. crassifolia
F. michailowskyi
F. minuta
F. olivieri
F. reuteri
F. alburyana
F. tubiformis
F. tubiformis
F. t. burnatii (SER)
F. t. moggridgei (MAR)
F. t. moggridgei (CRA)
F. t. moggridgei (FRO)
F. lusitanica
F. meleagris
F. tenella
F. japonica
F. gibbosa
F. karelini
F. raddeana
F. imperialis
F. chitralensis
F. serwerzowi
F. pallidiflora
F. persica
F. davidii
N. thomsonianum
C. giganteum
B1
B2
B3
B4
B5
70
96
71
92
65
98
81
81
61
86
83
95
59
74
95
95
76
80
95
81
99
95
98

Fig. 3

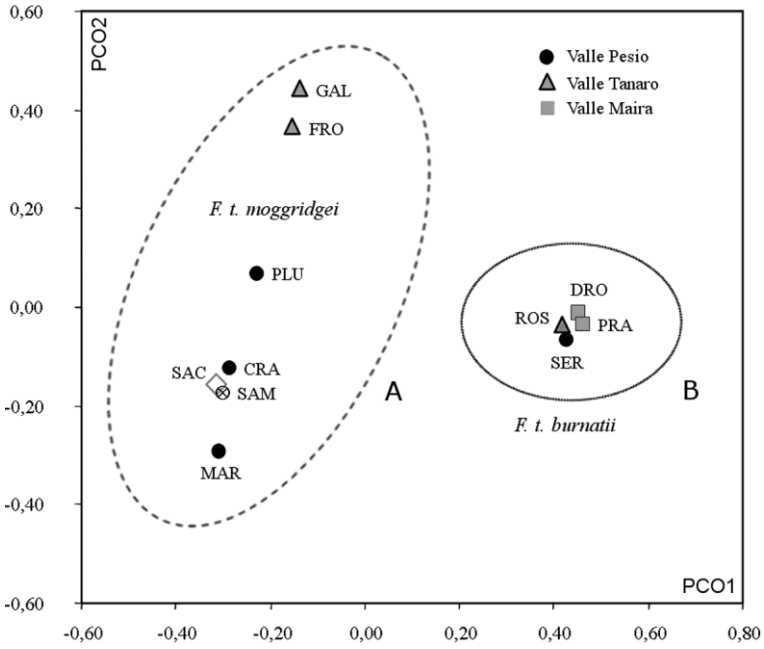
PCO2
PCO1
Valle Pesio
Valle Tanaro
Valle Maira
GAL
FRO
F. t. moggridgei
PLU
SAC
CRA
SAM
MAR
A
DRO
ROS
PRA
SER
B
F. t. burnatii
0,60
0,40
0,20
0,00
-0,20
-0,40
-0,60
-0,60
-0,40
-0,20
0,00
0,20
0,40
0,60
0,80

Fig. 4