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Analysis of genetic structure of *Ranunculus baudotii* in a Mediterranean wetland. Implications for selection of seeds and seedlings for conservation

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ABSTRACT

Seed collection and storage of wild species in *ex-situ* seed banks should be continued as an integrated tool for the conservation of plants in their habitats. Although seed-bank facilities are widely used today, their seed samples often suffer low genetic diversity. Consequently, reintroduced seeds and plant material may not have the resilience to cope with future environmental stress so leading to complete wastage of seeds. Molecular techniques allow the benefit of quantification of the genetic diversity of a seed collection in comparison with that of the natural population. In this study we focus on *ex-situ* seed bank samples and living collections of *Ranunculus peltatus* subsp. *baudotii*. We compare their genetic diversity and structure with that of the natural population before and after undertaking a restoration project on a natural pond in the Tuscan Archipelago National Park. ISSR analyses, carried out on a total of five sampling groups, shows a relatively high level of genetic diversity for the *ex-situ* cultivated groups. The analysis of molecular variance, in agreement with clustering obtained in the neighbour-joining dendrogram and with the pattern from cluster analysis, suggests dividing the samples analysed into two groups: one formed by individuals sampled before the pond restoration and the other formed by the subsequent pond population. The results highlight the importance of planning mixed propagation lines which can be obtained through the use of a range of germination conditions to exploit a novel source of genetic variability which may otherwise remain hidden within the seed collection.

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1. Introduction

Biodiversity decline is of primary concern throughout the world and its conservation presents a critical challenge to the natural sciences (Barnosky et al., 2011). The best place to conserve biodiversity is in the wild, through maintenance or restoration of habitats. Such interventions are sometimes sufficient for plants to recover without the reintroduction of propagules (Bunce et al., 2013). However, the spontaneous recovery of rare-plant populations may not occur under these conditions, as some species are severely dispersal-limited (Clark et al., 2007), and/or have transient seed banks (Thompson et al., 1997) and/or are unable to cope for long with the natural dynamics in the habitat (Stockwell and Ashley, 2004). Thus, restoration plans generally include a number of strategies that can best be considered in the context of contemporary evolution (Stockwell et al., 2006).

Restoration is recognised as a complex and high-risk activity which should include plant propagation and cultivation as well as habitat management. Prior to any restoration activity, the origin, variability and relatedness of the source materials should be considered carefully to avoid significant disadvantages and a further loss of biodiversity (Fenster and Dudash, 1994). However, it is not always the case that the desired plant materials are available in the wild. Thus, the collection of seeds of wild species, and their maintenance in *ex-situ* seed banks, should be included as an integrated tool along with *in habitat* plant conservation. Seed banks are facilities with a high technological content (Rao and Riley, 1994), which should take responsibility for the whole line of seed management all the way through to the reintroduction step (Hay and Probert, 2013).

To conserve seed samples able to be used many years into the future, much effort has been made to maximise the tolerance of desiccation and to predict seed storage behaviour (Merritt et al., 2014). This has important implications for the successful management of seed conservation collections that, along with the extensive literature on the dormancy-breaking and germination requirements of

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wild species, should lead to the development of effective propagation protocols for ecological restoration and species reintroduction (Merritt and Dixon, 2011). However, seed-bank samples often suffer low genetic diversity (Bedini and Carta, 2010; Godefroid et al., 2011) Consequently, introduced seeds and other plant materials may not have sufficient resilience to cope with future environmental stresses so possibly leading to a total wastage of seeds (Merritt and Dixon, 2011).

The identification of source material to ensure genetic similarity of the introduced, to the local populations, may be acquired by conducting an ecogeographic survey (Guarino et al., 2011). However, geographical distance is not always the best indicator of a population's genetic similarity (Allendorf and Luikart, 2007). In this context, a genetic molecular approach can provide the opportunity to identify the source of plant material and also to understand the effective level of genetic diversity of seed collections in respect to the natural population (Lauterbach et al., 2012). Furthermore, this approach should be applied at all stages of plant regeneration from seeds, measuring the genetic variation of living collections obtained through different laboratory germination conditions and/or propagation methods, and eventually providing an assessment of the likely success of the whole restoration protocol (Alonso et al., 2014). In this study, we refer to a restoration experience undertaken in a natural pond in the Tuscan Archipelago National Park involving habitat management (Foggi et al., 2011, 2014) and the constitution of seed bank collections and the cultivation of living plants in the Botanic Gardens of Florence and Pisa (Carta et al., 2012). We focus on these *ex-situ* samples, by comparing their genetic diversity and structures with those of the natural populations before and after restoration. The restoration of the pond was urgently required since the invasion by tall helophytes (such as *Typha latifolia* L., *Typha angustifolia* L. and *Phragmites australis* (Cav.) Trin ex Steud.) had substantially destroyed this fresh-water ecosystem and threatened, at the local scale, species such as *Myriophyllum alterniflorum* Dc. and *Ranunculus peltatus* Schrank subsp. *baudotii* (Godron) Meikle ex C.D.K. Cook (Ranunculaceae; hereinafter *R. baudotii*) (Foggi et al., 2011, 2014).

Restoration activities which included removal of these tall, expansive helophytes (see Foggi et al., 2014 for details), could perhaps have negatively affected the soil seed bank.

To understand the impact of pond restoration on the natural population of *R. baudotii*, its genetic structure was estimated on the basis of Inter Simple Sequence Repeats markers (ISSRs). This method is a PCR-based fingerprinting technique that involves amplification of a DNA segment between two inversely-oriented and closely-spaced microsatellite repeat regions (Reddy et al., 2002). This method is widely used in plant population genetics due to the reliability and reproducibility of the results (Rakoczy-Trojanowska and Bolibok 2004). In particular, the method has been used to assess genetic variation and population differentiation in a number of endangered plant species from a range of locations and climates (Luo et al., 2007).

Thus, our goal is to elucidate the following two questions:

1. Are there genetic differences or divergences between samplings at two temporal stages: before and after of the implementation of restoration activities of the pond?
2. Can a molecular genetic approach be effectively used to evaluate whether selected laboratory germination conditions could be responsible for genetic differences among propagation lines?

With these informations, the relatedness for reintroduction or reinforcement of the *ex-situ* propagated plant material and the practical implication a whenever ecological restoration are discussed.

2. Materials and methods

2.1. Plant materials and sampling protocol

The plants used in this study were sampled by the authors from the natural population of *R. baudotii* located in the Stagnone pond situated on Capraia Island (Tuscan Archipelago, north-central Mediterranean Sea; Fig. 1) at an altitude of 330 m a.s.l. The current population of *R. baudotii* occupies an area of ca. 0.4 ha. Since 1991 this population has undergone a drastic reduction in size reaching a minimum of ca. 0.03 ha in 2009.

R. baudotii is a hydrophyte belonging to the *Ranunculus* subgen. *Batrachium*. It is distributed mainly in brackish waters near the western and southern coasts of Europe and of the Baltic region, and, locally, inland in western and central Europe (Cook, 1993). This species can be annual or perennial (Cook, 1966), it is self-compatible and mainly self-pollinated (Dahlgren, 1995). Vegetative propagation can also play an important role in population maintenance/growth as sexual reproduction (Dahlgren, 1995). *R. baudotii* establishes communities considered of interest towards conservation according to the Habitats Directive 92/43/CEE. Because the species forms clumps by vegetative propagation, in order to avoid the sampling of "ramets" rather than "genets", we randomly selected 10–15 plants growing at least 10 m apart from one another. The samples were collected in 2010 (St1; pre-restoration) and 2014 (St2; post-restoration). A second collection (Ta) was also sampled in 2014, which represented an ephemeral population living in an artificial reservoir about 1.5 km away from Stagnone pond (Fig. 1).

To establish *ex-situ* collections for conservation purposes, the original material from Stagnone was collected in 2009. Approximately 20 plants were collected and cultivated in the Florence Botanical Garden and seed samples were stored in the Pisa Seed Bank (Carta et al., 2009, 2012).

In addition to these natural populations, samples for genetic analysis were also collected from the *ex-situ* collections in line with the following scheme (Fig. 2): plants cultivated in Florence were sampled in 2010, representing the second generation of the wild population sampled in 2009 (Fi); seedlings obtained from laboratory-germinated seeds collected in the wild were sampled in 2009 and used to establish a living collection in the Pisa Botanic Garden (Pi1); the seedlings constituting Pi1 were obtained by germinating seeds at both the near-optimal temperature (10 °C; PiTA) and at a supra-optimal temperature (20 °C; PiH) as indicated by Carta et al. (2012); seedlings obtained from germinated seeds produced in 2010 by Pi1 were also sampled (Pi2). For Pi2, seeds were germinated in the laboratory only at the near-optimal temperature (10 °C).

The groups of samples analysed and belonging to these various categories (reported in Table 1) are hereafter defined as Sampling Groups (SG).

2.2. Isolation of genomic DNA

Genomic DNA was extracted from silica-gel dried leaf tissue using the CTAB protocol (Mengoni et al., 2006). The quantity and quality of DNA was estimated spectrophotometrically using a BioPhotometer (Eppendorf).

2.3. ISSR profiling and analysis

Two of the six primers preliminarily screened, yielded clear and reproducible banding patterns. To test reproducibility a subset of samples was selected at random from the whole dataset. Primer sequences, size range and number of polymorphic sites detected in the entire dataset are reported in Table 2.

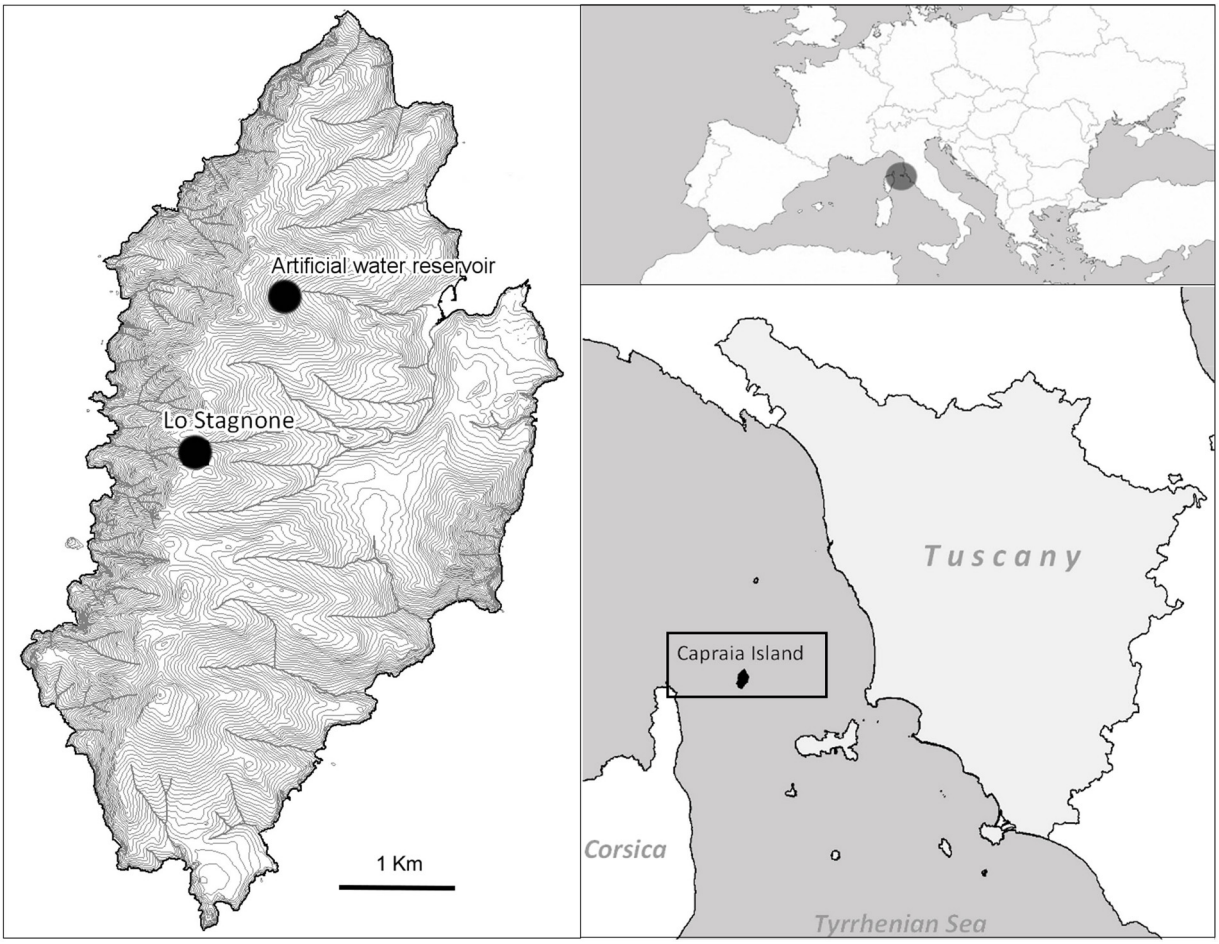

Fig. 1. The study area sampling sites. Stagnone pond and the artificial reservoir are shown with black dots.

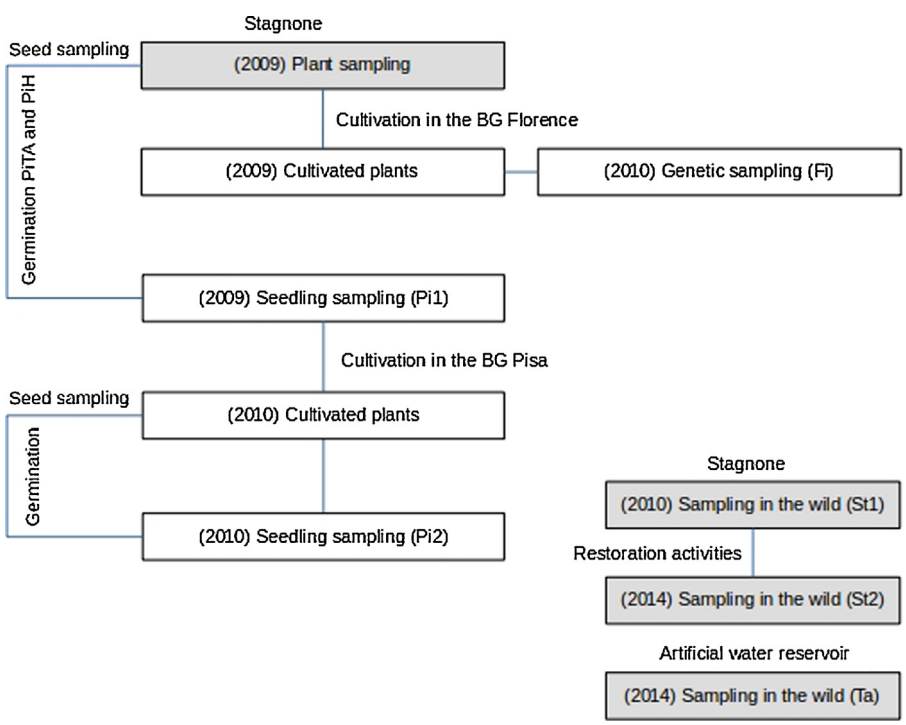

Fig. 2. Sampling design flowchart. Grey boxes indicate activities in the wild, white boxes indicate activities in the laboratory or botanical gardens. The sampling group code is also reported.

Table 1
List of examined accession and year of sampling. The following parameters are shown: percentages of polymorphic sites (Ps%). Number of rare bands (Nrf). Number of private bands (Npf). Average gene diversity over loci (genetic diversity), mean population heterozygosity (Hs) and total variation (Ht).

SG code	Year of sampling and location	Pol. (%)	Nrf	Npf		Genetic diversity
Pi1	2009; Pisa	85.4	23	2		0.33
Pi2	2010; Pisa	52.1	13	0		0.24
St1	2010; Stagnone	66.7	18	0		0.26
Fi	2010; Florence	72.9	20	1		0.29
St2	2014; Stagnone	82.6	9	2		0.18
Ta	2014; Reservoir for rainwater	22.9	7	0		0.12
					Hs	0.24
					Ht	0.34

Table 2
Primer sequences, size range and number of polymorphic sites detected by each primer in the whole dataset.

Primer	Primer sequence	Size range (bp)	Polymorphic sites (no.)
ISSR1	$(GT)_7$-YR	500–3000	23
CA6	$(CA)_6$.RG	250–2200	25

PCR were carried out in total volumes of 20 μl containing: 10 ng of DNA, 2 μl of reaction buffer (Dynazyme II, Finnzyme, Espoo, Finland), 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 2 μl of 10 mM Primer and 1.4 U of Taq DNA Polymerase (Dynazyme II, Finnzyme, Espoo, Finland). Thermocycling was carried out after an initial denaturing phase of 5 min at 94 °C followed by 35 cycles each of 40 s at 94 °C, 45 s at 43 °C and 90 s at 72 °C. A final cycle was set for 45 s at 94 °C, 45 s at 42 °C and a final extension step of 5 min at 72 °C. Amplification products were separated on 2% (W/v) agarose gel electrophoresis containing 1 μg ml^{-1} of ethidium bromide. Electrophoresis was carried out at a constant 75 W for 2 h. The resulting bands were visualised by a UV transilluminator and analysed by ImageJ software vs. 1.43f (Image Processing and Analysis in Java, http://rsbweb.nih.gov/ij/index.html).

2.4. Data analysis

All amplified bands were treated as dominant markers and all ISSR profiles obtained were translated in a 1-0 binary matrix. A matrix of genetic similarity between plants was computed using Jaccard's coefficient of similarity. This takes into account band sharing between individuals and is commonly used for the analysis of dominant markers (Lowe et al., 2004) such as AFLP or ISSR.

The percentage of polymorphic loci in the data set was first determined. The number of rare bands (Nrf), present in less than 20 individuals in the whole data set, and of private bands (Npf), namely those unique to one sampling group was calculated following Stehlik et al. (2001). An intrapopulation measure of genetic diversity was then computed as the "average gene diversity over loci" (Nei, 1987). The mean genetic diversity between SG (Hs) and the total genetic diversity (Ht) were also computed. All analyses at the intra-SG level were carried out using the program Arlequin 2.000 (Schneider et al., 2000).

The proportion of genetic variation explained by the differences among SG was estimated by means of the Slatkin's linearised FST's matrix (Slatkin, 1995 P-value in Appendix 1 of Supplementary information).

The Slatkin's matrix generated by Arlequin 2.000 software (Schneider et al., 2000) was used to generate a neighbour-joining dendrogram (Saitou and Nei, 1987) using the software Mega 6 (Tamura et al., 2013).

The binary matrix obtained from the ISSR profile was used to improve a bayesian phylogram by MrBayes. The best fitting models of binary matrix were "Binary Model" with coding bias option "noabsencesites" as suggested from MrBayes 3.1 manual (Ronquist and Huelsenbeck, 2003). The analyses were performed using four incrementally heated Markov chains (one cold, three heated) simultaneously started from random trees, and run for one million cycles sampling a tree every ten generations. The stationary phase was reached when the average standard deviation of split frequencies reached 0.01. Trees that preceded the stabilization of the likelihood value (the burn-in) were discarded, and the remaining trees were used to calculate a majority-rule consensus phylogram. The tree were showed with indication of Bayesian Posterior Probabilities (PP) values for the internal tree nodes.

Analysis of molecular variance (AMOVA; Excoffier et al., 1992) as implemented in Arlequin 2.000 (Schneider et al., 2000) was carried out to investigate genetic structure at different hierarchical levels: within SG and between SG. The AMOVA was computed three times separately for each of three hypothetical clusters of SG formations. The statistical support for the analysis was tested through 1023 permutations.

3. Results

ISSR analysis was carried out on a total of 57 samples. The two selected primer combinations produced a total of 48 analysable loci (100% polymorphic). Within each SG, the percentage of polymorphic loci ranged from 22.9% (Ta) to 85.4% (Pi1). The number of rare and private fragments is reported in Table 1. The three sampling groups Pi1, St1 and Fi showed a higher number of rare fragments (23, 18 and 20, respectively) whereas, the second generation from Pisa (Pi2), St2 and Ta showed a reduced number of rare fragments. Private fragments were observed only for two samples of Pi1, one sample for Fi and two samples for St2. Average gene diversity over loci ranged from 0.12 (Ta) to 0.33 (Pi1); total genetic diversity (Ht) was 0.34, whereas average gene diversity within SG (Hs) was 0.24 (Table 1). Otherwise, the genetic diversity of the current population in the pond (St2) was lower (0.18). For Pi1 the genetic diversities of samples germinated at near-optimal temperature (10 °C, PiTA) and at supra-optimal temperature (20 °C, PiTH) were 0.40 and 0.18, respectively.

3.1. Inter SG diversity and cluster analysis

The neighbour-joining dendrogram of *R. baudotii* accessions based on Slatkin's linearised pairwise FST matrix suggests the existence of two clusters, namely A and B (Fig. 3a). Cluster A consists of Pi1 accessions and a subcluster including St2 and Ta. Cluster B comprises the second generation of Pisa (Pi2) and a subcluster formed by the natural population of 2010 from Stagnone (St1) and Fi. A second dendrogram is shown where the two subsamples PiH and PiT are separated in the two clusters B and A respectively (Fig. 3b). The level of genetic similarity obtained from ISSR profile comparison was used to create a Bayesian phylogram (Fig. 4). Bayesian analysis partially confirms the patterns reported by the neighbour-joining dendrogram and only the samples from St2 and Ta (both sampled

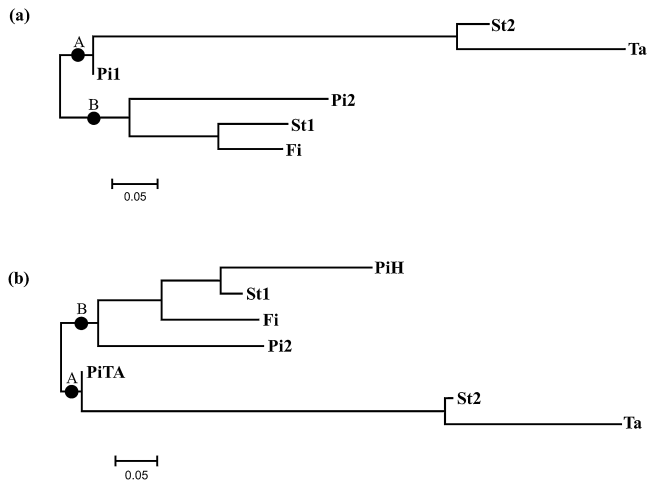

Fig. 3. (a) Neighbour-Joining dendrogram of sampling groups based on on Slatkin's linearised pairwise FST matrix and (b) Neighbour-Joining dendrogram where the samples constituting Pi1 were subdivided in PiTA and PiH. The scale bar indicates Slatkin's linearised FST value of genetic distance. The two main clusters are indicated. See Table 1 and Fig. 2 for the codes for the accessions examined and their positions in the sampling design.

in 2014) formed a weakly separated cluster (0.54 PP) with respect to the rest of dataset.

3.2. Genetic variation among SG

AMOVA analysis (Table 3) revealed that the greatest portion of genetic variation was due to within-SG differences (71.99% of variance) rather than to among-SG differences (28.01% of variance). Total among-SG differentiation (FST) was 0.280 (p-value <0.001). Among all hypothetical groupings of SG tested, only tree showed highly-significant results (see Table 3). The groups that explained the highest percentage of variation (22.07%) consisted of two groups, the first with SG sampled in 2014 (St2 and Ta) and the second constituted by PiH, PiTA, Pi2, St1 and Fi.

4. Discussion

4.1. Population genetic diversity in the natural environment

Stagnone pond hosts a small, isolated population of *R. baudotii* which is suffering severe contraction due to habitat change. The altering conditions likely affected the reproductive biology of this species that, similarly to other *Ranunculus* species of the subgen. *Batrachium* is probably able to adopt a mixed breeding system i.e. inbreeding or outbreeding (Dahlgren, 1995).

As suggested by Nybom (2004), species with self or mixed breeding systems should show mean values of within-population genetic diversity of 0.12 and 0.18, respectively. The population of *R. baudotii* examined here exhibits a moderately higher mean level of genetic diversity ($Hs = 0.24$) suggesting a significant population recruitment from cross-pollinated seeds. Whilst the mean value is moderately higher, the genetic diversity at the intra-SG level for Ta, is lower than the Nybom value (see Table 1) which suggests genetic drift and, most probably, a founder effect for the ephemeral population growing in the artificial reservoir. This phenomenon occurs reasonably often within the group of subgen. *Batrachium* due to the capacity of its achenes to be dispersed by birds (Dahlgren, 1995).

A first evaluation of the vegetation response after the restoration has shown a self-recovery of the former aquatic vegetation (Foggi et al., 2014). The population of *R. baudotii* (St2) has recolonised the Stagnone pond without any reintroduction and/or reinforcement activity. The ability of this species to form a soil seed bank has

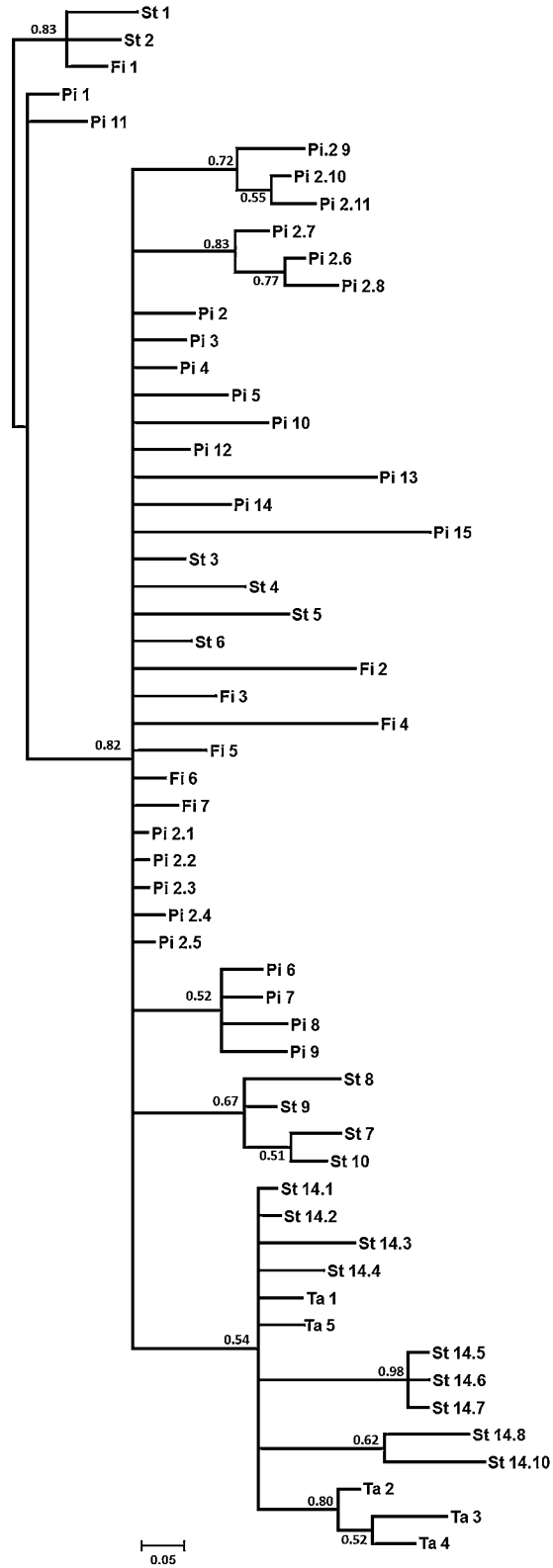

Fig. 4. Bayesian 50% majority-rule consensus tree from the ISSR binary matrix, with posterior probability values (PP) shown near statistically supported nodes. See Table 1 and Fig. 2 for the codes of the accessions analysed and their positions in the sampling design.

Table 3
Results of Analysis of molecular variance (AMOVA) performed to test the differentiation among 7 sampling groups and the partition among three hypothetical group formations. The table shows: degrees of freedom (df), sum of squared deviations, variance component estimates, percentages of total variance contributed by each component, and the probability of obtaining a more extreme component estimate by chance alone (p). *P*-values were estimated with 1023 permutations.

Source of variation	*df*	Sum of squares	Variance component	Percentage of variation	*P*-value
Within sampling groups	51	312.45	6.13	71.99	<0.001
Among sampling groups	5	141.38	2.38	28.01	<0.001
Total	56	453.83	8.51		
Structure of hypotetical groupings of SG					
(PiH;St1;Fi;Pi2) – (PiTA;St2;Ta)	1	47.31	0.90	10.14	<0.001
(PiH;PiTA;St1;Fi) – (Pi2) - (St2;Ta)	2	98.40	1.82	20.20	<0.001
(PiH;PiTA;Pi2;St1;Fi) – (St2;Ta)	1	64.67	2.13	22.07	<0.001

been largely reported for other geographical areas (Grillas et al., 1993). However, since the top 10–20 cm of soil was removed during the restoration work (Foggi et al., 2014), we suggest that the reappearance of *R. baudotii* in the pond was derived from a resilient seed bank of the soil, probably not completely damaged during the restoration or even encouraged by a mixing of the substrate by the earthmoving machinery. This hypothesis finds further support in evidence from the analyses of genetic diversity of SG collected before (Pi1, Pi2, St1 and Fi) and after the restoration (ST2). Indeed, the SG collected in 2009 (Pi1) and 2010 (Pi2, St1 and Fi), show a mean value for genetic diversity (0.28) slightly higher in than that observed for St2 (sampled in 2014; average gene diversity: 0.18). Future monitoring will likely determine whether the former level of genetic diversity will be re-established.

4.2. Genetic diversity and differentiation among natural populations and ex-situ plant material

The analysis of molecular variance (AMOVA) shows that the large percentage of variance was due to within-SG differences rather than to inter-SG differences (72% and 28%, respectively). It is clear that genetic differentiation of SG with respect to the complex of germplasm analysed, is negligible but not entirely absent. Among all the hypothetical groupings of SG tested to explain the observed proportion of molecular variation, three yielded highly significant results (see Table 3). The one that explained the highest percentage of variation consisted of two groups, the first formed by PiTA-PiH-St1-Fi and the second with the two SG of 2014 (St2 and Ta). This result is in agreement with the clustering obtained in the neighbour-joining dendrogram and with the pattern from Bayesian analysis described above (Figs. 3 and 4) suggesting that the SG should be analysed as two separate groups: the first constituted by the samples collected during the pre-restoration period and the second by the SG of 2014 (St2 and Ta). These results support the hypothesis that St2 and Ta are undergoing genetic drift. These SG likely originated in the impoverished soil seed bank subjected to a "bottle-neck" for St2 and/or a "founder effect" event for Ta.

The *ex-situ* cultivated populations showed relatively high genetic diversity and thus should be assessed as highly-valuable material, from a conservation point of view. Interestingly, the population cultivated in Florence exhibited a relatively high level of genetic diversity after the first generation and in Pisa this pattern was maintained through the second generation also, suggesting that a mid-term cultivation should not lead to severe genetic inbreeding processes. Indeed, it can be noted that in the wild (and also under cultivated conditions) *R. baudotii* behaves as an annual hydrophyte that regenerates each year through the germination of seeds (A. Carta, unpublished data).

Among the propagated lines obtained in the laboratory through seed germination, PiTA showed the highest genetic diversity value, while the PiH line showed one of the lowest although exhibiting genotypes apparently not present in the current population (see Figs. 3 and 4). This result suggests that the two different germination temperatures used may have been responsible for a genotype-selection process. That is to say, at temperatures reported as optimal for germination for this species (Carta et al., 2012) many seeds germinated and thus the whole range of genotypes was expressed (PiTA) whereas at the supra-optimal temperature, germination was strongly suppressed so only a few seeds germinated and a more limited range of genotypes was expressed (PiH). By considering the genetic variability expressed by PiTA we also confirm that our *ex-situ* seed collection does not suffer low genetic diversity. However, to maximise the expression of this diversity when *in-situ* species recovery is planned, the optimum germination conditions should be used.

4.3. Insight into population conservation

The results corroborate the usefulness of ISSR markers in setting priorities and planning optimal actions for the conservation of endangered plant species or populations in threatened biotopes (Thompson, 2005; Lopes et al., 2014).

The monitoring of the pond after the restoration shows widespread recolonisation of *R. baudotii*. However, if the diversity level will not be re-established autonomously, it may be appropriate to re-introduce individuals during subsequent years. Two strategies can be considered for reinforcements in this site. A breeding-oriented perspective suggests reinforcement of the impoverished population by introducing plants from other sites to enrich the genetic basis of the population. This strategy may reduce the risk of genetic drift and, ultimately, may help offset the negative effects of habitat change (Coppi et al., 2010). On the other hand, from a conservation perspective, the reintroduction would be better undertaken using the local seed sources in the *ex-situ* seed bank which are more likely to exhibit increased fitness over non-local genotypes (Xiao et al., 2006). Since the original material is available, our data would suggest that the second option should be preferred. In addition, we also suggest to increase the genetic diversity by mixing propagation lines obtained using a range of germination conditions based on the hypothesis, here demonstrated, that this should increase the genetic variability of this species.

Ex-situ management inevitably implies selection pressures different from the natural conditions (Lauterbach et al., 2012).

In general, it is preferable to reintroduce plants in the wild using seedlings (Godefroid et al., 2011). In the context of our study, there is evidence that increased genetic diversity can be obtained by selecting a range of germination conditions. Thus, when ecological restorations are planned, we recommend to considere the possible negative effects of germinating seeds under laboratory environments which may select a different range of genotypes than those expressed under a natural environment. Since it may not always be possible to have genetic data, an additional recommendation for *ex-situ* handling is to undertake both cultivation and propagation in a near-natural environment (Volis and Blecher, 2010). In line with the view of Smith et al. (2011), our results highlight the importance of considering *ex-situ* conservation as a complemen-

tary approach to *in-situ* conservation, additionally supported by the genetic monitoring.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2015.06.002

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