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*Reintroduction of a dioecious aquatic macrophyte (*Stratiotes aloides *L.) regionally extinct in the wild. Interesting answers from genetics*

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ABSTRACT

1. The reintroduction of a plant species regionally extinct in the wild poses a stimulating conservation challenge. If the species is dioecious and the *ex situ* preserved population is only of one sex, the challenge is even more difficult. To assess whether the female population of *Stratiotes aloides* originally studied requires a reinforcement to increase its genetic variation, and to determine from which source male individuals should be taken to re-establish a viable population, the genetic structure of nine different accessions of *S. aloides* across Europe and Asia were analysed – six native populations and the last three Italian populations, preserved *ex situ*.

2. Amplified fragment length polymorphism (AFLP) fingerprinting of 190 individuals from these populations was performed using six primer combinations and chromosome counts.

3. AFLP markers revealed medium to high values of genetic diversity at the population level, unexpectedly including residual *ex situ* accessions. Neighbour-joining tree, PCoA and STRUCTURE analyses indicate the presence of three genetic patterns identifiable in the central-western, central and eastern Europe–Asian populations. Chromosome counts revealed the presence of diploid (2n = 24) and tetraploid (2n = 48) populations.

4. Similarity between populations belonging to different hydrographical basins, and differences between neighbouring populations could be explained through long-distance bird-mediated dispersal events. Genetic analysis showed that reinforcement with female individuals from other European populations to increase the genetic diversity of the Italian female population is not necessary. Surprisingly, the geographically closest male population (Bavaria) to the Po basin is not the best option for male reintroduction. Instead, male individuals should be reintroduced from the Rhine basin (Netherlands) and eastern part of the Danube basin (Romania).
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KEY WORDS: lake; pond; endangered species; genetics; reintroduction; macrophytes; nutrient enrichment; pollution

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INTRODUCTION

The re-establishment of populations of species or genotypes extinct in the wild from *ex situ* preserved stocks of individuals is now quite common practice worldwide (Aguraiuja, 2011; Seddon *et al.*, 2014). A similar approach is currently needed for *Stratiotes aloides* L. (Hydrocharitaceae), an aquatic dioecious plant threatened in many European countries and extinct in the wild in Italy, but preserved *ex situ*. However, the number of genotypes is often low in *ex situ* collections, which complicates the re-establishment of the genetic properties of the original populations (Rucinska and Puchalski, 2011). When only part of the original genetic variation of a species is maintained *ex situ* it is important to maximize the number of founder individuals to increase the chance of survival of a reintroduced population (Maunder *et al.*, 2000). On the other hand, when local genotypes of widespread species are the subject of a conservation translocation, the selection of potential sources for population reinforcement may be more challenging owing to the genetic structure of the global population, the species' dispersal strategy and the limitations to dispersal. Pre-reintroduction genetic analyses allow the choice of best source populations (Maschinski and Haskins, 2012). In fact, widely-distributed species may show complex genetic patterns and the choice of a given source population may determine different dynamics and success (e.g. inbreeding and outbreeding depression; Pelabon *et al.*, 2005; Becker *et al.*, 2006). So, the genetic structure, the genetic variability and the effective population size of the source population(s) are important issues for a successful translocation.

As a general principle, the greater the genetic variability and effective population size, the higher the probability of successful population establishment (Forsman, 2014). Moreover, the right balance between inbreeding and outbreeding of translocated populations together with gene flow between populations should be assured (Godefroid *et al.*, 2011). It is well established that an understanding of the genetic structure of the source populations is very important for planning breeding programmes with an appropriate mix of source populations and gene flow management. This especially applies to animals, where the release of both sexes with a correct balance is also essential (Stanley-Price, 1991; Snyder and Snyder, 2000).

For plant species, dioecy may greatly complicate the re-establishment of viable self-sustaining populations (IUCN, 2013). The separation of sexes is rather rare in flowering plants, occurring in about 6% of all species (Renner and Ricklefs, 1995) and for this reason this aspect has rarely been considered in the literature on translocations (see Maschinski and Haskin, 2012 and references therein). For instance, in a reintroduction trial of the Australian dioecious species *Symonanthus bancroftii* (F. Muell.) L. Haegi, Ye *et al.* (2007) highlighted the need to determine the optimal sex ratio and the possibility of strong inbreeding depression following the *ex situ* cultivation of the two remnant male and female individuals. Moreover, they demonstrated that different crosses between remnant individuals may result in different reproductive performance and fitness of translocated individuals.

In this context, an interesting case is represented by the dioecious *Stratiotes aloides* L. This Euro-Siberian species is widely distributed in lakes, ponds, ditches and canals (Figure 1(A), (B)) where it often dominates macrophyte communities (Efremov and Sviridenko, 2008). Their stands harbour a high diversity of macroarthropod fauna, containing species of high conservation concern (Suutari *et al.*, 2009), including the larvae of *Aeshna viridis* (Rantala *et al.*, 2004), a Near Threatened dragonfly species (Kalkman *et al.*, 2010) protected by the nature conservation legislation of the European Union.

Despite its broad distribution, *Stratiotes aloides* is declining in Western Europe (Cook and Urmi-Konig, 1983; Smolders *et al.*, 2003; Zantout *et al.*, 2011), where it has recently become extinct at the southern edge of its range. *Stratiotes aloides* was abundant in wetlands of the eastern Po Plain (N. Italy), until the beginning of the twentieth century, with mostly female populations (Orsenigo *et al.*, 2012). The major reason for decline (and regional extinction) is the increased inorganic nitrogen (in particular nitrates) in the water, as a consequence of intensive agriculture and farming (Abeli *et al.*, 2014). Considered lost forever, remnants of the Italian population of *S. aloides* have recently been

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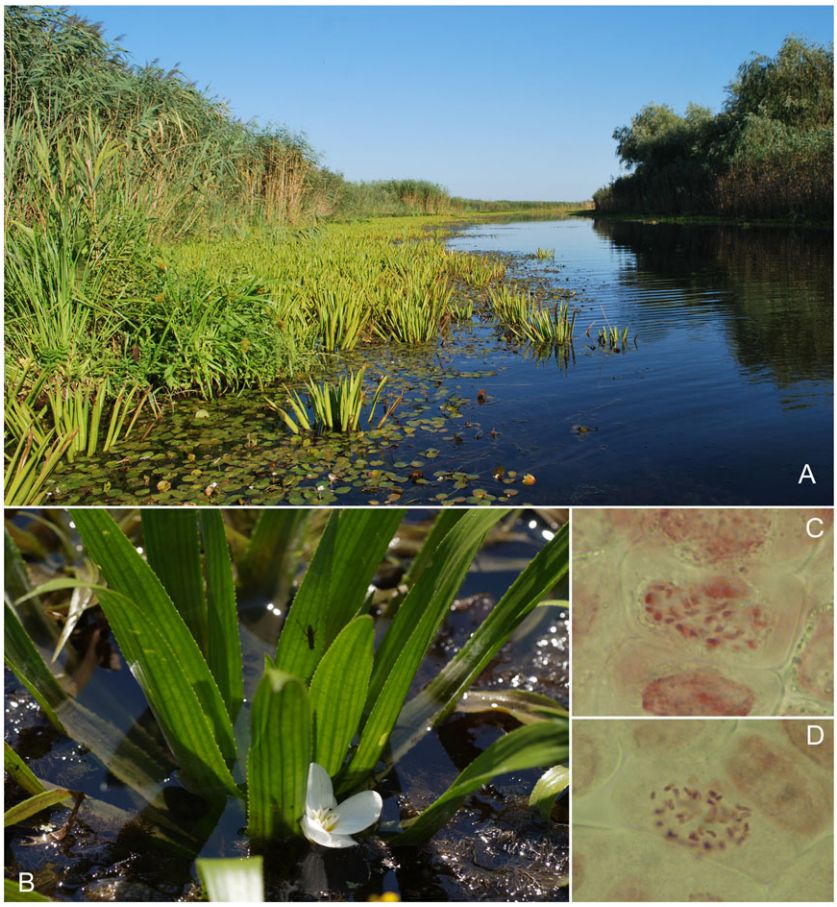

Figure 1. (A) Population of *Stratiotes aloides* in Sulina, Danube Delta (Romania). (B) A female individual of *Stratiotes aloides* from Bavarian population (Plattling, Germany). (C) Mitotic metaphase plate of BAV; 2n = 24 (×1000). (D) Mitotic metaphase plate of NED; 2n = 48 (×1000).

re-discovered *ex situ*. However, only female plants are preserved *ex situ* – offsprings of a few plants (exact number unknown) collected from the Mantua Lakes (Lombardy, Italy) at the beginning of the 20th century. These have reproduced vegetatively for many years, and are at present preserved in three *ex situ* private collections, while male individuals are considered definitively extinct. The discovery of surviving plants of *S. aloides* provides the opportunity to reintroduce the native Italian population in historical sites of occurrence or other sites in the historical distribution area (provided that conditions are suitable). It also raises several interesting questions concerning the recovery of plants extinct in the wild, but preserved *ex situ,* and the problem related to dioecy.

The aim of this study was to investigate the opportunities and problems of reintroducing *S. aloides,* a dioecious plant extinct in the wild in Italy, from plant individuals preserved *ex situ*. A DNA molecular analysis was used to study the genetic pattern of several *S. aloides* populations with the principal goal of selecting a gene pool that would be useful for reintroduction programmes (McKay *et al.*, 2005; Gentili *et al.*, 2010). Such analysis was based on the AFLP approach, which is considered an effective tool to reveal variability and population structure within a single species (Bruni *et al.*, 2013). The genetic variation of the Italian accessions was investigated in the broader context of the genetic variation and structure of nine populations of *S. aloides* in Europe and Asia, with the following specific aims: (1) to assess whether the original Italian female population requires reinforcement to increase its genetic variation; and (2) to determine from which source population male individuals should be taken to re-introduce the male population. Three hypotheses were proposed: (1) low genetic

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variation of the Italian *ex situ* population of *S. aloides*, as a consequence of long-term vegetative reproduction of the plants collected in the wild; (2) the *ex situ* population represents only a small portion of the species/population gene pool owing to the random collection of few individuals from the wild ('collector-mediated' founder effect and genetic drift); and (3) the geographically closest populations of *S. aloides* may represent the best source populations for male reintroduction.

MATERIAL AND METHODS

Sampling materials

DNA analyses were performed on nine accessions from six natural populations of *S. aloides* from the Netherlands (NED), Germany (BAV), Romania (populations ROM1 and ROM2), Western (EUR) and Central Russia (ASR) (Table 1), two *ex situ* populations (MN1 and MN2), remnants of the last Italian wild populations of Mantua, cultivated by two different amateur botanists, and one population cultivated at the Ferrara Botanical Garden (Italy), originating from specimens introduced from the Botanical Garden of Berlin (FE). Populations were sampled in five different river basins: the Rhine and the Danube are the closest to the Po basin, where the original Italian population occurred, and the Volga and the Ob from Russia (Table 1). Possibly the accession from Ferrara, originally derived from Berlin, may belong to a different basin. Within each population, 9–42 individuals were sampled, depending on the population size. Table 1 shows the locations and characteristics of the sampled populations. Male and female individuals of the mixed Dutch population were sampled and analysed separately, in order to determine differences in genetic diversity between sexes. The Italian female population of *S. aloides* is preserved *ex situ* at C.R.E.A. (Centro Riqualificazione Ecosistemi Autoctoni, Cornaredo, Milan, A. Nania), at three private floriculturists (Lilium Aquae, Castelfranco Veneto, Treviso Az. Agricola Beschi Alvaro & Giulio, Brescia, Italy, MN1, and at P. Vanetti, Inarzo, Varese, Italy, MN2) and more recently at the Botanical Garden of the University of Pavia.

DNA extraction and AFLP

DNA extraction and AFLP genotyping were conducted by Ecogenics GmbH, Schlieren (Switzerland), based on standard protocols. 200–500 ng genomic DNA was digested with EcoRI/MseI and ligated with corresponding AFLP adapters. Preselective PCR (pre amplification) was performed with the AFLP primers EcoRI-A (E01) and MseI-C (M02). Selective PCR (selective amplification) was done on 1:50 dilutions of the pre-amplification reactions using the specified selective primer combinations (Supplementary information, Table S1). In all the reactions, only the EcoRI primers were 5' labelled with a fluorescent dye (6-FAM). For fragment length analysis Applied Biosystems 3730xl DNA Analyzer was used. To assess the reproducibility of the analysis, the whole procedure (i.e. from DNA extraction to capillary electrophoresis) was repeated for 20 samples (about 10% of the total) and the error rate was calculated as the number of phenotypic differences over the total number of phenotypic comparisons (Bonin *et al.*, 2004; Dettori *et al.*, 2014).

Genetic diversity

The number and proportion of polymorphic loci (PPL-5% at the 5% level, corresponding to P95) were calculated using AFLP-SURV version 1.0 (Vekemans, 2002). With the same software Nei's gene diversity Hj (analogous to H or He in most publications; Nei, 1973), Ht (total gene diversity: gene diversity in the overall sample), Hw (average gene diversity within populations) were calculated. AFLP-SURV allowed the calculation of allele frequencies using the default Bayesian method with non-uniform prior distribution (Zhivotovsky, 1999). The effective allele number (ne) and Shannon's information index (I) at the population level, was determined using GenAlEx 6.5 (Peakall and Smouse, 2006). The number of locally common bands (restricted to a limited area and found in $\leq$25–50% of populations) was determined using GenAlEx 6.5 (Peakall and Smouse, 2006). The binary matrix generated with AFLP analysis was subjected to Principal Coordinates Analyses (PCoA) in PAST 2.1 software. A Neighbour-joining (NJ) analysis based on a matrix of Nei–Li

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Table 1. Sampled populations and estimates of genetic diversity in *Stratiotes aloides*; PPL5% = number and proportion of polymorphic fragments at the 5% level; I = Shannon's information index; Hj = Nei's heterozygosity; ne = effective number of alleles

Population	Location	Country	River basin	Coordinates	Pop.size	Habitat	Samples	Gender	Ploidy level	PPL-5%*	I	Hj	ne
BAV	Isar Mouth	Germany	Danube	48°46'N-12°55'E	<5000	Isolated oxbow lake	27	♀	2n = 2x = 24	64.5 (338)	0.315 ± 0.010	0.212 ± 0.006	1.269 ± 0.007
FE	*Ex situ*	Italy	Unknown	-	-	-	15	♀	-	68.9 (361)	0.327 ± 0.009	0.232 ± 0.004	1.301 ± 0.006
MN1	*Ex situ*	Italy	Po	-	-	-	15	♀	2n = 4x = 48	88.5 (464)	0.313 ± 0.008	0.213 ± 0.003	1.270 ± 0.005
MN2	*Ex situ*	Italy	Po	-	-	-	15	♀	-	92.2 (483)	0.365 ± 0.008	0.248 ± 0.003	1.329 ± 0.005
NED	Gieethorn	Netherlands	Rhine	52°44'N-06°06'E	> 10.000	Ditches between meadows	42	♀ + ♂	2n = 4x = 48	75.3 (395)	0.269 ± 0.002	0.181 ± 0.002	1221 ± 0.025
ROM1	Charaorman Channel	Romania	Danube	45°08'N-29°21'E	Ca. 10.000	Secondary river channels in the delta	30	♀	-	68.5 (359)	0.236 ± 0.006	0.147 ± 0.002	1.172 ± 0.003
ROM2	Sulina	Romania	Danube	45°13'N-29°18'E	Ca. 10.000	Secondary river channels in the delta	19	Unknown	-	79.4 (416)	0.318 ± 0.008	0.213 ± 0.004	1.271 ± 0.005
ASR	Rasvet	Russia	Ob'	55°13'N-73°01'E	> 10.000	Isolated oxbow lake	18	♀ + ♂	-	87.2 (457)	0.433 ± 0.010	0.319 ± 0.004	1.468 ± 0.008
EUR	Kachkashur	Russia	Volga	58°08'N-52°44'E	Ca. 5000	Isolated oxbow lake	9	♀ + ♂	-	89.3 (468)	0.411 ± 0.009	0.311 ± 0.007	1.452 ± 0.007

*5% criterion applied to Bayesian estimates of allele frequencies

Species level

Ht	Hw	Hb	Fst
0.245	0.226	0.019	0.077

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distance was conducted with TREECON 1.3b (Van de Peer and De Wachter, 1994). The tree was edited graphically using the program SplitsTree 4.13 software (Huson and Bryant, 2006); support of nodes was assessed with 1000 bootstrap replicates.

Ancestry of *S. aloides* samples was estimated to model population structure using Bayesian methods as described by Falush *et al.* (2007) in STRUCTURE v. 2.3.4 (see also Pritchard *et al.*, 2000). The allele frequencies of the different *S. aloides* populations were assumed to be correlated, which is a realistic model for populations that are likely to be similar because of common migration events or shared ancestry. To determine the best number of clusters, 20 independent runs of K ($K = 1$–10; see Supplementary File F1) were performed with an admixture model at 100 000 Markov chain Monte Carlo (MCMC) iterations and a 20 000 burn-in period (LOCPRIOR option; estimate λ). The admixture model assumes that each individual is supposed to have inherited some proportion of its ancestry from each population, so this is a ubiquitous approach to capture latent population structure in genetic samples. The ΔK, the second-order rate of change in ln P $(X|K)$ for successive values of K to determine the number of clusters (Evanno *et al.*, 2005) was used. The distribution map of STRUCTURE was plotted according to K value at the highest log likelihood. To estimate genetic structure and degree of genetic differentiation within populations, among populations and among biogeographic districts analysis of molecular variance (AMOVA) was performed using the Genalex software version 6.1 (Peakall and Smouse, 2006). The significance of the estimates was obtained through 999 data replications. Since no genetic differences were found among male and female Dutch populations, the results were grouped and statistically analysed together.

Chromosome counts

Chromosomes were counted by N. Ardenghi using individuals of the Italian remnant populations and those populations closest to Italy (MN1, ROM2, BAV and NED) growing *ex situ* at the Botanical Garden of the University of Pavia. Meristems from the tips of developing roots not yet penetrated in the sediment were collected. The root tips were pretreated in hydroxyquinoline for 3 h at room temperature, then fixed in Carnoy's solution (3:1, 3 parts of ethanol and 1 of glacial acetic acid) and preserved at 4°C until preparation. After hydrolysis in 1 N HCl for 6 – 7 min at 60°C, they were stained with lacto-propionic orcein overnight, dissected and squashed on clean glass slides with 1 or 2 drops of 45% acetic acid, before examination under a Zeiss Axiophot light microscope (1000×).

RESULTS

The six primer combinations averaged a low scoring error rate (3.67%; based on phenotypic comparisons among replicated individuals, Bonin *et al.*, 2004) which stressed the repeatability of the AFLP data set. The final data set consisted of 190 individuals from nine populations surveyed for AFLP variation, and about 524 fragments in the range of 50–600 bp, of which 92% were polymorphic overall across populations (Supplementary Table S1). All 190 individuals had a unique profile.

Genetic diversity

The results of the genetic diversity analyses of *S. aloides* populations are presented in Table 1. The percentage of polymorphism ranged from 64.5% (in pop. BAV) to 92.2% (in pop. MN2). The effective number of alleles (ne) ranged from 1.172 (ROM2) to 1.468 (ASR). The AFLP variation within populations, estimated as Hj, ranged from 0.147 (ROM1) to 0.319 (ASR); the average gene diversity within the nine investigated populations (Hw) was 0.226, and the total gene diversity (Ht) was 0.245. The lowest value for Shannon's information index (I) was also found in ROM1 (0.236), and the highest in ASR (0.433).

Population structure of *Stratiotes aloides*

In general, the neighbour-joining analysis conducted at the individual level using Nei and Li distances grouped individuals belonging to the same populations; support was medium to low (<50%) for basal branches (black ramifications) but quite

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high for upper levels branches (>90%; Figure 2(A)). The relationships among the populations of *S. aloides* were initially investigated by PCoA analysis (Dice index; transformation exponent, c = 2; Figure 2 (B)) and cluster analysis (Box). The first two main components in PCoA explained 17.7% and 10.7% of the total variation, respectively. PCoA analysis showed a relative clustering of the populations and a relative separation of populations BAV, FE and ROM1, in the lower part of the scatter plot (Figure 2 (B)). However, BAV and FE scored positive values along the first coordinate, while ROM1 showed a slightly separated distribution, with a degree of negative value along the first coordinate. In contrast, the individuals from the other six populations (MN1, MN2, NED, ROM2, ASR and EUR) were mostly located in the upper part of the scatter plot. MN1, MN2, NED, ROM2 displayed negative scores along

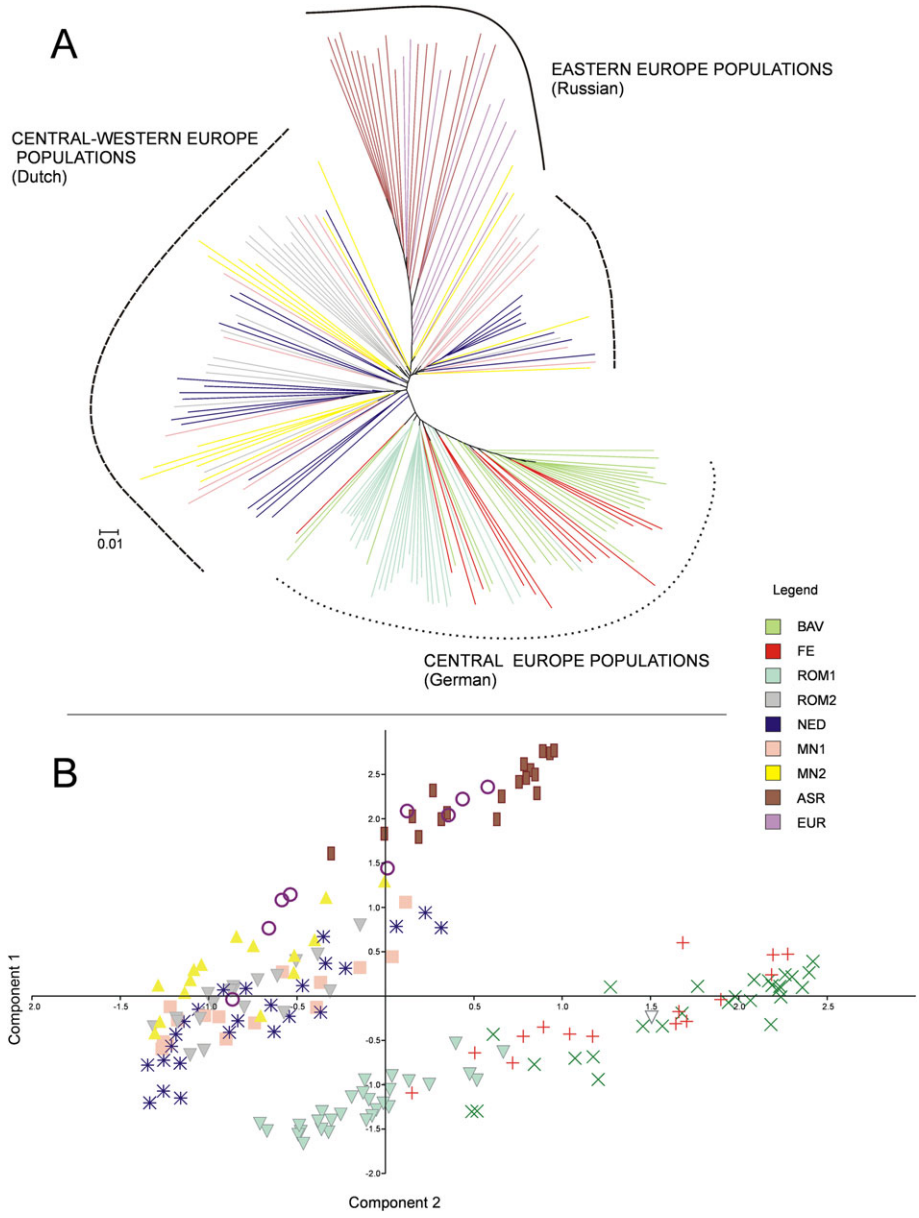

Figure 2. (A) Unrooted neighbour-joining tree based on Nei and Li distances. Bootstrap values were <50% for all branches. (B) PCoA based on Hamming genetic distances. The first two principal coordinates explained 27.2% and 15.9%, respectively, of the molecular variance.

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the first axis (Figure 2(B)); EUR and ASR populations showed positive values along the first and second axes.

Cluster analysis revealed that populations BAV and FE were related (both originating from Germany); populations MN1, MN2, NED and ROM2 formed a core group, while ROM1, EUR and ASR clustered in isolated ramifications.

STRUCTURE analysis estimated the highest mean log likelihood at K = 7 (ln P(D) (–177375.7)), indicating that populations of *S. aloides* are subdivided into seven distinct genetic clusters. The results are based on an Admixture model in which individuals may have mixed ancestors from different populations. Figure 3(A), (B) shows a degree of

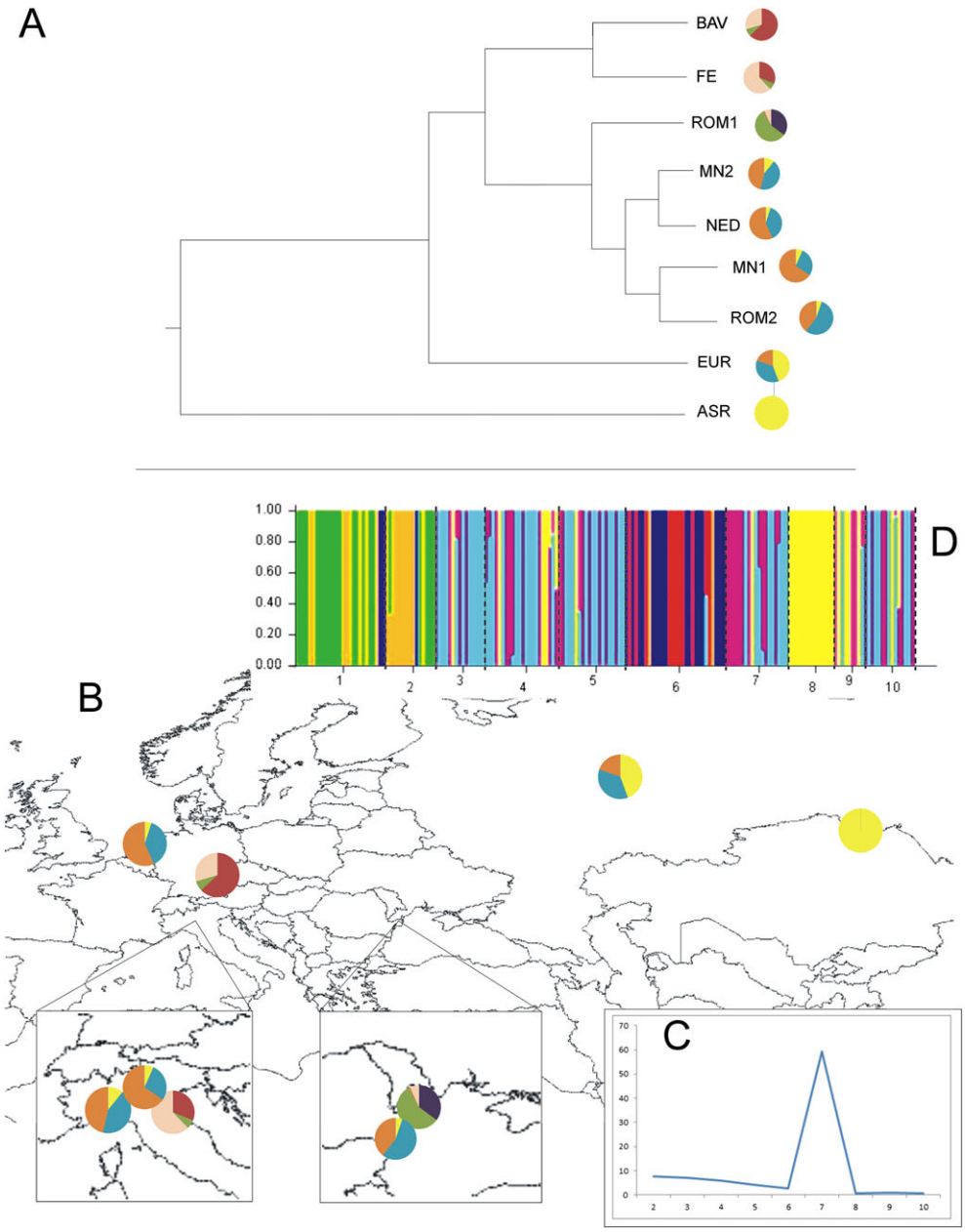

Figure 3. (A) Cluster analysis based on Nei's genetic (UPGMA) distance between *S. aloides* populations associated with results of STRUCTURE analysis. (B) Geographic location populations and STRUCTURE analysis. (C) Results of the ΔK calculation (see Methods for details). (D) In the bar diagram different colours represent the proportion of ancestry in each of the K populations.

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structure in *S. aloides* populations which seem to be subdivided into three main subsets: (a) BAV and FE (Germany); (b) MN2, NED, MN1 and ROM2; and (c) EUR and ASR (Russia). Population ROM1 appears to be the most distinct from the others.

The overall genetic differentiation among populations (F_{ST}) was 0.077. AMOVA (Table 2) showed that most of the genetic variation (about 87.6% in both the non-hierarchical and the hierarchical analysis) was allocated within populations, while a small, but substantial, proportion of the variation is explained by between-group differences. The between-group differences were evaluated for several combinations changing the position of the population ROM1, the most dissimilar from the others and then showing unclear attribution to the grouping revealed by the support of cluster, PcoA and STRUCTURE analyses. The percentage of genetic variation among regions (15.2%) and populations (6%) was higher (in total 21.2%) when considering the following grouping of the total variation (Table 2): [BAV, FE] [NED, MN1, MN2, ROM1, ROM2] [ASR, EUR]. Mantel's test between pairwise comparisons of population differentiation values from F_{ST} and Φ_{ST} matrices found significant correlation ($R = 0.92$; $P < 0.001$; Euclidean distance). Mantel's tests between the F_{ST} and Φ_{ST} population differentiation values and geographic distances were significant (F_{ST}: $R = 0.46$; $P = 0.049$; Φ_{ST}: $R = 0.65$; $P = 0.026$; see supplementary Table ST1 for the F_{ST}, Φ_{ST} and kilometric distance matrices).

Chromosome counts

Among the three populations analysed, only BAV proved to be diploid, with $2n = 2x = 24$ (Figure 1 (C)); MN1 and NED were shown to be tetraploid, with $2n = 4x = 48$ (Figure 1(D)). The data confirm the chromosome counts already reported in literature: $2n = 24$ (Schürhoff, 1926, origin unknown) and $2n = 48$ (Gadella and Kliphuis, 1973, from Loosdrecht, Netherlands). The count by Negodi (1929) ($2n =$ 'slightly higher than 20'), probably from Po Plain (Italy), can be interpreted as a diploid count rather than an aneuploid, as stated by Letz *et al.* (1999); no aneuploid counts (such as that by Letz *et al.*, 1999, $2n = 40$, from Veľké Leváre, Slovakia) occurred. Unfortunately, the count for the population ROM2 failed.

Table 2. Results of five analyses of molecular variance (AMOVA) of AFLP data (squared Euclidean distance) from nine populations of *S. aloides*. In the four groupings the relative positions of the population ROM1 were checked. The maximum diversity (variance among groups + variance among populations within groups) were obtained in the following combination [BAV, FE] [NED, MN1, MN2, ROM1, ROM2] [ASR, EUR]

Grouping	N	Source of variation	df	SS	variance (%)	*P*
no grouping	9	among populations	8	8246.67	16.0%	0.001
		within populations	164	36582.35	84.0%	0.001
[BAV, FE] [NED, MN1, MN2, ROM2] [ASR, EUR] [ROM1]	4	among groups	3	6341.1	15.2%	0.001
		among populations within groups	5	1905.5	3.4%	0.001
		within populations	164	36582.4	81.4%	0.001
[BAV, FE] [NED, MN1, MN2, ROM1, ROM2] [ASR, EUR]	3	among groups	2	5022.0	14.6%	0.001
		among populations within groups	6	3224.7	6.0%	0.001
		within populations	164	36582.4	79.5%	0.001
[BAV, FE, ROM1] [NED, MN1, MN2, ROM2] [ASR, EUR]	3	among groups	2	4687.5	11.8%	0.001
		among populations within groups	6	3559.1	7.2%	0.001
		within populations	164	36582.4	81.1%	0.001
[BAV, FE] [NED, MN1, MN2, ROM2] [ASR, EUR, ROM1]	3	among groups	2	3414.0	5.1%	0.001
		among populations within groups	6	4832.6	12.0%	0.001
		within populations	164	36582.4	82.8%	0.001

df = degrees of freedom; SS = mean sum of squares

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DISCUSSION

Genetic diversity

In this study the genetic diversity and structure of different populations of *S. aloides* were investigated in order to select an appropriate source to re-establish the male population of *S. aloides* in Italy, where the species is currently extinct in the wild and where only females are preserved *ex situ*. A low genetic variation was expected in the remnant females after several years of *ex situ* clonal reproduction and we hypothesized that the German populations, the closest to the historical area of occurrence of the species in Italy, best represent the original Italian male genotype.

The first investigation of the genetic diversity of *S. aloides*, based on nine populations of the species across its Eurasian range, showed medium to high values of genetic diversity at the population level using AFLP markers (Nei's gene diversity ranged from 0.147 in ROM1 to 0.319 in ASR; mean = 0.226). Such values were comparable with that of *Thalassia testudinum* Banks and Sol. ex K.D. Koenig, another clonal dioecious species belonging to the family Hydrocharitaceae: mean Nei's H = 0.35, detected by AFLP (Waycott and Barnes, 2001). Similar values were also found in *Halophila ovalis* (R. Brown) J. D. Hooker (Hydrocharitaceae), by means of SSR analyses ($H_E = 0.306$ and $H_E = 289$ in Indian and Pacific populations, respectively; Nguyen *et al.*, 2014). Hence, the expectation that genetic variability would be low in clonal populations (e.g. *Marsilea quadrifolia*; Bruni *et al.*, 2013) is not supported in some cases. Although vegetative reproduction is known to occur in *S. aloides* (Smolders *et al.*, 1995a), other specific processes may have led to such high values of genetic diversity even in small populations. In particular, the prevalent outcrossing mating system, the occasional occurrence of hermaphrodite plants (Forbes, 2000), somaclonal mutation events (often observed in aquatic plants) and a likely persistent gene flow (favoured by dispersal of vegetative floating propagules: Sarneel (2013)) may provide the basis of the unexpected high genetic diversity, including the genetic diversity of the female individuals of the remnant Italian population. In addition, the ploidy level (4x) found in the Italian remnant females may also explain their high genetic variation despite the founder effect and the many years of *ex situ* clonal growth. Regarding the other accessions analysed, the results confirm the karyological heterogeneity previously reported in the literature, where different chromosome numbers are indicated: $2n = 24$ (Schürhoff, 1926) and $2n = 48$ (Gadella and Kliphuis, 1973). The variable number of cytotypes within *S. aloides* suggested by the literature and confirmed by the experimental data, are in line with intrageneric and intraspecific chromosome number variation described by Les and Philbrick (1993) for the family Hydrocharitaceae and, in general, for most aquatic angiosperms. Possibly, the karyological variability evidenced by the results of the present study may also explain the different genetic clusters shown by the STRUCTURE analysis. In fact, somatic doubling of chromosome number is a common, if not predominant, mode of polyploidy in aquatic plants. Prevalence of clonal growth above sexual reproduction, associated with high vagility of asexual propagules in aquatic habitats, is effective in increasing the opportunity for somatic doubling to occur (Les and Philbrick, 1993).

Genetic structure

The results suggest that wild populations of *S. aloides* across its Euro-Asiatic range have different genetic patterns. Both PCoA, and NJ tree and cluster analysis indicated the presence of three main genetic patterns identifiable in the central-western (MN1, MN2, NED and ROM2), central (BAV and FE) and eastern (EUR and ASR) Eurasian populations. ROM1 is partially separated but seems more similar to the central-western Europe group according to clustering of populations and AMOVA. On the other hand, STRUCTURE analysis showed seven main ancestral populations that may be the result both of the high variability of AFLP analysis (detected by this highly sensitive analysis) and the sex separation. In any case PCoA, NJ and cluster analysis do not seem in conflict with

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STRUCTURE analysis as the former analyses showed a certain degree of grouping subsets. In all the analyses, the Russian populations (eastern range, in Asia) clearly show a distinct ancestry, while the genetic analyses confirmed the German origin of the specimens cultivated at the Botanical Garden of Ferrara. A reintroduction attempt with plants of unknown origin promoted in north-eastern Italy by an amateur botanist, if successful, may have introduced a non-native genotype in Italy. This suggests extreme caution in the use of plant sources of uncertain provenance and highlights the importance of genetic analysis before translocation.

Considering that most of the variation was detected within populations, the similarity between populations belonging to different hydrographical basins could be explained through long-distance bird-mediated dispersal events, as first proposed by Forbes (2000), but not proved owing to the lack of direct or indirect observation of birds feeding on *Stratiotes* seeds (Dessborn *et al.*, 2011; L. Dessborn personal communication). Recently, coots (*Fulica atra*), which in some cases may perform long-distance migrations, have been observed (in the Netherlands: Smolders, and in Russia: Efremov, personal observations) to eat *S. aloides.* Considering the timing of seed dispersal and bird migration events and the diet of different species of waterfowl other dispersal vectors might include teal, mallard, shoveler and moorhen (Cramp, 2000). It is interesting that germination of *S. aloides* is enhanced when seeds pass through the animal digestive tract (Smolders *et al.*, 1995b). Thus, the late summer migration of waterfowl, which generally occurs in north–south and east–west directions (Boere *et al.*, 2006) may help to explain the genetic structure of *S. aloides* in Europe. However, as demonstrated by the Mantel test between F_{ST}/Φ_{ST} matrices and geographic distances, unconnected populations of *S. aloides* (e.g. NED, BAV and ASR) have low or absent gene flow, resulting in a higher degree of differentiation over time. Consistently, aquatic macrophytes such as *S. aloides* that often occur in isolated hydrographic basins may be affected by recurring population bottlenecks, cycles of local colonization and extinction that together affect genetic patterns across populations (Incagnone *et al.*, 2015).

Previous genetic analyses on clonal dioecious aquatic species belonging to Hydrocharitaceae yielded discordant results. A high level of between-population differentiation has previously been reported in *Thalassia testudinum* using AFLP (Waycott and Barnes, 2001); on the contrary, low differentiation between populations was detected in *Vallisneria spinulosa* S. Z. Yan (Chen *et al.*, 2007) by using allozyme variation and in other aquatic species, such as the aquatic fern *Marsilea quadrifolia* L. using AFLP (Bruni *et al.*, 2013). Species-specific characteristics and different dispersal ability may explain such differences.

Implications for reintroduction

Stratiotes aloides is ecologically important because it supports a rich macroarthropod diversity and some invertebrate and vertebrate species are exclusively or largely dependent on its presence, such as the dragonfly *Aeshna viridis* (Suhonen *et al.*, 2013) and the black tern *Chlidonias niger* (Beintema, 1997). Therefore, its conservation is of increasing importance in Europe, and experience from Italy, where the species is preserved *ex situ*, can contribute to the elaboration of an integrated conservation strategy for this species throughout its whole range.

Ex situ preserved populations are often characterized by depauperate genetic diversity (Rucińska and Puchalski, 2011), which makes the reintroduction of species or genotypes extinct in the wild challenging. Restoration practices are generally successful in re-establishing populations that hold a degree of genetic diversity comparable with those of natural populations, especially when calibrated for the number of individuals sampled (Halbur *et al.*, 2014; Gentili *et al.*, 2015). In the case of *S. aloides*, our first hypothesis of low genetic diversity in the original Italian female population should be rejected, despite many years of *ex situ* clonal reproduction. In fact, AFLP analysis revealed a medium level of genetic variability suggesting that this population is not inbred. Whatever the reason (somaclonal mutation, residual diversity or polyploidy), reinforcement with female individuals from other European populations aimed

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at increasing the population's genetic diversity might not be necessary in a translocation trial. However, an important task for the re-establishment of a viable population in the long term is the reintroduction of male individuals. These must necessarily be introduced from a population outside of Italy, as the Italian males became extinct more than 50 years ago. Two strategies can be adopted: maximizing genetic diversity by mixing genotypes from different clusters, or preserving the original genotype by introducing male plants from populations belonging to the same cluster as the Italian females. In the first strategy, the risk of altering the adaptability of the original Italian population through outbreeding depression should be taken into account (Edmands, 2007). Moreover, the natural gene flow that was responsible for the present genetic structure of *S. aloides* may also be compromised. In the second strategy, the outbreeding depression is avoided but it may result in reduced within-population genetic variation. In any case, the present study proves that suppositions on the genetic structure of plant populations based on geographical proximity may be wrong and that the choice of the closest population as source material for translocation may not be the best solution.

An alternative option might be considered, in which male and female individuals are translocated from populations with ecological characteristics (e.g. water quality, turbidity; Boedeltje *et al.*, 2001) similar to the translocation release site, irrespective of the genotypic cluster. This option is interesting because *S. aloides* was shown to be extirpated in Italy by the degraded water quality in the Po Basin (Abeli *et al.*, 2014). The Po river basin in particular is characterized by high levels of inorganic nitrogen (i.e. nitrites and nitrates) so individual plants taken from localities with similar ecological conditions may be more tolerant of this type of water pollution. The choice of source populations, however, requires further detailed experimentation on the tolerance limits of *S. aloides* to different environmental stressors (Harpenslager *et al.*, 2016). Moreover, this option might lead to the artificial genetic breeding of the Italian population. Therefore, considering that the within-population variation is possibly at an acceptable level, reintroducing males from the same cluster of the Italian females would be the best practical option for restoring a viable population of *S. aloides*. In particular, male individuals should be taken from NED or from ROM2 populations. The choice of the source population for male reintroduction is not the only important consideration in the translocation of a dioecious species as sex ratio and ploidy should also be considered. An inappropriate sex ratio (related to the reproductive strategy of the species) may result in low reproductive success. Ploidy should be considered, especially when different chromosome numbers are found in different populations; in the present study polyploidy contributed to maintaining a high genetic variation in *ex situ* populations.

The case of *S. aloides* highlights the issues related to the reintroduction of dioecious species that although not very common, represent a particular challenge for conservationists (Rottenberg and Parker, 2003; Ye *et al.*, 2007). Problems regarding the genetic structure and ploidy of the source populations should be addressed with ad hoc studies, especially for those species characterized by a long-distance dispersal strategy, even if long-distance dispersal events occur only occasionally. In addition, the approach used here to identify the most appropriate source population(s) for reintroduction is relevant in the context of de-extinction, in which new wild populations are established from *ex situ* genetic materials. This practice is likely to become increasingly important in the future, especially for plants.

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