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Phylogenetics and phylogeography of the monocot genus *Baldellia* (Alismataceae): Mediterranean refugia, suture zones and implications for conservation

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ABSTRACT

Aquatic plants, and especially the emblematic genus *Baldellia* (Alismataceae), are among the most threatened organisms, due to unprecedented human-driven habitat destructions. Therefore protection plans are crucially needed and call for thoroughly documenting the genetic diversity and clarifying the taxonomy of this endangered genus. Our sampling included 282 individuals from 42 natural populations and covered the whole geographical range of the genus, across Europe and the Mediterranean. We combined sequencing of nuclear internal transcribed spacer (ITS) and chloroplastic *trn*L-*ndh*F regions with amplified fragment length polymorphism (AFLP) genotyping to investigate the Alismataceae phylogeny, and produce a phylogeography of *Baldellia*. Our phylogeny strongly supported the monophyly of *Baldellia* and placed it as the sister clade to *Luronium* and *Alisma*, therefore excluding, as previously supposed, a close genetic relatedness to the predominantly neotropical genus *Echinodorus*. The phylogeography of *Baldellia* outlined patterns consistent with a hypothesis considering glacial refugia located in the Iberian Peninsula and the Italy/Balkan region from which two distinct genetic lineages re-colonized Europe. These two lineages corresponded respectively to *Baldellia ranunculoides* (Italy/Balkan derived populations) and *Baldellia repens* (populations recovered from the Iberian Peninsula refuge), therefore supporting differences outlined between the two taxa in previous ecological and morphological studies. These results allowed clarifying taxonomic uncertainties by confirming the genetic distinctness of *B. repens* according to *B. ranunculoides*. A third lineage, *Baldellia alpestris*, originated and remained endemic to the mountainous regions of the Iberian Peninsula. Unexpectedly, *B. repens* populations collected in northern Africa, appeared to be genetically distinct from their European counterparts, this calls for further investigation to fully address their genetic and conservation status. Finally, we detected a large hybridization zone in northwestern Europe between *B. repens* and *B. ranunculoides*. These results were discussed in light of conservation approaches for *Baldellia* populations.

1. Introduction

Aquatic plants of Europe and the Mediterranean belong to the most threatened organisms, due to unprecedented human-driven habitat fragmentation and destruction (Preston and Croft, 2001). Investigating the phylogeography of these plants is of prime interest because it accurately depicts the complexity of these endangered organisms and allows optimizing protection plans for conservation purposes (Newton et al., 1999; Forest et al., 2007). The recent and most influential phylogeographies revealed characteristic patterns owing to Quaternary climatic oscillations, such as refuge areas, post-glacial re-colonization pathways and hybridization zones (e.g. Taberlet et al., 1998; Hewitt, 2000, 2001, 2004). However, these studies were carried out almost exclusively on land organisms and only fragmented insights are available for wetland plants (Kozlowski et al., 2009a).

Here we focus on the genus *Baldellia*, an exclusively aquatic Alismataceae (Cook, 1990; Les et al., 1997) that experienced Quaternary climatic oscillations in Europe, as attested by fossil remains found in Pliocene deposits from Germany and France (Mai and Walther, 1988; Kozlowski et al., 2008). All *Baldellia* taxa are stoloniferous, mostly perennial, rooted water plants and grow in lakes, ponds and slow streams (Vuille, 1988; Kozlowski et al., 2008). The genus *Baldellia* belongs to the Atlantic–Mediterranean element of the European flora (Kozlowski et al., 2009a). Plants under this biogeographical classification, comprising approximately 100 taxa (Roisin, 1969), grow mainly in coastal regions of western

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Europe but possess also some isolated populations in the Mediterranean, notably in areas with distinctive oceanic climates (elevated humidity and mild winters).

The taxonomic identity of *Baldellia* species presents continuing difficulty due to the large degree of phenotypic plasticity and ecological similarities, as observed in a majority of aquatic plants (Chase et al., 1993; Lehtonen and Myllys, 2008; Li and Zhou, 2009). The generic definition remains unclear, as some authors have intended to merge *Baldellia* within the mainly neotropical genus *Echinodorus* (Cook, 1990). Confusion also exists for circumscribing species within *Baldellia*. Generally, two taxa are recognized: *Baldellia ranunculoides* (L.) Parl. and *Baldellia alpestris* (Coss.) Vasc. (Cook, 1983; Kozlowski et al., 2008). Many studies have recognized, however, a third taxon, either called *B. ranunculoides* subsp. *repens* (Lam.) A. Löve & D. Löve or *Baldellia repens* (Lam.) van Ooststroom (Lawalrée, 1959). *Baldellia* is therefore an excellent example of the Linnean shortfall (uncertainty in species number and taxonomy: Bini et al., 2006; Kozlowski, 2008) among vascular plants in Europe, which adds further complexity to the assessment of its conservation status (Kozlowski et al., 2009b).

Most genetic studies including *Baldellia* species have focused on general patterns in Alismataceae and Alismatales phylogeny (Chase et al., 1993; Les et al., 1997; Chen et al., 2004; Jacobson and Hedrén, 2007; Li and Zhou, 2009). The order Alismatales is indeed of great interest because it has often been regarded as one of the most ancient group of the monocotyledons and therefore centers several studies investigating the systematics, biogeography and evolutionary processes of flowering plants (e.g. Les and Schneider, 1995; Les et al., 1997; Chen et al., 2004; Jannsen and Bremer, 2004). As a consequence, apart from a minor investigation of the isozyme variations between *Baldellia* taxa carried out by Triest and Vuille (1991), no detailed phylogeographic studies based on molecular data has previously been performed exclusively on the genus *Baldellia*. Furthermore, peripheral populations from the eastern Mediterranean and many isolated and today endangered populations have been rarely, if ever, visited and sampled (Kozlowski et al., 2009b).

The present study (i) clarifies the monophyly of the genus *Baldellia*, its position within the Alismataceae family and its species circumscription, (ii) provides a comprehensive phylogeographic framework taking into consideration the recent post-glacial history of Europe. Since aquatic plants are underrepresented in genetic analyses and surveys, our study presents an important completion to the land-plant dominated literature on European phylogeography. Additionally, it delivers insights about the Atlantic–Mediterranean element to which *Balldellia* belongs – a biogeographic plant group for which phylogeographic data is fragmented. Furthermore, our work will contribute to the conservation efforts and priority settings for all *Baldellia* taxa and populations throughout its area of distribution.

2. Materials and methods

2.1. Plant material, sampling and DNA extraction

We used the taxonomic division proposed by Triest and Vuille (1991), recognizing three distinct taxa (*B. alpestris*, *B. ranunculoides*, and *B. repens*). *B. ranunculoides* grows mainly in coastal regions of Western Europe and the eastern Mediterranean, *B. repens* appears to be restricted to more Atlantic regions and *B. alpestris* is endemic to mountainous regions in northern Portugal and northwest Spain (Kozlowski et al., 2008). For more details on the genus *Baldellia* see Casper and Krausch (1980), Vuille (1988), Triest and Vuille (1991), as well as our previous studies (Kozlowski et al., 2008, 2009b; Kozlowski and Matthies, 2009; Kozlowski and Vallelian, 2009).

Leaf material of *Baldellia* taxa was collected from 42 natural populations exhaustively covering the geographical range of the genus across Europe and the Mediterranean (see Table S1 in Supplementary Material for geographical coordinates and short site characterization): 21 populations have been attributed morphologically to *B. ranunculoides*, 11 to *B. repens*, and ten to *B. alpestris* using the detailed determination keys of Triest and Vuille (1991) and Kozlowski et al. (2008). In larger populations, at least ten individuals were collected, and in smaller populations, all plants were collected. Altogether, 282 individuals were included in the analysis (68 of *B. alpestris*, 129 of *B. ranunculoides* and 85 of *B. repens*). Collections were made in the years 2006–2008 at the height of the growing season (June/August). Leaves were washed thoroughly in water, dried with paper towels and stored in plastic bags with silica gel (Chase and Hills, 1991). DNA extractions were performed with approximately 100 mg of silica gel-dried leaf material using the CTAB protocol (Chen and Ronald, 1999). Three molecular genetic analyses were conducted using this material (the complete list of samples and analyses is presented in Table S1). First, in order to build a phylogeny of the family, we sequenced a portion of nuclear DNA for a subset of the three *Baldellia* taxa (two *B. alpestris* individuals, five *B. repens* and three *B. ranunculoides*). This dataset was completed with Alismataceae sequences available on GenBank (Table S2), including *Albidella*, *Alisma*, *Astonia*, *Baldellia*, *Caldesia*, *Echinodorus*, *Helanthium*, *Luronium*, *Ranalisma*, *Sagittaria*, *Wiesneria*, and Butomaceae (*Butomus umbellatus*, used as outgroup). Sequences of *Burnatia*, *Damasonium* and *Limnophyton* were not available and thus not included in the Alismataceae phylogeny. Second, the phylogeographic patterns within *Baldellia* taxa were unraveled using chloroplast DNA (cpDNA) sequencing for a subset of 89 individuals, including all three *Baldellia* taxa and *Alisma plantago-aquatica* (used as outgroup). Third, fine-scale phylogeography was investigated using amplified fragment length polymorphism (AFLP) analysis. This analysis was performed with all *Baldellia* samples (282 individuals) and completed with several outgroups from the Alismataceae (*A. plantago-aquatica*, 2 populations/9 individuals; *Sagittaria latifolia*, 2/9; *Echinodorus horizontalis*, 1/3) and Butomaceae (*Butomus umbellatus*, 1/2). All outgroup samples were collected from natural populations (see Table S1 for further details), and DNA was extracted as described above.

2.2. Nuclear and cpDNA sequencing

The nuclear DNA sequencing focused on the internal transcribed spacer (ITS), which is located in ribosomal DNA, using primers described in White et al. (1990). PCR amplifications were performed on 30 ng of genomic DNA in a 25 μl reaction mixture [1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM primers and 1 U Taq polymerase (Promega, Madison, WI, USA)] using the following amplification program: 95 °C for 2 min followed by 35 cycles of 95 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min, plus a final extension of 10 min at 72 °C.

The cpDNA sequencing used the trnL(UAG) and ndhF primers (Shaw et al., 2007) to investigate three contiguous loci: the *ndh*F-*rpl*32 inter-genic spacer (hereafter IGS), the *rpl*32 gene and the *rpl*32-*trn*L IGS. PCR amplifications were performed on 30 ng of genomic DNA in a 40-μl reaction mixture (1× PCR buffer, 0.2 mg/ml BSA, 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.2 mM primers and 2 U Taq polymerase) using the "*rpl*16" PCR cycle proposed by Shaw et al. (2005, 2007). PCR products of nuclear and cpDNA markers were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and fluorescent sequencing was performed by Macrogen Inc. (Seoul, South Korea) using the same primers used for PCR amplification. ChromasPro (version 1.34, Technelysium Ltd., Helensvale, Queensland, Australia) was used for checking

the base calling and assembling complementary sequence strands. Sequences were deposited in GenBank under accession numbers: HQ607769 to HQ607776 for ITS and HQ607679 to HQ607768 for *trnL-ndh*F. Sequence alignments of nuclear and cpDNA datasets were performed using ClustalX (Thompson et al., 1997) and thereafter manually adjusted with the program Bioedit (Hall, 1999) using the similarity criterion (Morrison, 2006).

2.3. Phylogenetic analyses (nuclear and cpDNA)

Phylogenetic analyses and their corresponding bootstrap analyses were performed on the nuclear and cpDNA datasets separately, using the maximum likelihood (ML). ML analyses were performed using RAxML version 7.0.0 (Stamatakis et al., 2008) with 1000 rapid bootstrap analyses followed by a search of the best-scoring ML tree in one single run. These analyses were done using the facilities offered by the CIPRES portal in San Diego, USA (http://8ball.sdsc.edu:8888/cipres-web/home). As recommended by Stamatakis et al. (2008), the general time reversible model was used with an alpha parameter for the shape of the gamma distribution to account for among-site rate heterogeneity for both datasets. Highly congruent topologies were obtained using ratchet parsimony (Nixon, 1999), following the same method as in Buerki et al. (2009).

2.4. AFLP dataset

Reactions were performed using two PCR-selective, fluorescently-labeled primer pairs (EATC-MCAC and EACA-MGTC, protocol in Gugerli et al., 2008) and recorded on an ABI 3730XL capillary sequencer (Applied Biosystems, foster service provided by Macrogen Inc.). AFLP reactions were conducted on 96-well plates on which samples were randomly distributed. Eight samples chosen randomly from each plate were replicated (intra-plate replicates, representing 10% of the final dataset) to calculate the error rate (Bonin et al., 2004). Bands that were clearly not reproducible were discarded from further analyses. To detect and calculate the size of AFLP bands, raw electropherograms were analyzed with PeakScanner (Applied Biosystems, using default peak detection parameters except a light peak smoothing), and the scoring was performed using the automated RawGeno R CRAN package (Arrigo et al., 2009) using the following settings: scoring range = 100–400 bp, minimum intensity = 100 rfu, minimum bin width = 0 bp, and maximum bin width = 1 bp. Finally, non-parsimony-informative bands were removed and, as recommended by Vekemans et al. (2002), the correlation between AFLP band size and frequency among samples was assessed to check for potential homoplasy.

2.5. Bayesian clustering and Neighbor-Net

Bayesian clustering, as implemented in STRUCTURE 2.2 (Pritchard et al., 2000), was used to unravel phylogeographic patterns within the AFLP dataset. STRUCTURE results were processed using the SIMIL R script collection (Alvarez et al., 2008). The analysis assumed admixture and correlated allele frequencies between groups and used the default parameters of the program. Five independent runs were performed for each value of *K*, ranging from 1 to 20, with model parameters estimated for 400,000 Monte-Carlo Chain generations (following a burn-in period of 100,000 generations). For each *K* value, only runs yielding the highest maximum likelihood value were considered for further analyses. The selection of the best *K* value was achieved following the recommendations of Evanno et al. (2005). Using the best STRUCTURE run, samples were assigned to their respective groups following the majority-rule criterion (>0.5 in assignment probability). The genetic distances among the obtained groups were estimated using the net nucleotide distance, a measure of allele-frequency divergence implemented in STRUCTURE 2.2. In order to visualize the genetic relationships between the samples, we compared the obtained STRUCTURE groups to a Neighbor-Net analysis of the AFLP genotypes. This latter approach used a Dice distance computed between samples as implemented in SplitsTree 4.6 (Huson and Bryant, 2006). Only samples that could be attributed to a STRUCTURE group were analyzed using Neighbor-Net. Finally, because Bayesian clustering offered little phylogeographic resolution for *B. ranunculoides*, we further analyzed these populations using a principal coordinates analysis, following the approach described in Cavalli-Sforza (1996). In short, we performed a principal coordinates analysis between *B. ranunculoides* individuals, using a jaccard similarity distance, and projected the first eigen-axis on a geographical map. Computations were performed using the "vegan" and "fields" R CRAN packages.

2.6. Regional AFLP diversity

The diversity patterns of *Baldellia* species were investigated by computing the Shannon index on AFLPs, using a geographical 'sliding window' approach (as in Arrigo et al., 2010). The analysis considers a 75 km grid over the whole sampling area (i.e., one grid point for each 75 km, in latitude and longitude) and computes the Shannon index by considering samples located within a 100 km perimeter around each grid point. Computations are bootstrapped 1000 times by resampling 10 samples per grid point, in order to provide an unbiased Shannon index under unequal sampling among areas. Computations were performed using custom R scripts (R Development Core Team, 2010, script available on request). Analyses were performed using the complete AFLP dataset (i.e., merging datasets from all *Baldellia* species) in order to unravel patterns shaping the diversity of the genus.

3. Results

3.1. The genus Baldellia in the phylogeny of Alismataceae

The aligned ITS dataset was 614 bp length. However, to avoid misleading relationships due to alignment uncertainties, the following regions were discarded from the alignment before performing the phylogenetic analyses: 152–191 bp, 307–355 bp and 543–578 bp. The final dataset was 516 bp in length with a total of 265 variable characters of which 196 were potentially parsimony-informative. The obtained ML and MP topologies were highly congruent and to avoid redundancy only the ML phylogenetic tree is presented here (Fig. 1, log-likelihood = −5931.63). Both topologies strongly supported the monophyly of *Baldellia* (BS: 92) and placed it as the sister clade to *Luronium* and *Alisma* (BS: 78). Although not strongly supported, these three genera were retrieved as sister to the remaining genera of Alismataceae (Fig. 1).

3.2. Baldellia cpDNA phylogeny and phylogeography

The aligned *trn*L-*ndh*F dataset was 819 bp in length (excluding gaps), with a total of 55 polymorphic characters of which 41 were parsimony-informative. Only the maximum likelihood phylogeny is presented here (log-likelihood = −1359.19).

A total of 20 distinct cpDNA sequences (hereafter referred to as haplotypes) were detected, forming five major clades (i.e., Fig. 2a, outgroup and clades A to D). The distinction between the outgroup and *Baldellia* samples appeared rather clear. Nevertheless, inconsistencies were detected with clade A including *B. repens* and *B. ranunculoides* samples that showed haplotypes distantly related to those observed in the remaining *Baldellia* samples (i.e., clades B, C and D). Insights from AFLPs revealed that at least two of these

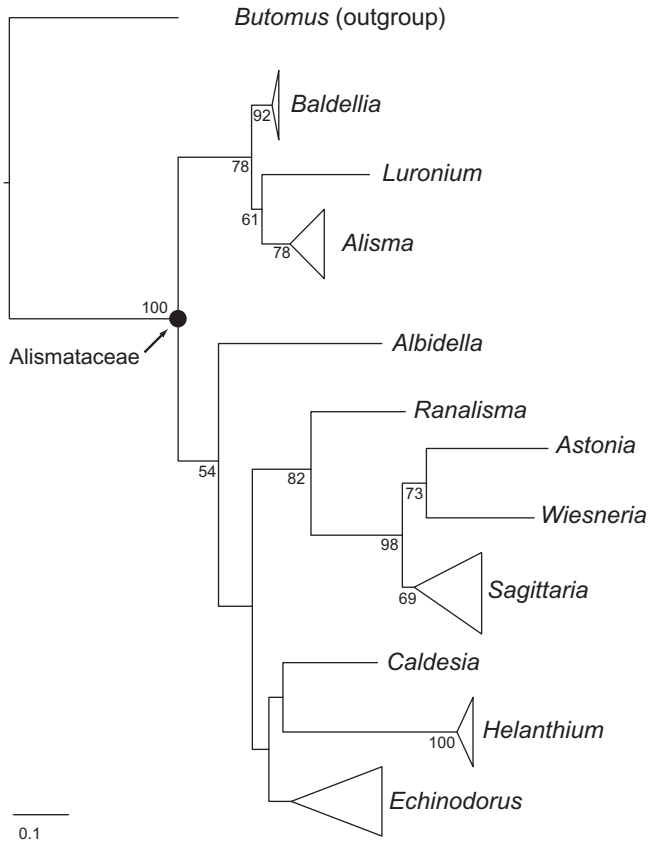

Fig. 1. Simplified phylogeny of the Alismataceae family, as revealed from the ITS nuclear sequencing (maximum likelihood tree, GTR + G model with estimation of invariable site proportions). Only supports greater than 50% are displayed (1000 bootstraps). The corresponding GenBank accession numbers are indicated in Table S2.

five samples were admixed with *A. plantago-aquatica* (i.e., Ran20_12 and Ran20_15, data not shown). On the other hand, one *A. plantago-aquatica* sample belonged to a *Baldellia* specific clade (i.e., clade B). We considered these six samples as evidences of inter-generic gene flow, and thus excluded them from phylogeography discussions. The congruence between the cpDNA topology and species circumscriptions within *Baldellia* was mitigated and varied between taxa (Table 1 and Fig. 2a). First, the majority of *B. alpestris* samples belonged to clade B (BS: 63) and appeared to be distinct from other taxa (only three samples unexpectedly clustered in clade C). Second, the sister group of *B. alpestris*, which included clades C and D, represented all the *B. ranunculoides/repens* samples (BS: 87). Within this sister group, the distinction between the two taxa appeared unclear. *B. ranunculoides* was mostly represented in clade D, with 32 samples in clade D versus six samples in clade C. Addressing *B. repens* required us to consider European and Tunisian samples separately: all eight Tunisian samples belonged to clade D while European *B. repens* samples were almost equally represented in clades C and D (with 6 and 7 samples, respectively). In addition, six Tunisian samples showed a specific variant nested within clade D haplotypes, diverging by three base pairs from its closest European relatives (Fig 2a: Rep9_4, Rep9_6, Rep9_12, Rep10_2, Rep10_6 and Rep10_15).

Phylogeographic patterns are outlined in Fig. 2b. Clade B was restricted to the Iberian Peninsula, clade C was represented in Portugal, western France and the British Isles, and clade D was largely spread across continental Europe, ranging from the Balkan Peninsula to the British Isles. Clades B, C and D matched the proposed taxonomical division for most of the samples that were collected in Portugal and Spain (populations Alp1–Alp10 associated to clade B, populations Rep1 and Rep2 associated to clade C) and southern Europe (Ran10 to Ran21, associated to D haplotypes). In contrast, most of the *B. ranunculoides* and *B. repens* samples collected in northwestern Europe (Ireland, United Kingdom, Benelux and France) were split between clades C and D. Finally, several connections between the Tunisian populations of *B. repens* and the southeastern Mediterranean populations of *B. ranunculoides* (Fig. 2a) were detected: (i) one Tunisian population (samples Rep11_11 and Rep11_17) showed European cpDNA haplotypes from clade D; and (ii) one South Italian population of *B. ranunculoides* (Ran17) showed the typical Tunisian cpDNA haplotypes of *B. repens*.

3.3. AFLP dataset

The two AFLP primer combinations produced an average of 71 bands per sample. In total, 177 polymorphic bands (95 and 82 for EACA-MCTG and EACT-MCAT, respectively) that were highly reproducible (3.16% and 4.27% of respective error rates) were analyzed. However, the potential presence of homoplasy in our dataset was revealed with a significant negative correlation between AFLP band size and frequency (correlation = −0.38, $p < 0.001$ and correlation = −0.4, $p < 0.001$ for EACA-MCTG and EACT-MCAT, respectively).

The Bayesian clustering reached an optimum with $K = 10$ groups (Fig. S1, log-likelihood = −8458.8). These ten groups, referred to as S1-S10, segregated the *Baldellia* samples (S1–S8 groups) from the *Alisma* (S9) and the *Sagittaria*/*Echinodorus*/*Butomus* individuals (S10). These clustering results matched almost perfectly with the morphological delimitations of *Baldellia* species (summarized in Table 1). Indeed, three groups characterized *B. alpestris* (S1–S3), four others were specific to *B. repens* (S4–S7), and only one group (S8) included all *B. ranunculoides* samples. However, several assignment inconsistencies were highlighted. Fourteen *Baldellia* samples were assigned to a genetic group different from that which would be expected according to their morphological identification. Among these unexpected genotypes, seven *Baldellia* samples were assigned to *Alisma* (S9) or to the outgroup with other Alismatales (S10). The remaining seven samples involved misaffiliations between *Baldellia* taxa: two *B. alpestris* samples were assigned to S4 (typical of *B. repens*), two *B. repens* were assigned to S1 and S8 (the groups characterizing *B. alpestris* and *B. ranunculoides*, respectively), and three *B. ranunculoides* belonged to S1, S4 and S6, the typical groups of *B. alpestris* and *B. repens*. Finally, a total of 39 additional samples could not be attributed to one of the ten groups because of their low assignment probabilities (Table 1 and Fig. S2, NA samples).

The Neighbor-Net (Fig. 3a) analysis matched the Bayesian clustering results and further outlined divergences between *Baldellia* species and the outgroup samples. In addition, it confirmed the distinct status of *Baldellia* taxa because *B. ranunculoides*, *B. repens* and *B. alpestris* samples belonged to distinct clusters. Furthermore, the analysis showed that Tunisian populations of *B. repens* diverged with their European counterparts as much as with the other *Baldellia* species.

The geographical distribution of Bayesian clustering results revealed consistent patterns (Fig. 3b). The groups typical of *B. alpestris* discriminated Spanish (S1) from Portuguese (S2, S3) populations. The S4–S7 groups, typical of *B. repens*, revealed populations from Portugal, the British Isles, Benelux and Tunisia as being four distinct genetic entities. Finally, the S8 group covered almost the complete sampling area (with the exception of the Iberian Peninsula) and included the complete genetic diversity of *B. ranunculoides*.

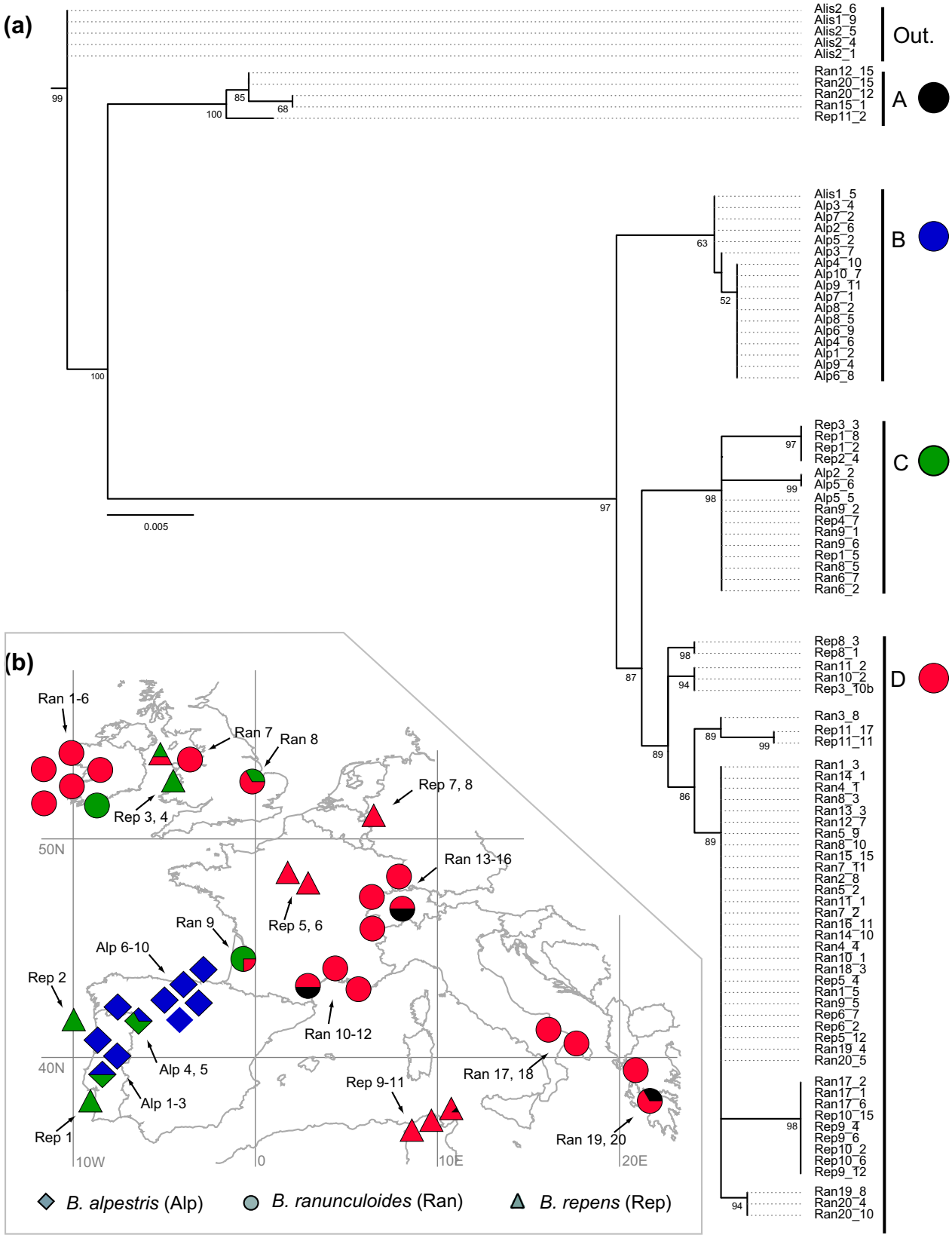

Fig. 2. (a) Phylogeny of the genus *Baldellia*, as revealed from sequences of chloroplast *trn*L-*ndh*F inter-genic spacer (maximum likelihood tree, GTR + G model with estimation of invariable site proportions). Tips are labeled according to their taxonomic status (with taxa names as acronyms) and numbered according to their population and individual number (corresponding to Table S1). Four supported clades (A–D) are defined based on the obtained topology. Only supports greater than 50% are displayed (1000 bootstraps). (b) Geographical representation of the four cpDNA clades defined in (a). The results are displayed for each sampled population. Pie-charts represent the proportions of observed cpDNA clades. The shape of pie-charts corresponds to the taxonomical status of populations: circles – *B. ranunculoides*, triangles – *B. repens*, and diamonds – *B. alpestris*.

Table 1
Comparison between the taxonomical status of individuals included in the present study and their cpDNA haplotypes and AFLP Bayesian groups. Outgroup taxa are outlined in grey.

	cpDNA[a]						Bayesian clustering[b]											
	Out	A	B	C	D	Total	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	NA	Total
B. alpestris			16	*3*		19	26	16	10	*2*							14	68
B. repens		*1*		6	*15*	22	*1*			11	17	13	26	*1*			16	85
B. ranunculoides		*4*		6	32	42	*1*			*1*		*1*		113	*5*	*2*	6	129
A. plantago-aquatica	5		*1*			6									8		1	9
S. latifolia																8	1	9
B. umbellatus																2		2
E. horizontalis																2	1	3

[a] Four highly supported clades (A–D) were defined according to the phylogeny the genus *Baldellia* (*Maximum likelihood* tree based on *trn*L-*ndh*F inter-genic spacer).
[b] Ten genetic groups were defined (S1–S10) using STRUCTURE 2.2. Individuals were attributed to S1–S10 when their respective assignment probability exceeded 50%. Samples that could not be attributed at 50% probability were labeled as NA.

Examining the genetic distances among these groups revealed fine-scale phylogeographic patterns. The groups S1–S3 (i.e., *B. alpestris*) showed the largest genetic differentiation in the *Baldellia* dataset (with an average allele-frequency divergence of 6.9 between the S1–S3 groups). The groups S4–S7 (corresponding to *B. repens*) outlined large-scale patterns. A close genetic relationship (3.40 allele-frequency divergence) was revealed between Portuguese samples (S4) and those collected in the British Isles (S5). Samples occurring in central Europe (S6) appeared to be the most genetically distant group of the European *B. repens* populations (with 4.78 and 5.06 allele-frequency divergence for S4 and S5, respectively). Finally, the Portuguese group (S4) represented the closest European relative of the Tunisian S7 group, with an allele-frequency divergence of only 4.25 (whereas S5 and S6 had 6.7 and 7.1 allele-frequency divergences with S7). Bayesian clustering revealed no fine-scale genetic patterns within *B. ranunculoides* (i.e., the S8 group). Nevertheless, the principal coordinates analysis revealed a gradient of increasingly divergent genotypes across Europe within this group, ranging from the Balkans to the British Isles (Fig. S3).

The sliding window revealed contrasting genetic diversity patterns across the different sampling areas (Fig. 4). The highest diversity values were observed on the southern margins of species distribution (i.e., northern Africa, southern Italy and Greece) or where several species co-occurred (i.e., the Iberian Peninsula and western England). Notably, populations from northern France, Benelux and Ireland were unexpectedly diversified (this pattern was consistent with analyses considering each species separately, data not shown). In contrast, populations collected from Southern England, Mediterranean France and Switzerland had low diversities.

4. Discussion

4.1. Monophyly of Baldellia and position in the phylogeny of Alismataceae

Our ITS phylogeny clearly places *Baldellia* in a clade composed of *Alisma* and *Luronium* (Fig. 1). This result corroborates several recent molecular, chromosomal and anatomical studies suggesting that *Baldellia* is actually closely related to *Alisma*, *Damasonium* and *Luronium* (Uchiyama, 1989; Les et al., 1997; Posluszny et al., 2000; Charlton, 2004; Chen et al., 2004; Lehtonen, 2009). Our study definitively ends the long standing discussion about the relationship of *Baldellia* with the predominantly neotropical genera *Echinodorus* and *Helanthium*, as suggested by Cook in his influential book on the aquatic plants of the world (Cook, 1990). Therefore, morphological similarities of these genera probably result from convergences and not from genetic relatedness.

In addition, our ITS analysis confirms the monophyly of *Baldellia* and validates the genus definition. However, this view is nuanced by our population-scale cpDNA and AFLP datasets, which both suggest gene flow between *Baldellia* and *Alisma* species (and possibly with other Alismataceae genera, Table 1, Fig. 2a and Fig. S2). These results corroborate ecological observations in which the only reported inter-generic hybrids of *Baldellia* in the wild have been with *A. plantago-aquatica* and *Alisma lanceolatum* (Kozlowski et al., 2008). The existence of *Alisma* × *Baldellia* hybrids could be explained by the phylogenetic relatedness of these two genera (Fig. 1).

4.2. European phylogeography of Baldellia: glacial refugia and recolonization routes

The early presence of *Baldellia* in Europe is attested by the numerous fossils reported in Pliocene deposits collected in central and western Europe (seed remains dated to 2.5–3.4 m.y. old, Mai and Walther, 1988; Kozlowski et al., 2008). Consequently, *Baldellia* clearly experienced Quaternary glaciations in European territories and probably underwent dramatic range contractions and/or expansions across Europe during the last two million years.

Accordingly, our chloroplast and AFLP datasets reflected genetic patterns that probably originated during and since the last glacial event. Insights from cpDNA revealed that three genetic lineages (clades B–D, Fig. 2a) shaped the distribution and diversity of *Baldellia* populations, with clades B and C being restricted to the Atlantic coasts of Europe (Fig. 2b) and clade D covering almost completely the geographical range of *Baldellia* (but absent from the Iberian Peninsula, Fig. 2b). Congruent insights were provided by AFLPs, as the groups S1–S3, S4–S6 and S8 broadly matched with the cpDNA clades B, C and D, respectively (but see below). These results strongly suggested that Atlantic lineages (S1–S3 and S4–S6 AFLP groups; cpDNA clades B and C) had been recovered from an Iberian refugium. On the other hand, populations from group S8 (cpDNA clade D) probably originated from an Italian or Balkanic refugium (our sampling did not allow for discriminating between these two potential origins; we therefore refer to the "Italy/Balkans refugia"). In support of this hypothesis, the Iberian Peninsula has long been recognized as a main glacial refugium for many plant and animal species (corresponding to R1 in Taberlet et al., 1998), including plants of the Atlantic phytogeographical element, the group to which *Baldellia* belongs (Kozlowski et al., 2008, 2009a). Similar observations were reported for the Italy/Balkans refugium. These regions correspond to R2 and R3 refugia in Taberlet et al. (1998) and were connected to the Iberian refugia during interglacial periods (as reported for European ivy, *Hedera* sp., populations, Grivet and Petit, 2002). While the Italy/Balkans refugium might appear counterintuitive when considering the current geographical distribution of *Baldellia* taxa, this may be explained by the fact that eastern Mediterranean populations of *Baldellia* suffered from dramatic climatic changes during the last millennia

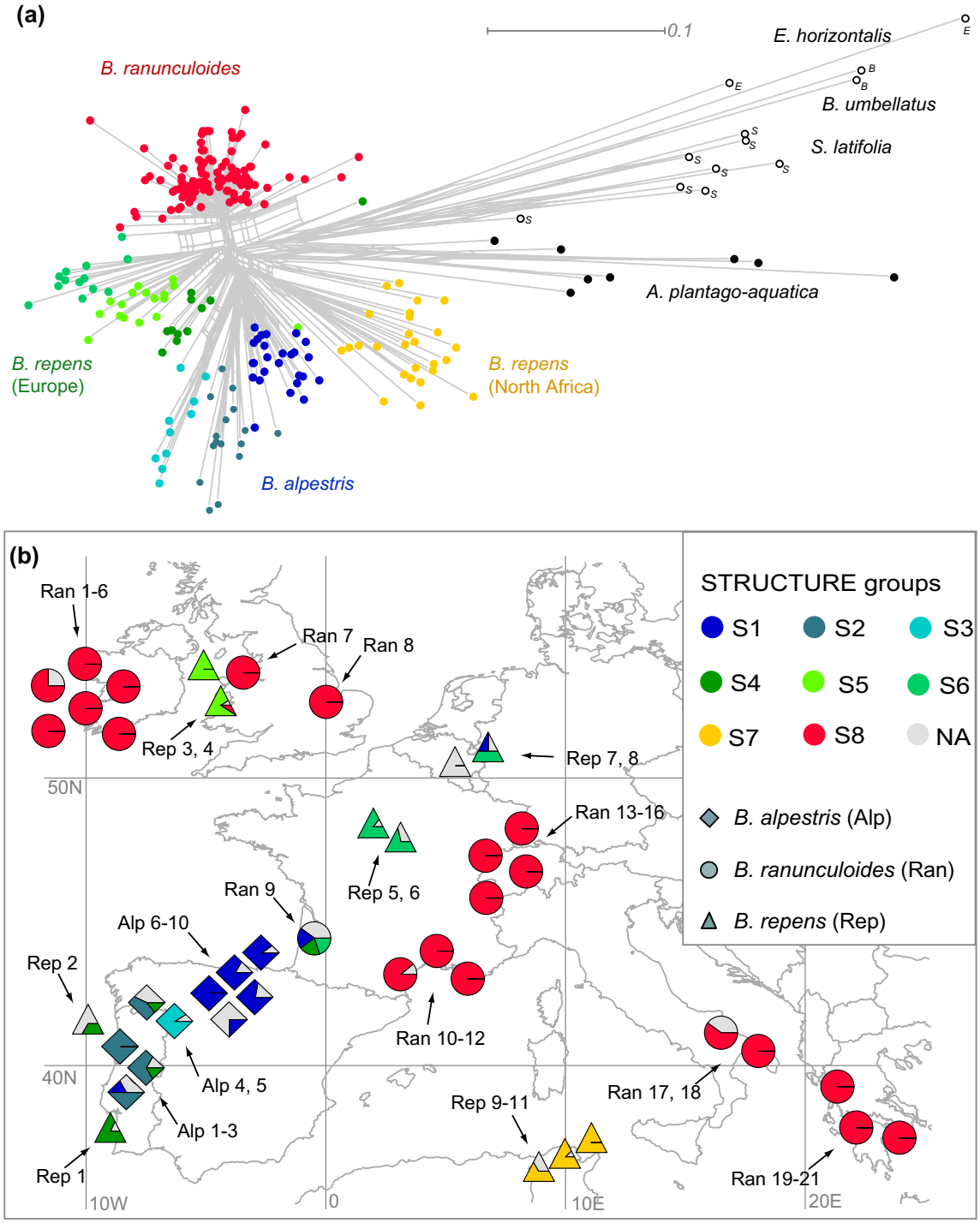

Fig. 3. (a) Neighbor-Net of *Baldellia* individuals (AFLP dataset, computed on a Dice distance), with individuals labeled according to groups (S1–S10) defined using Bayesian clustering (STRUCTURE 2.2, $K = 10$ groups, individuals are attributed to a group when exceeding 50% assignment probability). The taxonomic status of samples is indicated; blue – *B. alpestris*, green – European *B. repens*, orange – North-African *B. repens*, red – *B. ranunculoides*, black – *Alisma plantago-aquatica* and open circles – outgroups (*Sagittaria latifolia*, *Echinodorus horizontalis* and *Butomus umbellatus*). The individuals that could not be attributed at 50% probability were excluded from this display. (b) Geographical mapping of Bayesian clustering groups. The proportions of individuals observed among groups S1–S10 are reported for each population with pie charts. Individuals that could not be attributed at 50% probability (i.e., NA individuals) are represented in grey. The shape of pie-charts corresponds to the taxonomical status of populations: circles – *B. ranunculoides*, triangles – *B. repens,* and diamonds – *B. alpestris*.

and recently, from severe human-made habitat destructions. Thus, these current isolated populations probably represent relicts of former larger populations.

Further insights regarding the post-glacial re-colonization routes potentially followed by *Baldellia* populations were revealed by the AFLPs (i.e., groups S1–S8, Fig. 3a). As mentioned above, these groups broadly matched the cpDNA phylogeny and were notably highly corelated to the taxonomic status of samples (Table 1). First, S1–S3 groups were restricted to the Iberian Peninsula and largely matched with cpDNA clade B. They corresponded to *B. alpestris* populations and were restricted to high altitude ponds and rivers of northern Portugal and Spain. Their genetic

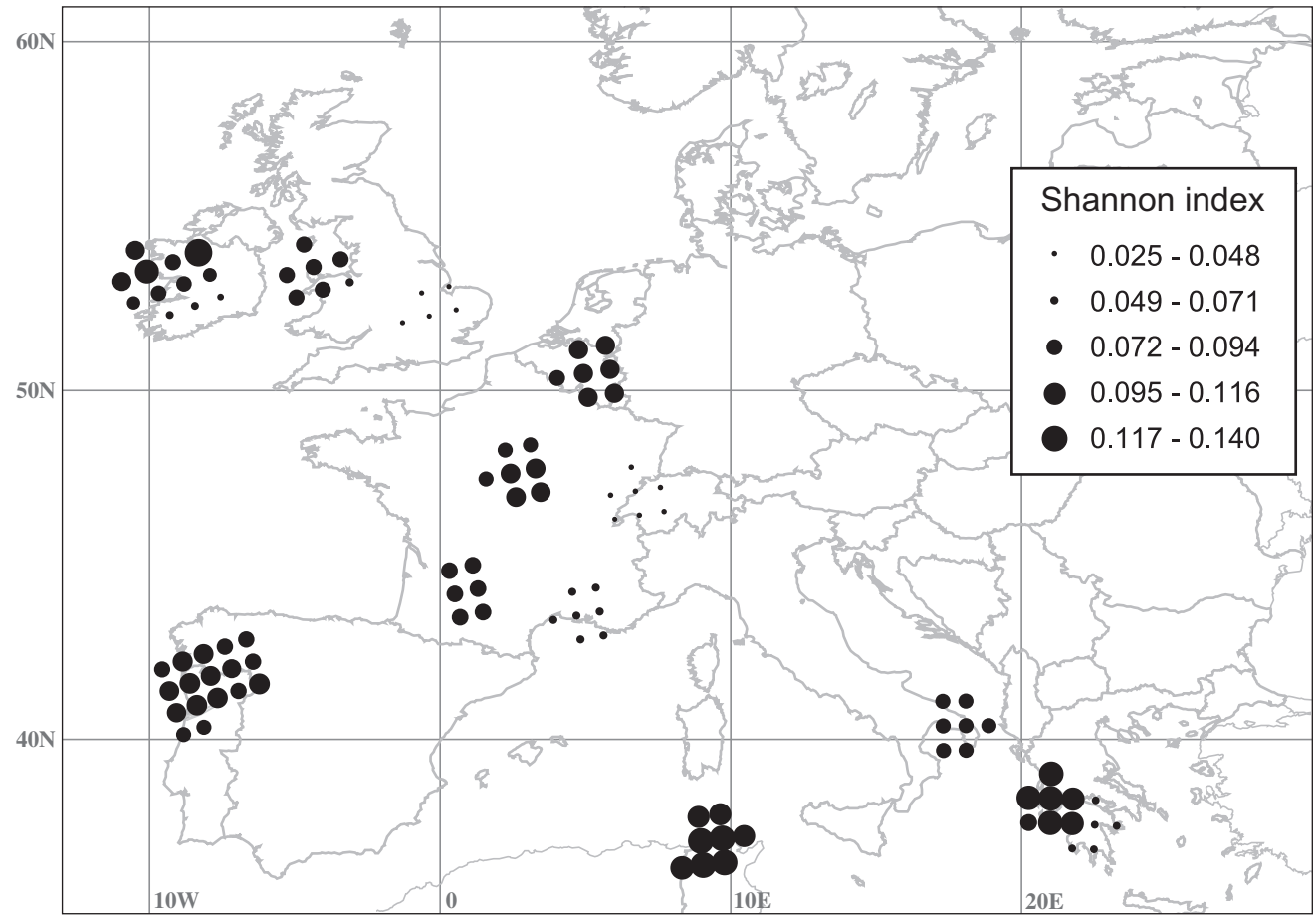

Fig. 4. Regional patterns of genetic diversity within the genus *Baldellia*, measured using a sliding window on a 75 km grid (Shannon index, 10 individuals are sampled per grid cell, the displayed results are averaged from 1000 bootstraps).

distinctness to other *Baldellia* taxa and their sister clade position in the genera phylogeny were in agreement with previous isozyme data (Triest and Vuille, 1991). This pattern probably reflected the geographical isolation inherent to the ecological niche of this *Baldellia* species. The S4–S6 groups corresponded to the European populations of *B. repens*. The genetic connections among these groups appeared to be complex because low allele-frequency divergences suggested genetic relatedness between populations from the Iberian Peninsula (i.e., S4) and those collected in the British Isles (S5). These results outline geographical patterns that are usually referred to as Hiberno-Cantabrian connections (Kingston and Waldren, 2006) and reflect former genetic exchanges between populations of these areas. Finally, the S8 group was more largely spread and showed less phylogeographic patterns (Fig. 3a), except a southeastern–northwestern genetic gradient, as further revealed from principal component analyses (Fig. S3). These populations included all *B. ranunculoides* samples and corresponded to haplotypes included in clade D.

4.3. Suture zones in northwestern Europe

Both cpDNA sequencing and AFLP genotyping revealed the presence of hybrid genotypes between *Baldellia* taxa (Fig. 2a, Table 1, Fig. S2). Indeed, not less than 53 *Baldellia* samples showed admixed AFLP genotypes (39 samples) or inconsistencies between genotypes and morphological identities (14 samples). These results suggested that hybridization events could blur the apparent limits between *Baldellia* taxa.

Our study revealed a large hybridization zone in geographical regions where *Baldellia* lineages deriving from the Iberian Peninsula and the Italy/Balkans refugia met each other. Accordingly, several samples from the British Isles (for example Rep3, belonging to S5) as well as populations collected in northern France and Benelux (for example Rep5–Rep8, belonging to S6) showed unexpected genotypes. Although all these samples belonged to S4 and S5 AFLP groups (deriving from the Iberian refugium and morphologically attributed to *B. repens*), they possessed cpDNA clade D haplotypes, haplotypes that were specific to the Italy/Balkans refugia and normally corresponded to *B. ranunculoides* lineages. These hybridization areas matched closely with observations from other plant and animal species that had experienced similar glacial and post-glacial histories (corresponding to the *suture zones* reported in Taberlet et al., 1998). Further focus on the Franco-Benelux suture zone (populations Rep5–Rep8) informed us about the relative speeds of post-glacial re-colonization of Europe by *Baldellia* populations. When a local species is excluded from its ecological niche by an inter-fertile relative, the organelles of the local species are generally introgressed within the invading one (Currat et al., 2008). As a result, organelles detected in the invading species can reveal the identity of taxa that formerly occupied the area. Our results highlighted introgressed populations in a territory colonized by *B. repens*, as attested by morphological identification and AFLP genotypes (i.e., S5–S6 groups, which are specific to the Iberian Peninsula refugium), but associated to chloroplasts specific to the Italy/Balkans refugium (cpDNA clade D haplotypes and corresponding to *B. ranunculoides*). This pattern suggested that Italy/Balkans-derived lineages were first present in Benelux and

northern France and were subsequently replaced by populations derived from the Iberian Peninsula refugium. This hypothesis was consistent with the reproductive strategy of *B. ranunculoides* – a short, intensive flowering period, followed by abundant fruits that are well adapted for waterfowl dispersal and high seedling viability (Vuille, 1988) – that potentially allowed an early re-colonization of Europe.

4.4. Baldellia in northern Africa

Populations collected in Tunisia were morphologically assigned to *B. repens*. Their evolutionary trajectory probably diverged early from European populations, as evidenced by their markedly divergent genotypes. Accordingly, AFLP analysis revealed Tunisian genotypes were members of a genetic group (S7) that strongly diverged from all European *Baldellia* samples (Fig. 3a). These divergences could possibly reflect different colonizations leading to the establishment of Tunisian and European populations. For instance, several inter-glacial periods interrupted the Quaternary glaciations so that Tunisian populations could have been isolated from European ones during more than one glacial period. However, the geographical origin of Tunisian genotypes remained in question. The cpDNA phylogeny suggests connections with populations currently derived from the Balkan/Italian refugium (Fig. 2a) because Tunisian cpDNA haplotypes were nested within clade D. In addition, these specific haplotypes were also observed in southern Italy (Ran17 population, Fig. 2a), and suggested trans-Mediterranean connections between northern Africa and the Italian refugium (R2), as proposed already by Braun-Blanquet (1953) through the so called Italo-African bridge. However, AFLP showed that Portuguese *B. repens* populations (i.e., the S4 group) were the closest European relatives of the African plants (Table 1). Further research is thus needed to properly solve the genetic status of the North-African populations.

4.5. Genetic diversity of Baldellia populations: implications for conservation

The regional patterns of genetic diversity of *Baldellia* were consistent with results outlined by cpDNA and Bayesian clustering of AFLPs (Fig. 4). High genetic diversity levels were highlighted in the Iberian Peninsula and the Italy/Balkans refugia. These results are consistent with other studies focusing on European species (Taberlet et al., 1998; Grivet and Petit, 2002). They outline the importance of these areas for the conservation of *Baldellia* populations, as well as populations of other plants of the Atlantic–Mediterranean biogeographic group. In contrast, populations from southern France and Switzerland were less diversified, rather suggesting a recent dispersal through post-glacial re-colonization. Finally, Tunisian populations were also highly diversified, thus calling for further investigation to fully address their genetic and conservation status.

Our study shows that conservation approaches should always include phylogeographic surveys. First, we detected numerous instances of hybridization with *Alisma* genotypes. Given that several *Alisma* species co-occur in European wetlands with *Baldellia* species, one can ask whether *Baldellia* populations could be threatened by an "extinction through introgression" risk (Mallet, 2005). This calls for further investigation regarding the fitness of *Alisma* × *Baldellia* hybrids. Second, molecular markers helped circumscribing species within the genus *Baldellia*. Our results outlined *B. repens* as genetically distinct from *B. ranunculoides*, owing to differential refugial and post-glacial re-colonization histories. These results support biological and ecological differences described recently between these two *Baldellia* taxa (e.g. Kozlowski et al., 2008), and further validate *B. repens* as a distinct taxonomic entity. These results should allow better application of taxonomy-based conservation measures and field surveys for these endangered species. Third, we show that phylogeographic data provide accurate insights for the selection of populations worth protecting. For instance, focusing on areas with maximized genetic and/or taxonomic diversity can be misleading, as these criteria, if used alone, cannot discriminate refuge areas from post-glacial suture zones. Indeed, we report high genetic diversities in northwestern Europe, where *B. repens* and *B. ranunculoides* hybridized. To what extent suture zones are worth of protection remains under question. On one hand, these populations no longer correspond to original species genotypes, but on the other hand, their hybrid status might allow new ecotypes to emerge and thus present a strong evolutionary interest. Finally, there is no doubt that much more efforts should be invested in conservation of genetically diverse, isolated and today threatened *Baldellia* populations in refugial regions of the Mediterranean basin, including northern Africa.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.11.009.

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