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Global mitochondrial and chloroplast genome diversity in the threatened aquatic carnivorous plant *Aldrovanda vesiculosa*

Helena Štorchová [a,*], Manuela Krüger [a], Ian Small [b], Lubomír Adamec [c], Paul G. Nevill [d], Katharine A. Howell [b,1], Kingsley W. Dixon [d], Mark Derbyshire [e], Xiao Zhong [b,2], Adam T. Cross [f,g,**]

[a] *Institute of Experimental Botany, Czech Academy of Sciences, Rozvojová 313, Prague CZ-16502, Czech Republic*
[b] *Australian Research Council Centre of Excellence in Plant Energy Biology, The University of Western Australia, Crawley, WA 6009, Australia*
[c] *Institute of Botany of the Czech Academy of Sciences, Dukelská 135, Třeboň CZ-379 82, Czech Republic*
[d] *Trace and Environmental DNA Laboratory, School of Life and Molecular Sciences, Curtin University, G.P.O. Box U1987, Perth, WA 6845, Australia*
[e] *Centre for Crop and Disease Management, School of Molecular and Life Sciences, Curtin University, Perth, Australia*
[f] *School of Molecular and Life Sciences, Curtin University, G.P.O. Box U1987, Bentley, WA 6102, Australia*
[g] *Ecological Health Network, Brookline, MA, USA*

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ABSTRACT

The submerged aquatic carnivorous plant *Aldrovanda vesiculosa* (Droseraceae) is threatened by rapid deterioration of wetlands and oligotrophic lake habitats. Its native distribution spans four continents, but many historic populations are now extinct. Previous genetic studies found distinction between populations from Australia and those from the rest of the world, but due to limited genetic markers, neither detailed phylogenetic relationships nor the migration routes of *A. vesiculosa* populations were revealed. We used a de novo assembly of the *A. vesiculosa* mitochondrial genome and a previously published plastid genome as references for mapping short DNA sequence reads from 17 globally distributed populations. Phylogenetic trees were constructed based on detected polymorphisms. Genetic diversity of both the mitochondrial and plastid genome was low (Pi 0.55×10^{-4} and 0.7×10^{-4}, respectively). Greater polymorphisms were found in the mitochondrial compared with the plastid genome, owing to its larger size (1.27 Mb). Australian populations formed a monophyletic clade in both plastid and mitochondrial trees, while the mitochondrial tree also distinguished populations from southern and northern Europe. *Aldrovanda vesiculosa* likely migrated to Australia and Africa from a southern European refuge during the last interglacial period~100,000 years ago. When the last glaciation started, some populations could have survived in eastern Europe and moved north, when the continental glacier retreated. *Aldrovanda vesiculosa* experienced repeated population bottlenecks that reduced its genetic diversity.

1. Introduction

It is now widely accepted that more than 50% of the world's wetlands have been lost in the last century, and that inland freshwater wetlands are rapidly becoming one of the world's most at-risk ecosystems (Spiers, 1999; Amezaga et al., 2002; Davidson, 2014). The impact of multiple stressors (e.g., heat, drought, salinity, eutrophication) on freshwater habitats is reflected in increasing declines in the biodiversity of inhabitant flora and fauna, often implied from the loss of indicator taxa (e.g., Croonquist and Brooks, 1991; Collins and Storfer, 2003; Zedler and Kercher, 2005; Robledano et al., 2010). Highly sensitive and ecologically specialized plant species are usually among the first to disappear following environmental change and degradation (Swift and Hannon, 2010; Dawson et al., 2011; Moritz and Agudo, 2013),

* Corresponding author.
** Corresponding author at: School of Molecular and Life Sciences, Curtin University, G.P.O. Box U1987, Bentley, WA 6102, Australia.
E-mail addresses: storchova@ueb.cas.cz (H. Štorchová), adam.cross@curtin.edu.au (A.T. Cross).
[1] Present address: Graduate Research, Edith Cowan University, Joondalup, WA, Australia
[2] Present address: Genomics WA, Harry Perkins Institute of Medical Research, Telethon Kids Institute, The University of Western Australia, Nedlands, WA 6009, Australia

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particularly species that are slow-growing or exhibit low fecundity (Quesnelle et al., 2014) or those characterized by limited genetic diversity (Lacy, 1997; Hughes and Stachowicz, 2004). These characteristics typify many species of carnivorous plants (Clarke et al., 2018), and a recent study suggests that up to a quarter of the ca. 860 species of carnivorous plants that are currently described may be threatened with extinction (Cross et al., 2020).

A prime example of a highly sensitive carnivorous plant species considered an indicator of habitat quality is *Aldrovanda vesiculosa* L. (Droseraceae), a submerged, free-floating, rootless carnivorous macrophyte exhibiting an expansive bioclimatic envelope but considered Critically Endangered (Cross, 2012; Cross and Adamec, 2020). Despite exhibiting a narrow ecological niche, occurring only in nutrient-poor, acidic-neutral freshwater environments where competition from emergent macrophytes is limited (Cross et al., 2015; Adamec, 2018), the species was historically highly successful (as evidenced by its weed-like status following human introduction in north-eastern USA; Cross et al., 2015): the recorded historical distribution of *A. vesiculosa* encompassed an enormous latitudinal and longitudinal range spanning equatorial to cold temperate climatic zones from southern Australia to northern Russia and from Japan to West Africa (Cross, 2012). There is also fossil evidence from sediments buried by lava in east central Alaska indicating the species once occurred in subarctic North America (Matthews and Ovenden, 1990). *Aldrovanda vesiculosa* exhibits a strong reliance upon clonal reproduction at the expense of sexual fecundity (Cross et al., 2016; Adamec, 2018), and previous studies have indicated the species displays remarkably low genetic variation throughout its global range (Adamec and Tichý, 1997; Maldonado San Martín et al., 2003; Hoshi et al., 2006; Elansary et al., 2010). However, while these studies suggested a degree of genetic distinction between populations from Australia and those from the rest of the world, the limited genetic markers employed were unable to inform detailed phylogenetic relationships and it is unclear whether the apparent genetic uniformity can be attributed to a highly clonal life history, a great reduction of generative reproduction (Onelli et al., 2021), or the high likelihood of one or more bottlenecks followed by relatively recent post-glacial dispersal in its recent evolutionary history. It has also been suggested that *A. vesiculosa* possesses a slow mutation rate in nuclear and plastid genomes compared with other members of the Droseraceae (Hoshi et al., 2006; Elansary et al., 2010).

The genus *Aldrovanda* appears to be of early Tertiary origin and is believed to have experienced numerous significant bottleneck events during periods of glacial maxima leading to speciation in isolated refugia and rapid expansion during inter-glacial periods (reviewed in Cross, 2012). It has been suggested that *A. vesiculosa* persisted through the most recent glacial period in southern Europe, Africa, or Australia before recolonizing the European continent (Huber, 1961; Sculthorpe, 1967), likely within the last 50,000–200,000 years (Cross, 2012). However, genetic evidence supporting this hypothesis is lacking, and without sufficient evidence of phylogenetic relatedness it has not yet been possible to determine the possible routes by which *A. vesiculosa* might have migrated from one or more glacial refugia.

Although *A. vesiculosa* was once common and widespread across four continents in pre-agrarian/pre-industrial times, the species has diminished worldwide throughout the last century and has become increasingly rare (Adamec, 1995, 1999, 2005, 2018; Cross, 2012). The decline of *A. vesiculosa* closely matches the decline in the global wetland inventory both in rate, extent, and regional occurrence (Amezaga et al., 2002; Davidson, 2014), and the species is thus considered a bellwether of the way humans have progressively degraded freshwater environments (Cross, 2012). A strict specialist of medium-dystrophic, mesotrophic waters, *A. vesiculosa* appears extremely vulnerable to ecological change. Eutrophication of soft water habitats together with increased likelihood and duration of drought periods have been implicated as principal causes of the species global decline (Adamec, 1995; Jennings and Rohr, 2011; Cross, 2012; Cross et al., 2020). For example, > 60% of soft water habitats throughout Europe have disappeared or been significantly degraded over the last century (Amezaga et al., 2002). Europe represents a historical locus of *A. vesiculosa* distribution, and the timing of decline in the region's soft water habitats corresponds strongly with the decline of *A. vesiculosa* in the region as inferred from likely dates of extinction for historical populations (Cross, 2012). Fewer than 13% of the 292 historical *A. vesiculosa* subpopulations recorded from Europe remain extant, and the species has been confirmed extinct in six of the 19 European countries it is recorded from and is believed extinct in at least another four (Cross, 2012). Two-thirds of all remaining European *A. vesiculosa* subpopulations occur in a single river basin of the Danube and Dnieper Rivers and may be threatened by ongoing conflict in the region. The species has also been confirmed extinct in Bangladesh, India (though potentially rediscovered in 2019; Ngangbam et al., 2019), Uzbekistan, and East Timor, and its occurrence remains unverified in another 17 countries throughout Asia and Africa (Cross, 2012; Adamec, 2018). Without active conservation interventions it appears that this once widespread species may be on a trajectory toward European, and possibly global, extinction.

This study investigates genetic variation in the plastid and mitochondrial (mt) genomes among 17 disjunct populations of *A. vesiculosa* from four continents, improving the resolution of previous studies that were based on limited isozyme or DNA sequence data sets (Adamec and Tichý, 1997; Maldonado San Martín et al., 2003; Hoshi et al., 2006; Elansary et al., 2010). High-throughput sequencing has significantly increased the efficiency of studies examining genetic variation, particularly in species with very low levels of diversity, by enabling whole genome (typically organelle) sequencing of non-model species, resulting in a vast increase in the data available (Tonti-Filippini et al., 2017). This allows more detailed investigation of the relatedness and similarity of *A. vesiculosa* populations than has been previously possible, potentially offering greater insight into the ecology, historical distribution, and future conservation requirements of this iconic and unique species. We firstly investigate the possible impact of transoceanic dispersals in shaping the biogeography of *A. vesiculosa*, and then test the hypothesis that very recent expansion from temperate glacial refuges combined with life history traits/reproductive biology is the cause of genetic uniformity in the species.

2. Methods

2.1. Plant material

Vegetative *A. vesiculosa* material originating from nine European, one Asian, six Australian, and one African population was used for total DNA shotgun-sequencing. All sampled populations represent over one-third of all known recent sites (Adamec, 2018). They have been maintained as ex situ outdoor or indoor scientific collections at the Institute of Botany (Czech Academy of Sciences) at Třeboň, Czech Republic, since 1993 (see Elansary et al., 2010 for technical details). Individuals from each population were cultivated in discrete aquaria, maintained covered and physically separated to prevent mixing of individuals from different populations, and propagation within each aquarium was strictly via asexual clonal growth as is typical of the species (Cross, 2012; Onelli et al., 2021). One DNA sample was prepared from each population, representing ca. 5–6 individuals. Most accessions in the conservation collection represent material sent from botanists around the world and collected during field studies over the period 1993–2013, and as such, only two have official IPEN voucher codes. However, most of *A. vesiculosa* accessions maintained in the collection have been utilized for previously published analyses (Maldonado San Martín et al., 2003; Elansary et al., 2010) and have internal official institutional voucher codes. Both voucher systems are shown in Table 1.

Table 1
The populations of Aldrovanda vesiculosa examined in this study: collection locality and habitat; voucher IBOT, unofficial voucher codes used in the Institute of Botany, Třeboň, Czech Rep. (in italics); voucher IPEN, IPEN voucher codes.

Population (Continent)	Location	Collection site	Latitude Longitude	Year of collection	Antho-cyanin expression	Turion	Reference	*IBOT Voucher* IPEN Voucher
Botswana (Africa)	Okavango Delta	Shallow unnamed swamp	19° 33′ S 23° 13′ E	2005	Yes	No	Obermeyer (1963)-(1988)	*AV-TR-15*
NSW Armidale (Australia)	Armidale, New South Wales	Billybung lagoon	30° 06′ S 151° 47′ E	2006	Yes	Yes	Elansary et al. (2010)	*AV-TR-17*
NSW Broulee (Australia)	Broulee, New South Wales	Longvale Swamp	35° 35′ S 150° 09′ E	1997	Yes	No	Adamec (1999)	*AV-TR-04*
NT Katherine (Australia)	Katherine, Northern Territory	Leach Lagoon	14° 38′ S 132° 37′ E	2003	Yes	Yes	Elansary et al. (2010)	*AV-TR-12*
NT Darwin (Australia)	Darwin, Northern Territory	Girraween lagoon	12° 31′ S 131° 05′ E	1994	Yes	No	Wilson (1995)	*AV-TR-09*
WA Kimberley (Australia)	Kimberley, Western Australia	Little Mertens Falls	14° 50′ S 125° 41′ E	2000	Yes	No	Adamec (2018)	*AV-TR-10*
WA Cape le Grande (Australia)	Cape le Grande, Western Australia	Shallow unnamed swamp *	33° 48′ S 121° 49′ E	2002	Yes	No	Gibson (2004)	*AV-TR-11*
North Russia (Europe)	Lake Ladoga, northern Russia	Shallow lake near Sviri river estuary	60° 29′ N 32° 57′ E	1997	No	Yes	Afanas'ev (1953)	*AV-TR-06*
South Russia (Europe)	Lipeck, southern Russia	Shallow Lake Mokhovo'e	52° 24′ N 39° 34′ E	2005?	No	Yes	Adamec (2018)	*AV-TR-16*
EastPoland (Europe)	Łeczna-Włodawa lake district, eastern Poland	Lake Długie	51° 26′ N 23° 06′ E	1993	No	Yes	Kamiński (1987)	*AV-TR-01* PL 0 HBT 2017.04079
NortEast Poland (Europe)	Augustow lake district, North- Eastern Poland	Lake Kruglak	53° 54′ N 23° 19′ E	1994	No	Yes	Kamiński (1987)	*AV-TR-02*
Hungary (Europe)	Somody County, southwest Hungary	Lake Baláta-tó	46° 19′ N 17° 21′ E	2003	Yes	Yes	Elansary et al. (2010)	*AV-TR-13* HU 0 HBT 2017.03654
Lithuania (Europe)	Ignalina District, northeast Lithuania	Lake Ruzhas	55° 30′ N 25° 28′ E	2003	No	Yes	Vilkonis (2003)	*AV-TR-14*
Ukraine (Europe)	Kiev Reservoir, northern Ukraine	Tet'erev River estuary	51° 03′ N 30° 25′ E	1997	No	Yes	Adamec (1995)	*AV-TR-05*
Romania (Europe)	Danube Delta, eastern Romania	Shallow lake on Obretim Island	45° 11′ N 29° 19′ E	1998	No	Yes	Elansary et al. (2010)	*AV-TR-07*
Germany (Europe)	Lindau, southwest Germany	Lake Constance **	47° 34′ N 09° 41′ E	1994	No	Yes	Koch (1950)	*AV-TR-03*
Japan (Asia)	Hanyu City, Saitama Prefecture	Lake Hozoji Pond	36° 12′ N 139° 42′ E	1980	No	Yes	Komiya (1966)	*AV-TR-08*

* Plants at this location may have been introduced from individuals collected at Little Mertens Falls, Kimberley, Western Australia, according to anecdotal evidence.
** Plants originate from this location, but were introduced to Lake Metmenhaslisee, Switzerland, in 1908 and were collected from this location.

2.2. DNA extraction

Total genomic DNA was extracted by the sorbitol method (Štorchová et al., 2000), modified for use with *A. vesiculosa* (Maldonado San Martín et al., 2003). High molecular genomic DNA for PacBio long read sequencing was prepared with Genomic Tips G20 (Qiagen) according to the manufacturer and dissolved in T buffer (10 mM Tris-HCl buffer, pH 8.5). All samples were treated with RNAse (Qiagen), and DNA quality and quantity were assessed using a NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific, USA), a Qubit 2.0 fluorometer (Invitrogen, Life technologies), and via agarose gel electrophoresis. DNA samples were purified and concentrated using a DNA Clean & Concentrator-5 kit (Zymo Research).

2.3. Shotgun illumina sequencing

Total genomic DNA (30–150 ng) was fragmented with a Covaris S2 to a mean fragment size of 550 bp. Individual genome library preparations were performed using a the TruSeq DNA Nano Library Prep kit (Illumina), according to the manufacturer's instructions. Libraries were quantified by quantitative polymerase chain reaction, using a KAPA Illumina Library Quantification kit (KAPA Biosystems) and LightCycler 480 (Roche), and then pooled in approximately equimolar amounts. The pooled libraries were sequenced (2 × 150 nt) using Illumina HiSeq 1500 in rapid run mode using a TruSeq Rapid PE Cluster kit, for on-board cluster generation, and TruSeq Rapid SBS kits.

2.4. PacBio sequencing

High-molecular genomic DNA was measured by using a NanoDrop spectrophotometer and checked by 0.8% agarose gel electrophoresis. It was sent to Eurofins Genomics (Ebersberg, Germany) and sequenced on the PacBio RS II sequencer. Consensus sequences were generated by the SMRT analysis software by the Eurofins Genomics company.

2.5. Mitochondrial genome assembly

The Illumina HiSeq short read dataset from *A. vesiculosa* individuals originating from the Kimberley region of Western Australia, in combination with PacBio long reads (0.6 Gbp high quality reads sequenced at Eurofins Genomics) acquired from the same population, were used to assemble the *A. vesiculosa* mt genome. Short reads were initially subsampled by 50% using the seqtk toolkit sample command to reduce data size, with the same random seed (-s 100). Subsampled fastq files were converted to fasta with a custom perl script (and input to Newbler 3.0). The output contained the contigs corresponding to the repeats and the regions between repeats in the mt genome, as well as the information about the connectivity among particular contigs in ContigGraph. Mitochondrial contigs were distinguished from nuclear and plastid contigs based on their coverages. The connection among the contigs and

scaffolds were confirmed by long PacBio reads, corrected with Illumina reads by Proovread (Hackl et al., 2014). The final mt genome assembly was polished by remapping the Illumina short reads. The annotation was performed through the comparison with the *Nepenthes* (Gruzdev et al., 2018) and *Liriodendron* (Richardson et al., 2013) mt genomes in Geneious 10.1, whilst tRNA genes were identified by means of tRNAscan (Chan and Löwe, 2019). The annotated mt genome was submitted to GenBank under the accession numbers OQ324918–20.

2.6. Read mapping and identification of polymorphisms

Raw Illumina HiSeq reads were quality checked and trimmed in Trimmomatic 0.39 (Bolger et al., 2014). For each specimen, overlapping paired-end reads were merged using the software FLASH v. 1.2.7 (Magoč and Salzberg, 2011) to maximize the effective read length. Clean reads were mapped against the plastid (NC_035416.1, Nevill et al., 2019) or mt (OQ324918–20) reference genomes. The aligner bowtie2 (Langmead and Salzberg, 2012) with default stringency (–local) was used with 17 populations, and the resulting.bam files were treated by mpileup (mpileup -t DPR), which generated vcf file summarizing the positions and frequencies of variant nucleotides. The numbers of raw, clean, and mapped reads are given in Supporting Table S1.

2.7. Phylogenetic analyses

The bam files were thoroughly examined in Interactive Genomic Viewer (IGV) (Robinson et al., 2023) and all the polymorphisms were verified. The sequences of organellar genomes from *A. vesiculosa* individuals originating from Romania contained ambiguities at many single nucleotides polymorphisms (SNP) positions, and this population was excluded from further analyses. Two data matrices were created based mt.bam files of remaining 16 *A. vesiculosa* populations. The first one consisted of SNPs, and the second one represented short inversions and indels with binary values 1 (presence) or 0 (absence). Owing to the absence of evolution model describing indel and inversions rates, MP method was preferred to ML. Nexus files made of the matrices were uploaded to PAUP 4a.0 (Swofford, 2002). A single matrix consisting of SNPs was prepared from plastid.bam files. The phylogenetic tree was constructed by MP with branch and bound algorithm and 1000 bootstrap pseudoreplicates. We also retrieved and aligned nuclear rDNA ITS sequences for all 16 specimens. However, no genetic variation was observed among *A. vesiculosa* sequences, all being identical to the nuclear genome sequence for *A. vesiculosa* previously published (Palfalvi et al., 2020), and as such these sequences were not included in later analyses. Coverage of the nuclear genome was too low to estimate the sequences of non-repetitive markers.

2.8. Nucleotide diversity calculation

Nucleotide diversity was estimated from nucleotide alignments of plastid and mt sequences using DnaSP v.6 (Rozas et al., 2017) with the Jukes-Cantor evolutionary model.

2.9. Estimation of intraspecific divergence time based on plastid and mt substitutions

In the absence of external information for the clock calibration we used the assumption of uniform substitution rate (Zuckerkandl and Pauling, 1962) to calculate the divergence time between the European and Australian populations. As the substitutions are located mostly or entirely in intergenic regions of organellar genomes of *A. vesiculosa*, the substitution rates specific for this genomic area were utilized. The substitution rate 1.52×10^{-9} s/s/y was estimated by Yamane et al. (2006) for plastid intergenic regions in grasses, whereas the rate 9.6×10^{-10} s/s/y was assessed for mt intergenic regions at the intraspecific level in *Arabidopsis thaliana* (Christensen, 2013). The divergence time was calculated according to the formula: D/ (2 R × S), where D was the observed number of substitutions, R the size of the region in bp, and S the substitution rate.

3. Results

3.1. Mitochondrial genome of A. vesiculosa

The mt genome of this species was assembled de novo. The mt genome consists of three fragments (hereafter referred to as chromosomes), which could not be connected with reliable support from our data. Chromosome 1 (481,467 bp) may exist in a circular form, while chromosomes 2 (675,742 bp) and 3 (116,705) are linear. Although further improvement of the current assembly of mt genome is expected in the future, the current version served sufficiently well as the reference for mapping the reads of the 16 populations. The total size of the *A. vesiculosa* mt genome was 1,273,914 bp.

The mt genome comprises a standard set of genes present in many angiosperms. It contains 32 protein coding genes (5 *atp,* 8 *nad*, *3 cox*, 1 *cob*, 1 *mttB*, 1 *matR*, 2 *rpl*, and 7 *rps* genes), the *sdh4* pseudogene, 3 *rrn*, and 18 *trn* genes.

3.2. Within-species variation in organellar genomes of A. vesiculosa

The 16 *A. vesiculosa* populations analyzed (i.e., excluding the Romanian population), despite originating from four continents, varied in only 49 plastid genome polymorphisms (36 single nucleotide substitutions, 10 single nucleotide indels, and 3 larger indels). In contrast, comparison of mt genomes revealed 1250 reliable polymorphisms comprising 263 single nucleotide substitutions, 72 larger indels, and 915 inversions or substitutions, most often consisting of 2 or 3 bp. The most frequent events were GTA/TAC inversions (285 cases), followed by GA/TC inversions (131 cases). The nucleotide diversity Pi calculated from plastid substitutions was 0.00007. A matrix of all polymorphisms is given in Supporting Table S2**.** The remaining polymorphisms were represented, for example, by the substitutions GTA/TCC, GCA/TAT, TTT/GAG, AAC/TTA, or TA/GCC. To assess the utility of the inversions for phylogenetic reconstruction, the number of GTA/TAC and GA/TC inversions in pairwise comparisons of individual populations with the reference were estimated and compared with the numbers of SNPs in the same pairwise comparisons. The numbers of inversions and SNPs correlated well in closely related populations (Pearson $r = 0.99$), while the more distant populations showed proportionally fewer inversions than SNPs [Supporting Figure S1]. The nucleotide diversity Pi calculated from mt substitutions was 0.000055.

3.3. Phylogenetic relationships among A. vesiculosa populations reconstructed from plastid genomes

The MP phylogenetic tree created from the alignment of plastid reads exhibited two prominent clusters, the first including all populations of European origin and the second including Australian populations and the population from Botswana (Fig. 1). While the tree did not resolve relationships among European populations, it did support clear separation of European and non-European populations and further separated the latter group into two clades (the Botswana population on the same branch as, but distinct from, all Australian populations). The East Poland population was identical to the Japanese (Hanyu) population.

3.4. Phylogenetic relationships among A. vesiculosa populations reconstructed from the mt genome

Comparative analysis of the mt genome among 16 populations of *A. vesiculosa* yielded about 20 times more polymorphisms than alignment of plastid genomes. The number of SNPs was about eight times higher in the mt data compared with the plastid data. The more

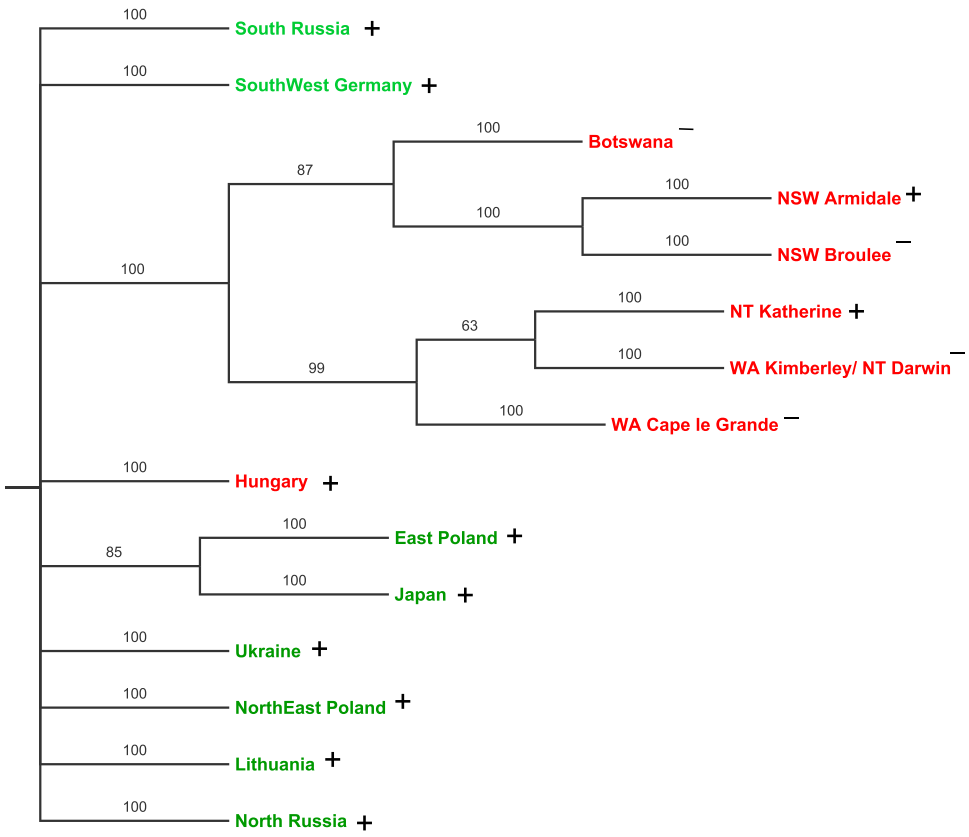

Fig. 1. The MP phylogenetic tree constructed from plastid genomic sequences of 16 populations of *A. vesiculosa*. Bootstrap values (in %) calculated from 10,000 pseudoreplicates are given under each branch. Red color marks the production of anthocyanin, green color means the absence of it. The " + " and " - " signs refer to the capability to form or not form overwintering turions.

abundant mt characters resulted in a robust tree with high bootstrap support. Unlike plastid-based reconstruction, mt-based reconstruction also provided resolution for the relatedness of European populations. Because of the specific repetitive nature of short inversions and substitutions, the origin of which is not known, mt phylogenetic trees were constructed separately from inversions/substitutions and from SNPs (Figs. 2, 3).

The MP tree based on mt SNPs showed the deepest split between the branch comprising the southern European populations and the branch comprising the northern European, Botswana, and all Australian populations (Fig. 2). As no close living relative of *A. vesiculosa* exists, the southern European clade was used as the outgroup to root the tree. The second split separated the populations from northern Europe and Botswana from all Australian populations. The northern European populations were not resolved, but populations from Ukraine and Botswana diverged earlier in this clade. East Poland and Japanese populations again did not differ in any SNP. The New South Wales and Katherine (Northern Territory) populations were basal in the Australian clade and populations from Darwin (Northern Territory) and Western Australia formed a cluster. However, this branch had low support (53%), and the accurate position of the Darwin population is uncertain as it missed about 43% of data.

The MP tree constructed from a binary matrix of mt inversions and substitutions agreed with the tree made of mt SNPs (Fig. 3), placing three South European populations together as a sister clade to the Australian and North European populations, with Ukraine and Botswana forming the sister tips. The Japanese and East Poland populations differed in a single TAC/GTA inversion only. The Australian clade was structured, with the Katherine population basal followed by populations from New South Wales and Western Australia.

The plastid- and mt genome-based trees differed mainly in the phylogeny of European populations. The mt tree strongly separated populations from northern and southern Europe while these were not resolved in the plastid tree, potentially due to the markedly lower plastid polymorphic data compared with mt data. The other discrepancy was the position of the Botswana population, which was the only African *A. vesiculosa* representative in our sampling. The Botswana population was clearly placed in the northern European clade by mt data, and in the Australian clade by the less abundant plastid data. The Botswana branch diverged early in the clade in both the mt SNP- and inversion- based trees.

3.5. *The time of divergence between European and Australian populations*

The split between Australian and European populations, which differed in 13 substitutions in about 40 kb of plastid intergenic region, assessed with the assumption of constant substitution rate for plastid intergenic regions, suggests a divergence time approximately 100,000 years ago. A similar calculation based on 190 substitutions occurring in about 1250 kb mt intergenic sequences and constant substitution rate yielded a divergence time estimate of approximately 80,000 years ago. The divergence time estimates calculated from the substitutions in two organellar genomes are in a good agreement.

4. Discussion

4.1. *The phylogenetic relationships among populations of A. vesiculosa, and possible migration history*

Both the mt and the plastid phylogenetic trees clearly separated Australian populations of *A. vesiculosa* from all others (e.g., European, Botswana, and Japanese populations; Figs. 1, 2, 3). Australian

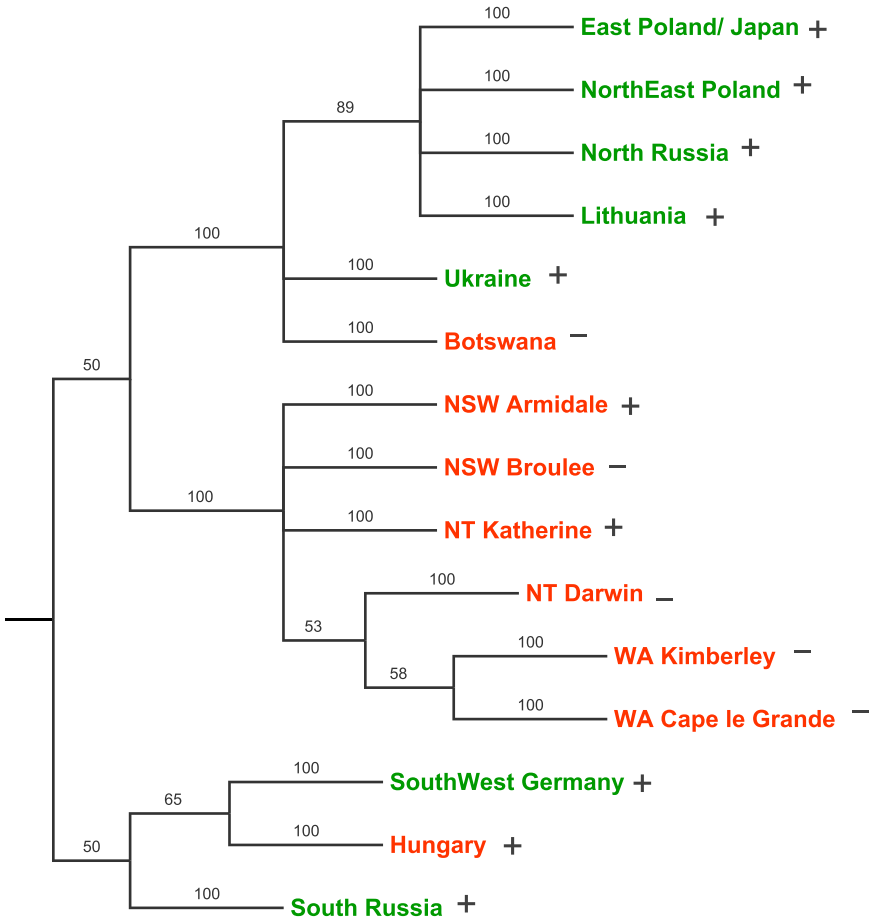

Fig. 2. The MP phylogenetic tree constructed from mitochondrial SNPs in 16 populations of *A. vesiculosa*. Bootstrap values (in %) calculated from 10,000 pseudoreplicates are given under each branch. Red color marks the production of anthocyanin, green color means the absence of it. The " + " and " - " signs refer to the capability to form or not form overwintering turions.

populations formed a monophyletic clade, suggesting that all the Australian populations originated from a single common ancestor. While the plastid tree did not distinguish European populations, the mt phylogenetic tree revealed a clade comprising southern European populations as the most distant from Australian populations. This basal position of southern Europe could be explained by the persistence of *A. vesiculosa* in one or more glacial refuges in this region, from which the plants dispersed throughout northern Europe, Asia, and finally to Africa and Australia. It appears probable that Australian and African populations resulted from two discrete migration events.

Previous discussion of the potential evolutionary history and global dispersal patterns by Cross (2012) hypothesized that extinction of ancestral *Aldrovanda* species appears to have occurred during repeated glaciation bottleneck events during the mid-Quaternary, during which *A. vesiculosa* (or, possibly, its likely precursor *A. praevesiculosa*) persisted at one or more refuges (most likely in southern Europe) before rapidly radiating throughout Europe and colonizing other localities in Asia, Africa, and Australia likely within the last 120,000–50,000 years. It was proposed this radiation most likely occurred stepwise via two major pathways probably representing two discrete events: i) east, from southern Europe into Kazakhstan, Bangladesh, and China and subsequently into the Koreas, Japan, southeast Asia, and northern Australia via the East Asia Flyway, and ii) south, from central or southern Europe into equatorial and then eastern and southern Africa (Cross, 2012).

Our data appear to support previous migration hypotheses, with *A. vesiculosa* likely persisting in a southern European refuge during the last glacial maxima before recolonizing Europe and migrating to Australia and Africa during the late Pleistocene (80–100,000 years ago). Confirmation of the hypothesized intermediate steps on this route would require DNA sequence information from populations in central and southeast Asia or Japan, which is unlikely to be possible in many cases as most populations recorded from these areas are now extinct (e.g., Bangladesh, India, Kazakhstan, Uzbekistan, Japan) or have remained unverified for many decades (e.g., North and South Korea, East Timor; Cross, 2012). Similarly, the vast majority of historical *A. vesiculosa* populations from Africa, including from Burundi, Cameroon, Chad, Ghana, Malawi, Mozambique, Rwanda, South Sudan, Tanzania, Togo, Uganda, and Zambia are considered unverified and are likely extinct (Cross, 2012). However, recently discovered populations, such as in eastern India, central Myanmar, eastern Russia, western Mongolia and eastern China (Ngangbam et al., 2019; Shiga et al., 2020; Verkhoziana et al., 2022; https://biotopeaquariumproject.com/bin/lake-inle-myanmar/; www.plantarium.ru/page/image/id/542035.html; Y. Yunlong, personal observation), may offer some insights into dispersal routes. We sequenced only one Japanese population (Hanyu), which was nearly identical to plants from East Poland, indicating a very recent dispersal most likely by waterfowl as has been previously hypothesized (Kamiya and Ozaki, 2002).

Distinction in the position of the Botswana population between the mt and plastid phylogenetic trees could be the consequence of the low number of polymorphisms in plastid genomic sequences. Another possibility is a potential cross between the populations associated with paternal transmission of only one of the two organelles. However, the probability of this event is very low considering the typical low frequency of sexual reproduction in *A. vesiculosa* and huge geographic distances among the populations.

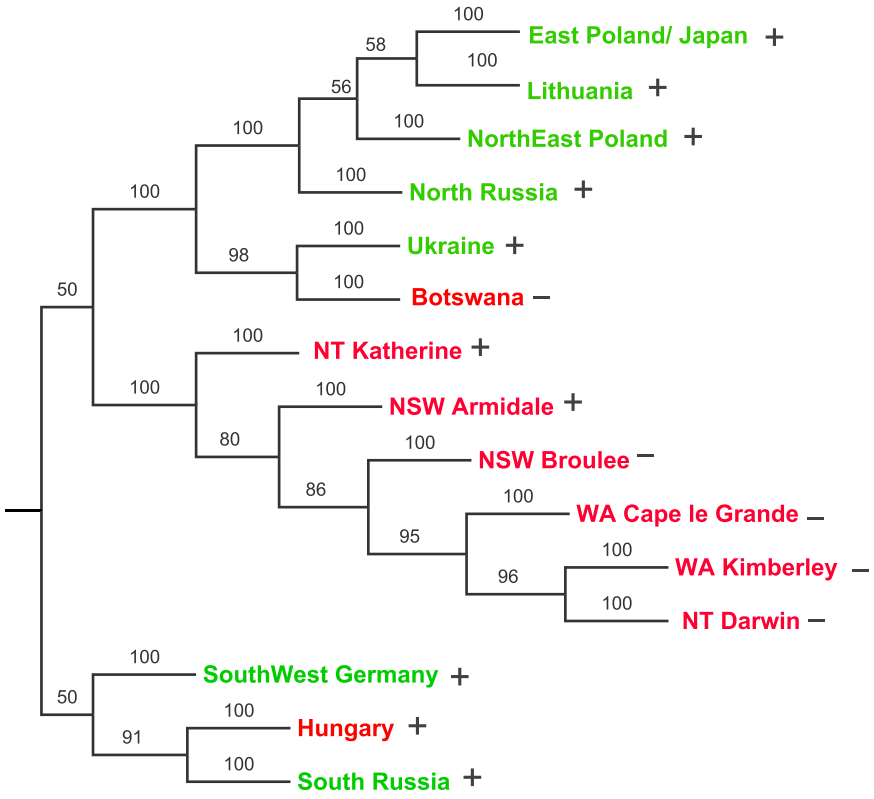

Fig. 3. The MP phylogenetic tree constructed from mitochondrial inversions and two- and three-nucleotide substitutions. in 16 populations of *A. vesiculosa*. Bootstrap values (in %) calculated from 10,000 pseudoreplicates are given under each branch. Red color marks the production of anthocyanin, green color means the absence of it. The " + " and " - " signs refer to the capability to form or not form overwintering turions.

4.2. Parallel gains and losses of turion formation and anthocyanin production

The analyzed *A. vesiculosa* populations differ in two distinct morphological characteristics – the capacity for turion (overwintering vegetative buds) formation and ability to produce the red pigment anthocyanin (Cross, 2012; Adamec, 2018). European and Asian populations are well-documented to produce dormant turions facilitating persistence through cold winter periods (Fig. 4), but most populations from Australia and Africa are unable to develop true, dormant turions (Table 1; Adamec, 2018). This may suggest the trait is ancestral and was lost at some stage during migration into the southern hemisphere, apparently independently on at least two occasions given individuals from Botswana and the four Australian populations did not cluster together in any of the constructed phylograms (see Table 1). It is also possible that turion production represents a pliable, genetically linked character that can be turned on or off under certain conditions, although the lack of turion formation in most Australian and African plants maintained in horticulture under cool temperate conditions even after several decades might not support this hypothesis (Adamec, 2018).

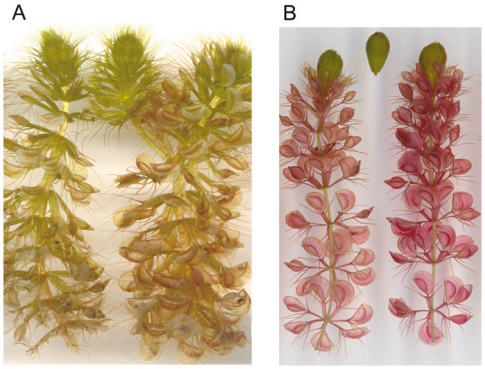

Fig. 4. *Aldrovanda vesiculosa* from Botswana grown in greenhouse at about 10 °C under short days in January. This population does not form dormant turions, but non-dormant winter shoot apices capable of growing after transferring to warm water (A). True dormant turions of *Aldrovanda vesiculosa* from NSW, Australia, Armidale. The turions were formed in an aquarium in laboratory near window at about 20 °C during winter cultivation in January (B). Photos by L. Adamec.

In contrast to turion formation, individuals from all Australian populations (apparently including historical records from East Timor), as well as plants from Hungary and Botswana, readily produce red anthocyanin; individuals from some European and Asian populations appear to lack this capacity (Cross, 2012; Adamec, 2018). Anthocyanin production likely affords photosystem protection against the high solar radiation experienced in tropical and subtropical latitudes (Cross, 2012). Anthocyanin production is likely an ancestral state given its wide occurrence in the Droseraceae and the convergence of red flavonoid pigments in land plants generally (Fleischmann et al., 2018; Piatkowski et al., 2020). If the character is ancestral, its loss in all northern hemisphere populations except individuals from the sampled Hungarian population, which is firmly embedded among European populations in both plastid and mt trees (Figs. 1, 2, 3), implies that this location may have been the area of refuge during the last post-glacial maxima. While it is possible that the anthocyanin production trait might have been lost and regained multiple times independently during the evolutionary history of the species, it is also likely that an anthocyanin-producing population persisted through the last glacial maxima and provided the source material to migrations into Australia and Africa, with another anthocyanin-lacking population dispersing throughout Europe and Asia. Such clear geographic structuring in anthocyanin production, and a lack of new populations displaying this characteristic in Europe, is curious given the apparent efficacy of *A. vesiculosa* in spreading rapidly around

the globe during the late Pleistocene and the persistence of an anthocyanin-producing population in the region to provide propagules for dispersal. However, the abundance and suitability of habitats capable of supporting *A. vesiculosa,* and the abundance of waterfowl as likely vectors of its dispersal (Cross, 2012), were probably much greater during the period of rapid postglacial expansion than in the most recent few centuries.

4.3. Sequence variation in the plastid and mt genomes of A. vesiculosa

The observed variation in *A. vesiculosa* plastid and mt DNA sequences for populations sampled across the global range of this species was lower than has been reported previously for other angiosperms with similar large distribution ranges (reviewed by Kan et al., 2022). Although our data are somewhat constrained by the limited number of populations sampled (17 distinct populations originating from four continents), given the well-reported propensity for almost exclusive asexual clonal reproduction in this species (see Cross, 2012; Adamec, 2018; Onelli et al., 2021) we are hopeful that the significant geographic scope of our sampling, particularly the inclusion of most populations at the periphery of the species' known distribution, is likely to have captured much of the potential genetic variation in the species. Our data also strongly support the results of several previous studies utilizing much lower resolution methods (e.g., isozyme analysis, RAPD, short plastid sequences) to examine genetic variation in *A. vesiculosa* (Adamec and Tichý, 1997; Maldonado San Martín et al., 2003; Hoshi et al., 2006; Elansary et al., 2010), all of which reported low levels of variation among different populations examined. Our data support previously proposed hypotheses that the limited genetic diversity found in *A. vesiculosa* likely reflects multiple significant historical bottleneck events followed by rapid expansion from one or few refugial populations.

Reports of intraspecific plastid DNA variation calculated from complete plastid genomic sequences of aquatic plants are rare so far. Huotari and Korpelainen (2013)) found 186 SNPs and 47 indels in the plastid genome in American and European populations of *Elodea canadensis,* an invasive submerged aquatic species that is similarly clonal and restricted in sexual reproduction to *A. vesiculosa.* The authors did not give Pi to enable nucleotide diversity comparison. The SNP and indel numbers are about four times higher than observed in *A. vesiculosa*, likely due to the invasive nature and much higher effective population sizes of *E. canadensis.* Supporting Table S3 summarizes the data on intraspecific plastid DNA variation in some aquatic angiosperms collected across continents but is mostly restricted to short genomic regions only. Plastid variation in *A. vesiculosa* (2.6 SNPs, 0.9 indels per 10,000 bp, nucleotide diversity Pi = 0.00007) is lower than has been reported in other aquatic plants, but more data from complete plastid genome sequences are needed for reliable comparison. Nucleotide diversity Pi was not often calculated in previously published reports but was consistently orders of magnitude higher for other species than in *A. vesiculosa* where reported (0.0038 – Zhu et al., 2015; 0.0022 – Fehrmann et al., 2012; 0.0035 – Tan et al., 2008).

Plastid DNA has been employed previously in intraspecific population analyses of *A. vesiculosa* (Elansary et al., 2010), and to infer phylogenetic relationships among four related carnivorous plants (Nevill et al., 2019) and Droseraceae species – for example, Rivadavia et al. (2012) proposed long-distance dispersal of an ancestor of *Drosera meristocaulis* from Western Australia to northern South America based on shared plastid markers *rbcL* and *rps16*. Intraspecific studies using plastid DNA appear to be scarce for other families with carnivorous representatives, with some examples in Nepenthaceae: Kurata et al. (2008) identified 17 haplotypes in *Nepenthes vieillardii* in New Caledonia using five plastid regions with a total length of 4660 bp, and a more comprehensive study utilized genome skimming and the sequences of 81 plastid genes to clarify reticulate evolution in *Nepenthes* (Nauheimer et al., 2019). To the best of our knowledge, no analysis has previously used the mitochondrial genome to decipher phylogenetic relationships in *A. vesiculosa* or any of its near or distant carnivorous relatives, and this study provides a pioneering effort to better understand the phylogenetic relatedness of carnivorous plants (Duminil and Besnard, 2021).

The assembly of complete mt genomes in flowering plants is a challenging task. The mt genomes are often fragmented, vary in size, gene number and order, contain many repeats, and exhibit intramolecular recombination (Gualberto et al., 2014; Christensen, 2021). The mt genome of *A. vesiculosa* has a large size of about 1274 kb, which places *A. vesiculosa* close to the species possessing the largest mt genomes, such as *Cucumis melo* (2740 kb; Rodriguez-Moreno et al., 2011) or *Silene conica* (11,000 kb; Sloan et al., 2012). Unlike the *A. vesiculosa* plastid genome, in which all *ndh* genes have been lost (Nevill et al., 2019), the standard set of 32 protein coding genes is present in its mt genome. There is no other set of multiple completely sequenced mt genomes available within aquatic angiosperm species, but we may compare *A. vesiculosa* data to rice (616 SNPs, 181 indels) mt genomes from the same study as cited above (Kan et al., 2022). This value largely outnumbered *A. vesiculosa* with only 263 mt SNPs. The large mt genome of *A. vesiculosa* is more than three times larger than the *A. thaliana* or rice mt genomes, therefore the number of *A. vesiculosa* SNPs must be divided by three for the comparison. The nucleotide diversity Pi estimated from the substitutions in the mt genome was 0.000055, being comparable with Pi calculated from plastid substitutions.

Unlike SNPs, the total numbers of 987 indels, substitutions and inversions in *A. vesiculosa* are more like the counts in rice (Kan et al., 2022). However, the nature of 915 inversions in the *A. vesiculosa* mt genome is enigmatic. They affect specific sequences (e.g., GCA/TAC) and are preferentially clustered in some regions, which may be considered "hot spots" for these inversions. We are not aware of similar polymorphisms in any other plant mt genome, and we neither know how frequently they originate, nor the mechanism by which they arise. Thus, we could compare to other species only the number of standard indels, which were 72 in the *A. vesiculosa* mt genome.

4.4. Divergence times in A. vesiculosa

Our plastid sequence data suggest Australian populations split from European populations about 100,000 years ago, and about 80,000 years ago based on mt data. This divergence time likely refers to the last glacial bottleneck and provides the explanation for lower intraspecific genetic diversity of *A. vesiculosa*. It corresponds with the last interglacial period, matching well with paleontological records. *Aldrovanda* is one of the aquatic genera best represented in the fossil record and may represent one of the oldest lineages in the carnivorous plants (Cross, 2012; Fleischmann et al., 2018). Paleontological records for the species include fossilized pollen and seeds, and even a single fossilized leaf; as reviewed in Cross (2012) it is probable that *Aldrovanda* is a relictual genus from the early–mid Tertiary period based on fossilized pollen possibly of Paleocene origin recovered from sediments in Germany (65–55 Ma) and fossilized seeds recovered from Eocene sediments in southern England, the Isle of Wight, eastern Germany, and Bulgaria (55–38 Ma). Numerous extinct ancestral *Aldrovanda* species have been described, and fossil records attributed to *A. vesiculosa* have been recovered around the world including across the European continent, from Asia, from North America, and from eastern Africa (e.g., Matthews and Ovenden, 1990; Worobiec, 2011; Cross, 2012; Cross et al., 2015; Gałka et al., 2015; Huang et al., 2017). These have been dated to the 130,000–115,000 BC interglacial period at various locations in Europe (Nikitin, 1927; Kuijper, 1988), 126,000–120,000 BC from central Russia (Zyuganova, 2009), 118,200–6300 BC from Germany (Rösch, 2019), to as recent as 760–550 BC from Bulgaria (Beug and Tonkov, 2017), 440 AD (Tanzania) and 1600 AD (Poland; Gałka et al., 2015).

4.5. Glacial refuges

In many cases, areas believed to have escaped glaciation have been

implicated in preventing the extinction of numerous flora and fauna throughout Europe (Svenning, 2003; Feliner, 2011), and a number of these refugial areas appear to overlap regions from which *Aldrovanda* fossils have been recovered. Certainly, the idea of isolated, refugial populations surviving glacial periods in the face of widespread extinction is not novel to *Aldrovanda*, though the number and severity of these events, as indicated by the apparent progression of speciation throughout the genus' history, is historically significant. Among the global aquatic flora *Aldrovanda* is perhaps the only genus to exhibit such strong cyclical evolutionary patterns, so much so that several species are commonly used by European paleobotanists as markers for dating soil samples (especially *A. praevesiculosa*; Degreef, 1997).

4.6. Conservation implications

Despite once being widespread across four (possibly five) continents and tolerating the different seasonal extremes found in climates ranging from subarctic and cold temperate to tropical and sub-tropical, *A. vesiculosa* has become extinct across much of its historical range as a result of human-induced eutrophication and degradation of wetland ecosystems. Although *A. vesiculosa* exhibits a hardiness to an extraordinary climatic range almost unparalleled by other aquatic species, it is remarkably sensitive to even small changes in water quality and habitat degradation. Restoring and maintaining suitable water quality and habitat structure (i.e., water depth, plant density) remains crucial to long-term in situ conservation efforts of the few populations that remain, and ex situ cultivation provides a necessary custodial resource in the event of stochastic population decline and extinction events (Adamec, 2005, 2018). Reintroductions of *A. vesiculosa* to locations where the species previously occurred from ex situ conservation populations may be technically feasible once natural hydrological regimes are reinstated and water quality controls are in place. However, introductions to new locations must be undertaken with caution as *A. vesiculosa* has the potential to become locally abundant in suitable habitat, and the impact of its establishment on local aquatic invertebrate communities remains unknown (Cross et al., 2015). Although the markers deployed here and in other studies indicate global populations of *A. vesiculosa* are characterized by high levels of genetic uniformity, it is recommended that individuals still be sourced from the closest available natural populations where reintroduction programs are implemented to conserve the minimal structuring present.

5. Conclusions

It appears that *A. vesiculosa* has recently radiated, then diverged in place but exhibits only minor divergence. The species is likely to have experienced numerous significant population bottlenecks resulting from range collapses during periods of glacial maxima, leading to persistence in isolated refuges and subsequent expansion during more favorable inter-glacial periods following glacial retreat. The knowledge on phylogeography of *A. vesiculosa* helps to protect this very interesting endangered species.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Raw PacBio reads (AV-TR-10) are saved in the BioProject PRJNA918534 for the BioSample SAMN32594937 under the accession SRR22981876. The raw Illumina reads are deposited in the BioProject PRJNA453847.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2023.103742.

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