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Uniformity of organellar DNA in *Aldrovanda vesiculosa*, an endangered aquatic carnivorous species, distributed across four continents

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ABSTRACT

Organellar DNA from the widely distributed but rare and critically endangered aquatic carnivorous plant *Aldrovanda vesiculosa* (Droseraceae) was examined. Six chloroplast intergenic regions (3700 nt in total) were sequenced before analyzing the Southern-RFLP (Restriction Fragment Length Polymorphism) of 2 mt gene flanking regions. Only two different chloroplast haplotypes among 15 *A. vesiculosa* accessions from Africa, Australia, Europe, and Japan were found, generally distinguishing European and non-European plants, with two exceptions. Genetic variation observed in *A. vesiculosa* appears to be even lower than in other aquatic species with a similar world-wide distribution. A recent bottleneck followed by long-distance dispersal by water birds or low mutation rates could be responsible for the observed genetic uniformity. Estimation of genetic distances based on six chloroplast intergenic regions led to the conclusion that the chloroplast genome of *A. vesiculosa* matches more closely to that of *Drosera regia* than *Dionaea muscipula*, a sister genus sharing snapping traps. The inconsistency between genetic distance estimates based on nuclear and cytoplasmic markers may reflect a chloroplast capture. In *A. vesiculosa*, a four amino acids substitution (TGWS) in the amino acid sequence of ATP synthase alpha subunit (ATP1), highly conserved mitochondrial protein, was discovered, unique among all organisms based on current knowledge.

1. Introduction

The aquatic carnivorous plant species *Aldrovanda vesiculosa* L. (Droseraceae) is widely, but patchily distributed across all continents of the Old World and in Australia (Maldonado San Martín et al., 2003). Becoming increasingly rare, the species is known only from several dozens sites (Adamec, 1995, 1999), and is therefore considered critically endangered in all countries of its current distribution (Walters, 1979). Major contributors to recent population declines may be eutrophication of required dystrophic sites or general land-use changes (Adamec, 1995). The species shows a very broad tolerance of climate, occurring in tropical zones (Australia, Africa, Asia) as well as at temperate sites with strong winter frosts (Poland, North Russia). Unlike plants from (sub)tropical locations, temperate populations of *A. vesiculosa* form overwintering dormant buds (turions) as a climatic ecotype, and often differ in color. Euro-Asian populations are green, whereas all Australian populations can exhibit red coloration due to anthocyanin (Adamec, 1999). An exception is a recently discovered red Hungarian population (Borhidi and Járai-Komlódy, 1959; Adamec, 2005). The temperate populations predominantly propagate vegetatively by branching (Adamec and Tichý, 1997).

The vast geographical range of *A. vesiculosa*, isolation of populations, and distinct morphological and physiological differences among populations have led to the expectation of some intraspecific genetic variation, however, recent molecular studies revealed very low polymorphism in the species. Only one out of 15 loci in seven enzymatic systems was variable among European populations (Adamec and Tichý, 1997). Maldonado San Martín et al. (2003) applied a RAPD technique to *A. vesiculosa* accessions from Europe, Asia, and Australia using 151 primers, with only 14% producing polymorphic banding patterns. Finally, the DNA sequence of ITS (internal transcribed spacer) in 45S rDNA was identical among six populations of *A. vesiculosa* from Australia, Japan, and Europe (Hoshi et al., 2006).

Many aquatic plants are spread across extraordinarily broad geographic ranges. Dispersal can potentially take place through movement with waterfowl (Darwin, 1859; Les et al., 2003; Charalambidou and Santamaria, 2005; Soons et al., 2008), but successful intercontinental transfer of propagules by birds should have a low probability (Green et al., 2002). Still, distance may not represent a substantial barrier to gene flow, diminishing genetic variation among populations of aquatic angiosperms across large areas (Madeira et al., 2007; Les et al., 2003).

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A. vesiculosa represents the only species of the genus, and lacks close relatives. A terrestrial carnivorous species *Dionaea muscipula*, possessing snapping traps similar to those of *A. vesiculosa*, was shown to be the species closest relative and this pair to be sister taxa to *Drosera* (Cameron et al., 2002; Rivadavia et al., 2003).

In the present paper, we used DNA sequences of chloroplast (cp) intergenic regions (*trnS-trnG*, *trnH-psbA*, *rpl20-rps12*, *trnT-trnL*, *atpB-rbcL* and *trnP-trnW*) and RFLP of mt gene flanking regions to detect genetic variation in *A. vesiculosa* at the within-species level. Because the world-wide collection of its accessions kept at the Institute of Botany in Třeboň (Czech Republic), has been recently supplemented with plants from Botswana (Africa), these also be included in our survey. We could therefore compare 15 *A. vesiculosa* specimens collected across four continents, the largest range achieved in the molecular analysis of this species so far. Chloroplast intergenic spacers are not imposed to evolutionary constraints, showing a high mutation rate, and as such they are able to detect genetic variation at the intraspecific level in aquatic plants (Madeira et al., 2007; Štorchová and Olson, 2007; Koga et al., 2008).

Plant mitochondrial (mt) DNA generally has a slow rate of nucleotide substitutions, with an elevation in some lineages e.g. in *Plantago* (Mower et al., 2007). However, it undergoes frequent intramolecular recombinations resulting in rearrangements of intergenic regions. The large distances between mt genes prevent the design of suitable primers similar to universal cp primers, and thus RFLP of gene flanking regions by means of traditional Southern hybridization remains a suitable method to analyze the polymorphism of mt genomes in plants (Abe et al., 1999; Olson and McCauley, 2002; Štorchová and Olson, 2004). In this study, we analyzed variation in flanking regions of two mt genes, *atp1* and *cox1*, among various accessions of *A. vesiculosa*.

We sequenced the same cp intergenic regions as in *A. vesiculosa* in the sister taxa *D. muscipula* and *Drosera regia* with the aim of estimating their genetic distances and relatedness between these species with snapping traps.

A unique substitution in mt enzyme cytochrome *c* oxidase (COX1) was found in *Utricularia* (Lentibulariacea), possibly affecting properties of this essential enzyme (Jobson et al., 2004). *Utricularia* traps need high energy supply for active ion pumping (Adamec, 2006). The *cox1* gene of *A. vesiculosa* lacked this substitution (Jobson et al., 2004), which may reflect the distinct character of *A. vesiculosa* trap. The sequence of the *atp1* gene has not yet been determined in *A. vesiculosa*. We have therefore sequenced this mt gene in *A. vesiculosa*, and two related species—*D. muscipula*, and *D. regia*.

2. Materials and methods

2.1. Plant materials

A. vesiculosa plants originating from seven European, two Japanese, five Australian, and one African population were used for molecular analyses (Table 1). Plants from East Poland were grown outdoors in a 1 m^2 plastic container at the Institute of Botany at Třeboň, Czech Republic (Adamec, 1997). Plants from all other populations were grown outdoors in small 3–20 l aquaria, which stood in a 2.5 m^2 plastic container filled with water for cooling. In addition, plants from all Australian and African populations were grown in 3 l aquaria indoors (Adamec, 1999). As plants from each population are grown separately in covered aquaria, any incidental mixing of the individuals was excluded. Plants in all cultures propagated strictly vegetatively. The other two species, *D. muscipula* and *D. regia*, were grown in a peaty soil at the Botanical Garden at Liberec, Czech Republic.

2.2. DNA extraction, amplification, and sequencing

Whole plants of *A. vesiculosa* and leaves of *D. regia* and *D. muscipula* were thoroughly washed and the traps with captured prey cut off to eliminate possible contamination of other DNA. Total DNA was extracted from fresh material exactly as described by Maldonado San Martín et al. (2003). Each of six cp intergenic regions (Table 2) was amplified by 35 cycles of PCR using the primers and annealing temperatures shown in Table 2. To get a complete sequence of a *trnT*(UGU)-*trnL*(UAA) region in *A. vesiculosa*, internal primers were designed (aldro_a and aldro_c). New primers were developed to amplify the *atpB-rbcL* region in all three species under study. An attempt was made at amplifying the *trnL*(UAA)-*trnF*(GAA) region and the *psbB-psbF* region using e and f primers (Taberlet et al., 1991; Hamilton, 1999), but without success. The partial sequence of the mt *atp1* gene was determined using the primers described in Table 2. PCR products were cleaned using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), sequenced directly with BigDye terminator reaction mix (Applied Biosystems), and run on an ABI377 sequencer (Applied Biosystems). The sequences are deposited in GenBank under the following accession numbers: *trnS*(GCU)-*trnG*(UCC) – FJ764823 to FJ764826, *trnH* (*GUG*)-*psbA* – FJ764827 to FJ7648, *rpl20-5′-rps12* – FJ764811 to FJ764814, *trnT*(UGU)-*trnL*(UAA) – FJ764819 to FJ764822, *trnP*(UGG)-*trnW*(CCA) – FJ764831 to FJ764834, *atpB-rbcL* – FJ764815 to FJ764818, *atp1* – FJ764808 to FJ764810.

2.3. Southern hybridization

RFLP variation was assessed for the HindIII restriction sites flanking the *atp1* (*adenosine 5′ triphosphate synthetase* subunit 1) and *cox1* (*cytochrome oxidase* 1) mitochondrial genes. One μg of DNA was digested with HindIII, electrophoresed overnight on a 0.7% agarose gel and transferred to a positively charged membrane (Hybond N+, Amersham) by capillary blotting. A PCR fragment of the *atp1* gene was amplified using the primers atp1 lo and atp1 up (Table 1) and labelled with digoxigenin (DIG) using a PCR labelling kit (Roche Applied Science, Germany). Primers described by Olson and McCauley (2002) were applied to amplify the *cox1* gene. The blots were hybridized in EasyHyb buffer (Roche) with a DIG labelled probe at 42 °C overnight, washed twice at high stringency (0.1 × SSC, 65 °C), and detected using CDPStar (Roche) as substrate. An exposure time of <30 min was sufficient to visualise mt genes on Hyperfilm (Amersham).

2.4. Data analysis

Chloroplast DNA sequences were aligned using AlignX in Vector NTI Suite9 (InforMax, Invitrogen); additional manual editing was necessary to improve gap placement. Nucleotide substitutions identified by the alignment among *A. vesiculosa*, *D. regia*, and *D. muscipula* were used to calculate genetic distances among those three species in PAUP* 4 b10 (Swofford, 2003). The HKY85 substitution model was applied according to Model test 3.7 (Posada and Crandall, 1998). A neighbor-joining (NJ) tree was constructed on the basis of non-coding cp regions in *D. regia*, *D. muscipula* and in two haplotypes of *A. vesiculosa* using PAUP* 4 b10 (Swofford, 2003).

The conservative nature of the ATP1 protein made it possible to align the *A. vesiculosa* ATP1 amino acid sequence with orthologous proteins across the plant kingdom and also with ATPA in yeast. Multiple alignment of ATP1 protein sequences was generated by AlignX in Vector NTI Suite9 (InforMax, Invitrogen). The size of alignment was 520 amino acid. Yeast ATPA sequence (1QO1_A) was included to the alignment, because a three-dimensional model of ATP synthase was available for this organism. The comparison of *A. vesiculosa* and yeast ATP1 sequences made it possible to locate a

Table 1
Plant material used for the analyses. (Sub)tropical *Aldrovanda* sites are labelled by bold letter.

Abbreviation or species	Country of origin	Site of collection	Latitude	Longitude	Year of collection	No. of collected plants	Start of the cultivation at Třebon	No. of stocked plants	Turion formation	Anthocyanin formation	Reference or vaucher
NR	North Russia	Shallow lake near the estuary of the Sviri river on the southeastern bank of Lake Ladoga	60°29N	32°57E	1997	8	1997	8	Y	N	Afanas'ev (1953)
LI	Lithuania	Lake Ruzhas in the Ignalina district, NE Lithuania	55°30N	25°28E	2003	10	2003	10	Y	N	Vilkonis (2003)
EP	East Poland	Lake Długie, Łeczna-Włodawa Lake District	51°26N	23°06E	1993	31	1993	31	Y	N	Kamiński (1987)
UK	North Ukraine	Western bank of the Kiev reservoir (on the Dn'epr river) near Stracholes' village, at the T'et'erev river estuary	51°03N	30°25E	1997	6	1997	6	Y	N	Adamec (1995)
SW	Germany (Switzerland)	Artificial site, Lake Metmenhaslisee near Kloten in the Zurich Canton, Switzerland; plant origin from pool Bühlweiher at Lake Constance near Lindau, SW Bavaria, Germany	47°34N	9°41E	1994	2	1994	2	Y	N	Koch (1950)
HU	SW Hungary	Lake Balâta-tó, Somody County	46°19N	17°12E	2003	12	2003	12	Y	Y	Borhidi and Járai-Komlódy (1959)
RO	Romania	Shallow lake on Obretim island near the Sulina branch in the Danube delta	45°11N	29°19E	1998	20	1998	20	Y	N	Maldonado San Martín et al. (2003)
JPT	Japan, Tokyo	Hozoji pond near Hanyu City in Saitama Prefecture, Honshu island	36°12N	139°42E	1980s	?	1993	12	Y	N	Komiya (1966)
JPK	Japan, Kyoto	Lake Ogura-Ike, Uji near Kyoto (plants from Osaka Botanical Garden, Japan)	35°01N	135°47E	1955	?	2001	8	Y	N	–
NA	**North Australia**	Girraween Lagoon, ca. 30 km SE of Darwin, NT	12°31S	131°05E	1994	2	1998	3	N	Y	Wilson (1995); Adamec (1999)
KA	**North Australia**	Leach Lagoon near Katherine, NT	14°38S	132°37E	2003	5	2003	5	N	Y	–
AF	**Botswana**	Shallow swamp in Okavango delta	19°33S	23°13E	2005	12	2005	12	N	Y	Obermeyer (1963-1988)
AR	**Australia Armidale**	Billybung Lagoon near Gyura, Armidale County, NSW	30°06S	151°47E	2006	3	2006	3	N	Y	–
SWA	**SW Australia**	Coastal lake near Esperance, W Australia	33°48S	121°49E	2002	5	2002	5	N	Y	Gibson (2004)
SEA	**SE Australia**	Longvale Swamp near Broulee at Batemans Bay at the East Coast, NSW	35°35S	150°09E	1997	12	1997	12	N	Y	Adamec (1999)
DM *Dionaea muscipula*	SE USA	Liberec Botanical Garden, Czech Rep.	?	?	?	?	–	–	–	–	CPV1d
DR *Drosera regia*	South Africa	Liberec Botanical Garden, Czech Rep.	?	?	?	?	–	–	–	–	CPD74

Table 2
The primers (names in bold) used in this study and the size of PCR fragments amplified from cp and mt genomes.

Region	Primer name	Sequence 5′–3′	Anneal. temp.	Length (bp)			Reference
				Aldrovanda vesiculosa	*Drosera regia*	*Dionea muscipula*	
trnS(GCU)-*trnG*(UCC)	**trnS**	GCCGCTTTAGTCCACTCAGC	60 °C	**780**	**750**	**630**	Hamilton (1999)
cp	**trnG**	GAACGAATCACACTTTTACCAC					
trnH (*GUG*)-*psbA*	**trnH**	ACT GCCTTGATCCACTTGGC	55 °C	**190**	**400**	**370**	Hamilton (1999)
cp	**psbA**	CGAAGCTCCATCTACAAATGG					
rpl20–5′-rps12	**rpl20**	TTTGTTCTACGTCTCCGAGC	60 °C	**760**	**750**	**730**	Hamilton (1999)
cp	**rps12**	GTCGAGGAACATGTACTAGG					
trnT(UGU)-*trnL*(UAA)	**cpa**	CATTACAAATGCGATGCTCT	55 °C	**920**	**780**	**640**	Taberlet et al. (1991)
cp	**cpb**	TCTACCGATTTCGCCATATC					
	aldro_cpaF1	CCCCTATTTGGTGCAATCAG	55 °C	**650**	**–**	**–**	This study
	aldro_cpbR1	CATAATCTCGAATATGAGTTCAA					
trnP(UGG)-*trnW*(CCA)	**trnP**	GATTTGAAC CTA CGA CAT CG	60 °C	**260**	**270**	**280**	Ichihashi and Minami (2007)
cp	**trnW**	GATGTGGCGCAGCTTGGTAG					GenBank AB298100
atpB-rbcL	**atpB**	GAAGTAGTAGGATTGATTCTCAT	50 °C	**800**	**–**	**–**	Savolainen et al. (1994)
cp	**Rbc60L**	CAGGAGTATAATAAGTCATTG					This study
	AtpRbcF1	TTAGCACTCGATTTCGTTGG	50 °C	**580**	**530**	**615**	This study
	AtpRbcR2	CGACATGAATTAGGCGTTACTG					
atp1 coding region	**Atp1 lo**	TCTAGTGGCATTCGATCACAGA	55 °C	**1277**	**1277**	**1277**	McCauley et al. (2005)
mt	**Atp1 up**	TACACGAATTTTCAAGTGGATGA					

substitution in *A. vesiculosa* ATP1 protein in a three-dimensional model.

Protein blast and tblastn searches were performed using a partial sequence (425 amino acid) of ATP1 from *A. vesiculosa* (Botswana, GenBank accession number ACW82493) as a query.

3. Results

3.1. Uniformity of chloroplast and mitochondrial DNA in A. vesiculosa

The DNA sequence of six cp intergenic regions, totally 3700 nt, in 15 accessions of *A. vesiculosa* originating from four continents (Table 3) was determined. Only two differences among cp sequences, the presence/absence of T in a T array (10/11 nt) in the intergenic regions *rpl20-rps12* and *atpB-rbcL* (Table 3) were found. Two polymorphisms identified two haplotypes A and B and divided the set of *A. vesiculosa* samples into two subsets, European and non-European accessions, with two exceptions. One Japanese sample from Tokyo had cp sequences identical to European accessions and the plants from Romania (Europe) shared the two polymorphisms with non-European representatives. There was no correlation between cp haplotype and anthocyanin or turion production. Non-European accessions originating from both temperate and tropical zones shared the same cp haplotype.

No differences were found in Southern-RFLP of flanking regions of the mitochondrial genes *atp1* and *cox1* among six representatives of *A. vesiculosa* from four continents (Fig. 1). We also estimated the partial sequence of the *atp1* coding region.

3.2. Genetic distances among A. vesiculosa, D. regia and D. muscipula based on cp DNA

Sequences of the same cp regions as in *A. vesiculosa* were also estimated in *D. muscipula* and *D. regia*. Four cp regions produced reliable alignment between *A. vesiculosa*, *D. muscipula* and *D. regia*, whereas only 570 nt from the *cpa-cpb* region, and 300 nt from the

Table 3
Sequence variation in two cp intergenic regions among *A. vesiculosa* accessions from four continents. The only difference was the size of one T array between A (11 T) and B (10 T) haplotypes. The intergenic regions *trnS-trnG*, *trnP-trnW*, *Cpa-Cpb*, *psbA-trnH* were identical among the accessions.

Abbreviation	Geographic origin	*rpl20–rps12*	*atpB–rbcL*
EP	Europe – East Poland	A	B
UK	Europe – North Ukraine	A	B
HU	Europe – Hungary	A	B
LI	Europe – Lithuania	A	B
SW	Europe – Switzerland	A	B
NR	Europe – North Russia	A	B
RO	Europe – Romania	B	A
SEA	SE Australia – Broulee	B	A
NA	North Australia – Darwin	B	A
SWA	SW Australia – Esperance	B	A
KA	North Australia -Katherine	B	A
AR	Australia – Armidale	B	A
JPK	Asia – Japan Kyoto	B	A
JPT	Asia – Japan Tokyo	A	B
AF	Africa – Botswana	B	A

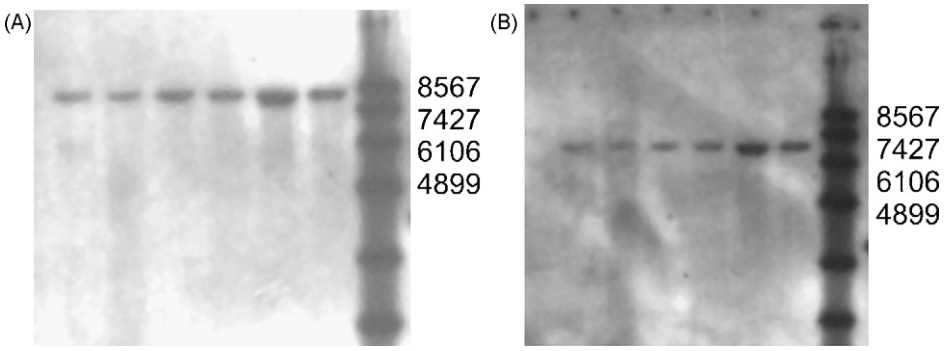

Fig. 1. Uniformity of mt Southern-RFLP patterns among six accessions (AF, NA, JPK, SWA, UK and LI) of *A. vesiculosa* from four continents. Total DNA was digested by EcoRI and hybridized with digoxigenin labeled *atp1* (**a**) and *cox1* (**b**) probe.

Table 4
Genetic distances calculated from nt substitutions in six chloroplast intergenic regions of *A. vesiculosa*, *D. muscipula* and *D. regia*. Empirical nt frequencies and a substitution model HKY85 were applied.

Chloroplast region	Length of alignment (nt)	*A. vesiculosa–D. muscipula*	*A. vesiculosa–D. regia*	*D. muscipula–D. regia*
atpB-rbcL	671	0.12916	0.07999	0.10471
cpa-cpb	987 (568)	0.16493	0.15485	0.13024
psbA-trnH	471	0.14824	0.16078	0.15353
trnS-trnG	305	0.13837	0.13775	0.08475
trnP-trnW	338	0.09017	0.07574	0.06902
rpl20-rps12	797	0.09966	0.06080	0.06796
Combined data	3150	0.12218	0.09869	0.09667

trnS-trnG region could be aligned unambiguously. Using the HKY evolution model, five cp intergenic regions as well as the combined data set suggested a shorter genetic distance between *A. vesiculosa* and *D. regia* than that between *A. vesiculosa* and *D. muscipula* (Table 4, Fig. 2). The intergenic region *psbA-trnH* was the only one showing the opposite result, however, the species under study differentiated by indels (insertion or deletions) rather than by nt substitutions in this region. Genetic distance was calculated using nt substitutions only. *D. muscipula* and *D. regia*, but not *A. vesiculosa*, possessed 80 nt insertion in the *psbA-trnH* intergenic region, similar (75%) to the chloroplast *trnY* sequence from *Nepenthes vieillardii* (GenBank AB103315).

3.3. The sequence of the atp1 gene in A. vesiculosa

The *atp1* sequence was identical in all *A. vesiculosa* accessions and harbored a short unique sequence motif. This motif composed of four amino acids (TGWS) was found at the positions 73–76 of the partial *A. vesiculosa* ATP1 amino acid sequence, which corresponded to the positions 121–124 of yeast ATPA (Fig. 3). The sequence of this region may vary at the broadest taxonomic scale (Fig. 3) but the consensus amino acid sequence LSDH, from which some species differ in one or two substitutions, was found in almost all angiosperms (Fig. 3a). The TGWS motif, present in *A. vesiculosa* Atp1, was exceptional not only among plants, but also among all living organisms, as was documented by BLAST search. ATP1 proteins of closely related *D. regia* and *D. muscipula* contained the angiosperm consensus LSDH (Fig. 3) at corresponding positions. Interestingly, the only other difference outside this region between *A. vesiculosa* and *D. regia* or *D. muscipula* was one substitution in a 400 amino acid long alignment of three partial ATP1 sequences (FJ764808, FJ764809, FJ764810).

Nucleotide alignment of the region of interest of *atp1* genes is shown in Fig. 3. The stretch of 12 nt, completely different from the *atp1* sequences of related species, encoded TGWS amino acids in *A. vesiculosa*. DNA sequence similarity between *atp1* genes from *A. vesiculosa* and *D. regia* or *D. muscipula*, calculated from a 1300-nt long alignment (after exclusion of TGW region), was very high—99.3% of identical nt.

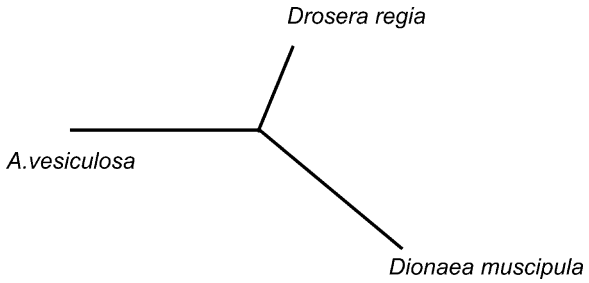

Fig. 2. Neighbor-joining (NJ) tree based on six non-coding cp regions showing relationship among *A. vesiculosa*, *Drosera regia* and *Dionaea muscipula*. Two haplotypes of *A. vesiculosa* are very close and are represented by the same branch.

4. Discussion

4.1. Genetic uniformity of A. vesiculosa

We detected no substitution and only two mononucleotide indels in a combined alignment (3700 nt) of six cp intergenic regions in *A. vesiculosa*, in spite of the fact that the specimens of this rare aquatic carnivorous species were collected within the vast range of four continents. Both indels were represented by one T in a T array. The number of Ts in T stretches is considered to be the most variable feature in non-coding organellar DNA (Hamilton et al., 2003), which are prone to extensive homoplasy (Ingvarsson et al., 2003) and may vary within the same population (Lia et al., 2007). However, we do not suppose that T indels arise repeatedly in *A. vesiculosa*. Two indels may occur in four combinations (AA, AB, BA, BB), but we found only two of them—AA and BB among 15 accessions. These haplotypes correlated, with two exceptions, with the geographic origin of specimens, distinguishing European and non-European accessions of *A. vesiculosa*.

Genetic uniformity of *A. vesiculosa* contrasts with polymorphism detected in cp genomes of other aquatic plant species with similar global distributions. Nine haplotypes were found in the *trnL-trnF* spacer and the trnL intron in *Hydrilla verticillata* collected in four continents (Madeira et al., 2007). Two nt substitutions and one indel in *trnK* intron separated *Ceratophyllum demersum* from North America and Australia (Les et al., 2003).

Uniformity of cp intergenic regions, accompanied by invariant RFLPs of mt gene flanking regions, seems to be more prominent in *A. vesiculosa* than in other aquatic species. A recent and severe bottleneck followed by long-distance dispersal by migratory birds was suggested by previous studies (Maldonado San Martín et al., 2003; Hoshi et al., 2006) to explain the low genetic variation in *A. vesiculosa*. Our results are consistent with this hypothesis. The plants from Tokyo (Japan) and Romania (Europe) had different cp haplotypes than accessions from the same continent. This deviation could also be explained by recent (Holocene) intercontinental transfer of *A. vesiculosa* mediated by water birds. An example of long-distance migration provides Bewick's swan which follows a 2000 km long route from southern Japan to northern Russia (Kamiya and Ozaki, 2002). This species also migrates from Siberia to Denmark, Poland and England (Klaassen et al., 2004). The possibility exists, that Bewick's swan may transfer *A. vesiculosa* propagule from Europe to Japan.

On the other hand, the possibility that a slow mutation rate contributes to DNA sequence uniformity in *A. vesiculosa* cannot be excluded. The genus *Aldrovanda* is very ancient, with the split between ancestors of *Aldrovanda* and *Drosera* estimated to have occurred about 50 Mya before present (Yesson and Culham, 2006). More extensive knowledge of DNA sequences in *A. vesiculosa* will be necessary to calculate mutation rate in this interesting species. Another explanation could be a selective sweep at the level of cp genome in *A. vesiculosa*. As recombination does not occur among cp

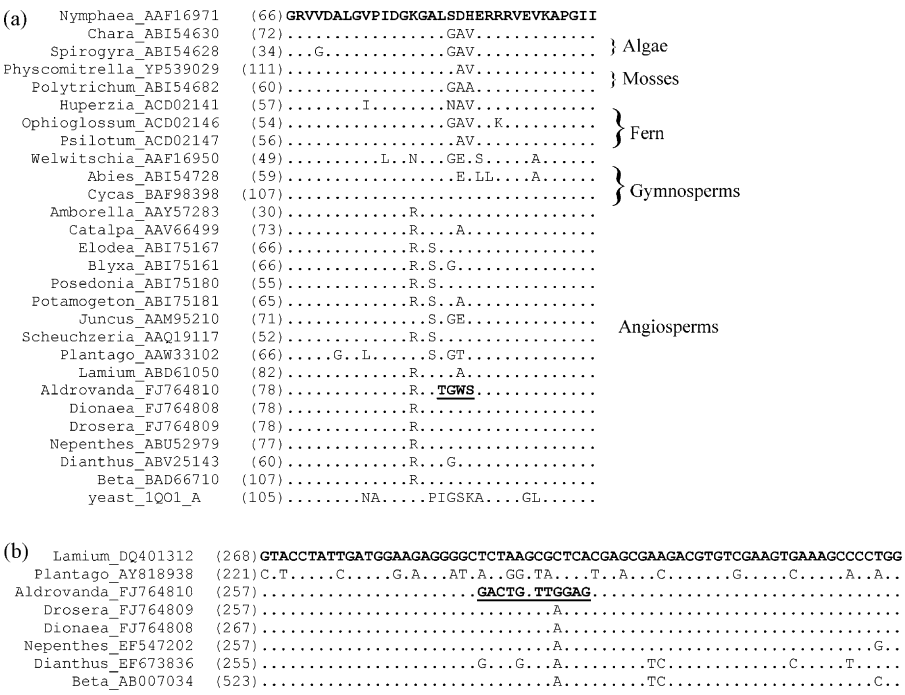

Fig. 3. (a) The alignment of partial (33 amino acid) sequences of ATP synthase alpha from green plants and yeast. The substitution in *A. vesiculosa* is underlined. (b) The alignment of partial (70 nt) sequences of *atp1* gene in Caryophyllales. Two outgroups are included—*Lamium* showing slow rate of nt substitutions, and *Plantago* with elevated rate of nt substitutions in mt DNA. The substitution in *A. vesiculosa* is underlined.

genomes, selection on single gene affects an entire genome. However, very low variation of nuclear markers (Maldonado San Martín et al., 2003; Hoshi et al., 2006) suggests that both cp and nuclear genomes were influenced by some factor reducing genetic variation.

Very low genetic variation was revealed in *A. vesiculosa* stands, contrasting with the variation in morphological and physiological characters (colour and production of turions) which typically distinguish Australian and Euro-Asian accessions. We suggest that the observed morphological and physiological differences could be caused by rare point mutations leading to the loss of anthocyanin production or turion formation. The same can apply also for the red Hungarian plants (Adamec, 2005). The recent observation that some Australian *A. vesiculosa* plants are able to produce dormant turions (Adamec, unpubl.) suggests that intrinsic capability to form turions has not been lost in Australian populations. The differences in turion formation between European and Australian accessions of *A. vesiculosa* are therefore rather quantitative. As the efficiency of turion production in plants cultivated under the same conditions depends on their origin, we assume that genetic differences in the components of ontogenetic signalling pathways are responsible for the observed morphological and physiological differences rather than phenotypic plasticity.

4.2. Chloroplast markers suggest short genetic distance between *A. vesiculosa* and *D. regia*

In previous studies, *D. muscipula* was shown to be the closest relative to *A. vesiculosa* and this pair to be a sister to *Drosera* (Cameron et al., 2002; Rivadavia et al., 2003). Our finding, based on six non-coding cp regions, that *A. vesiculosa* had smaller genetic distance to *D. regia* than to *D. muscipula*, seems to be contradictory to previous observations. However, a closer inspection of the published analyses (Cameron et al., 2002; Rivadavia et al., 2003) showed that trees placing *D. muscipula* as a sister to *A. vesiculosa* relied mostly on nuclear DNA, and that if cp DNA alone was considered the tree was either not resolved, or *A. vesiculosa* was closer to *D. regia*. Thus, our results are in agreement with the previous studies (Cameron et al., 2002; Rivadavia et al., 2003) and suggest that the cp DNA in *A. vesiculosa* has different origins than the nuclear genes. Similar inconsistencies between gene trees based on nuclear and cytoplasmic markers have been explained by chloroplast capture (Tsitrone et al., 2003), and this phenomenon has been documented in several plant species (Gaskin and Wilson, 2007; Barrett and Case, 2006).

4.3. Unique substitution in the atp1 gene of *A. vesiculosa*

Some carnivorous plant species possess unique molecular adaptations associated with the active trapping of prey. The mitochondrial enzyme COX1 in *Utricularia* (Lentibulariaceae) has two unusual amino acid substitutions (Cys-113 and Cys-114) which could contribute to the increased respiratory capacity needed for extensive active ion pumping in the traps (Jobson et al., 2004). The substitution of four amino acids (TGWS), found in the ATP synthase alpha subunit (ATP1) of *A. vesiculosa*, was located on the surface of the central domain in the positions corresponding to positions 121–124 of yeast ATPA (Stock et al., 1999). These positions are variable at the highest taxonomic level, but conserved among angiosperms (Fig. 3a). We cannot predict whether the substitution revealed in *A. vesiculosa* may affect the functioning of ATP1, a subunit of a key complex responsible for energy conversion in mitochondria. However, the substitution was unique, not found in ATP1 of any other organism, including the closest relatives of *A. vesiculosa*–*D. muscipula* and *D. regia*. As nt alignment (Fig. 3b) suggested, replacement of 12 nt in the *atp1* gene of *A. vesiculo*sa rather than gradual changes of four neighbouring codons were responsible for this amino acid substitution. The stretch of 12 nt is too short to allow the identification of its origin. It may be derived from mt genome, but the possibility of horizontal gene transfer from different species, frequent in mt DNA of some plants (Bergthorsson et al., 2004) cannot be excluded.

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