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Research Article

Phylogenetic relationships among *Orostachys* subsection *Orostachys* species (Crassulaceae) based on nuclear and chloroplast DNA data

[1]Marina M. KOZYRENKO [2]Svetlana B. GONTCHAROVA [1]Andrey A. GONTCHAROV*

[1](*Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences*, Vladivostok 690022, Russia)
[2](*Botanical Garden-Institute, Far Eastern Branch of the Russian Academy of Sciences*, Vladivostok 690024, Russia)

Abstract Nuclear ribosomal ITS and four cpDNA intergenic spacer sequences were used to assess how the patterns of molecular differentiation are related to taxonomic boundaries and geographic distribution in polymorphic and taxonomically complex *Orostachys* subsection *Orostachys* (Crassulaceae). Two major cpDNA lineages were identified in a set of *Orostachys* populations, lineage A, comprising 13 closely related haplotypes found in 11 populations of monocarpic *O. malacophylla* var. *malacophylla*, *O. maximowiczii*, and *O. gorovoii* and lineage B that included 9 out of 10 divergent haplotypes found in five populations of *O. paradoxa*, distinct in perennial stoloniferous habit. Our data suggest that the current concepts of *O. malacophylla* var. *malacophylla*, *O. maximowiczii*, and *O. gorovoii* are incompatible with the differentiation at the cpDNA level. Neither of these taxa could be allied to a particular haplotype or haplotype clade. The pattern of relationships between 7 ITS ribotypes found in 17 populations supported neither the morphology-based taxonomic subdivision in the subsection *Orostachys* nor grouping according to geographical origin of the populations or lineages recovered with cpDNA data. A high level of similarity of ITS rDNA sequences between the subsection members suggests their relatively recent and rapid divergence from a common ancestor.

Key words cpDNA, Crassulaceae, ITS rDNA, *Orostachys*, phylogeny.

The genus *Orostachys* Fisch. (Crassulaceae DC.) is characterized by a rosette vegetative morphology and terminal dense thyrsoid-racemose inflorescence. It accounts for ca. 20–25 species having a preliminary East-Asian distribution and likely origin (Ohba, 1978, 2003; Byalt, 1999, 2000). Only two members of the genus, *O. spinosa* (L.) C. A. Meyer ex Berger and *O. malacophylla* (Pallas) Fischer, have broad distribution areas, spreading from the Pacific coast to Eastern Europe (South Urals) and Eastern Siberia, respectively. Most other taxa are restricted to Central Asia, NW China, Southern Siberian Mts., and Mongolia or northeastern provinces of China and the Sea of Japan shores.

Current taxonomy recognizes two sections in the genus, *Orostachys* and *Schoenlandia* Ohba (see, however, Fu & Ohba, 2001; Ohba, 2003), differing in inflorescence and flower morphology. The former section accommodates most members of the genus and is split into two subsections, *Orostachys* and *Appendiculata* (Boriss.) Ohba, differing in blunt or cuspidate leafs, respectively. Recent molecular phylogenetic studies have only revealed distant relationship between the subsections and placed the robust subsect. *Orostachys* clade within the genus *Hylotelephium* Ohba, having similar flower features (Stephensen, 1994) but a distinct growth habit (Mayuzumi & Ohba, 2004; Gontcharova, 2006; Gontcharova et al., 2006, 2008). The subsect. *Appendiculata* (including the genus *Meterostachys* Nakai) was resolved as a sister to the *Hylotelephium*/*Orostachys* lineage.

The Sea of Japan shores and mountains of the Korean Peninsula, Japan, and Eastern Russia are an apparent center of the subsection *Orostachys* diversity and likely origin. Up to 10 species are recognized in the subsection based on differences in leaf' shape, size and color, presence of stolones, inflorescence shape, and features of the flower parts (Ohwi, 1965; Ohba, 1990, 2001, 2003; Ohba & Tagawa, 1990; Byalt, 1999; Gontcharova, 2006). These morphological characteristics are known for their plasticity in the natural populations, which allow them to often bridge boundaries between species and hampers their confident discrimination (Fig. 1). Morphological alterations (e.g., change of leaf color, leaf flattening, etc.) and frequent fragmentation of herbarium specimens of the naturally fleshy *Orostachys* plants often make their comparison with the protologs difficult. As a result,

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* Author for correspondence. E-mail: gontcharov@biosoil.ru. Tel.: 7-4232-338765. Fax: 7-4232-310193.

Fig. 1. Phenotypic diversity in *Orostachys* subsect. *Orostachys* members/populations. **A**, *O.* cf. *maximowiczii* P8; **B**, *O.* cf. *maximowiczii* P6; **C**, *O.* cf. *malacophylla* var. *malacophylla* P11; **D**, *O.* cf. *paradoxa* P15; **E**, *O. malacophylla* var. *malacophylla* P3; **F**, *O. gorovoii* P10; **G**, *O.* cf. *paradoxa* P13; **H**, *O. paradoxa* P16.

species composition and relationship within the subsection *Orostachys* remain controversial (Ohba, 1990, 2001, 2003; Byalt, 1999; Gontcharova, 2006).

The most recent taxonomic treatments recognized five species (*O. boehmeri* (Makino) H. Hara, *O. gorovoii* Dudkin & S. Gontch., *O. malacophylla* (Pall.) Fisch., *O. maximowiczii* Byalt, and *O. paradoxa* (Khokhr. & Vorosch.) Czerep.), and three infraspecific taxa (*O. malacophylla* ssp. *lioutchenngoi* Ohba, *O. malacophylla* var. *aggregata* (Makino) Ohba, and

O. malacophylla var. *iwarenge* (Makino) Ohba) in the subsection (Byalt, 1999; Dudkin et al., 2001; Ohba, 2003; Gontcharova, 2006). However, it is unclear whether these taxa represent distinct evolutionary entities or if the morphological diversity within the subsection is a product of a frequent local adaptation or phenotypic plasticity. A recent comparison of ITS rDNA sequence divergence in the crassulacean lineages revealed only minor genetic divergence between *Orostachys* subsect. *Orostachys* members and was unable to resolve patterns of relationship in the subsection (Gontcharova et al., 2006, 2008; Gontcharova & Gontcharov, 2009). Frequent hybridization in cultivation between generally nonsympatric subsection members further complicates our understanding of relationships between them.

This study provides the first molecular data for a better understanding of the evolutionary history of the Far-Eastern crassulacean flora, specifically the evolutionary relationship between members of polymorphic and taxonomically complex *Orostachys* subsect. *Orostachys*. We use the sequence data of the internal transcribed spacer (ITS) of nuclear ribosomal DNA and four intergenic spacers of the chloroplast DNA (cpDNA) (1) to investigate genetic differentiation among *Orostachys* subsect. *Orostachys* species and populations, (2) to test whether genetic data support the recognition of current species, and (3) how distinct lineages relate to geographical distribution.

1 Material and methods

1.1 Population sampling

To evaluate the population genetic features and monophyly of species with substantial morphological variation, 5–12 specimens were sampled from each population, and altogether 152 accessions were used for DNA analysis. The 17 natural populations sampled represented four species—*O. gorovoii*, *O. malacophylla* var. *malacophylla* (hereafter referred as *O. malacophylla*), *O. maximowiczii*, and *O. paradoxa*, and different habitats: coastal sandy terraces, (coastal) stony terraces and cliffs, inland limestone outcrops, and the Sikhote-Alin Mts. slopes. Sample size, population code, and the geographic coordinates for each population are given in Table 1 and shown in Fig. 2. The specimens were taken as far apart from one another as possible; however, neighboring individuals were sampled when the population was small (P9 and P13).

1.2 DNA extraction, polymerase chain reaction (PCR), and sequencing

Leaf tissue or inflorescence samples were shipped to the laboratory where they were stored in a −80 °C freezer until extraction. Total genomic DNA was extracted using DNeasy Plant Mini Kit (QIAGEN, Germantown, MD, USA), following the manufacturer's instruction. To amplify and direct sequencing ITS rDNA and four regions of cpDNA, we used universal primer pairs (Table 2) and thermocycling conditions that were recommended for the regions. The PCR products were sequenced using a BigDye terminator v. 3.1 sequencing standard kit (Applied Biosystems, Foster City, CA, USA). Sequencing was carried out in both directions under cyclic sequencing conditions described by Shaw et al. (2005, 2007). Sequences were analyzed on an ABI 3130 genetic analyzer (Applied Biosystems). Forward and reverse sequences were assembled using the Staden Package v. 1.4 (Bonfield et al., 1995) and aligned manually with the SeaView program (Galtier et al., 1996).

Table 1 Sampling sites locations, codes and sample sizes (*n*) of *Orostachys* subsect. *Orostachys* populations

Species	Region, locality, habitat	Codes	Latitude, N	Longitude, E	*n*
O. malacophylla	Zabaikalski Krai, Kira, steppe	P1	49°34′17.03″	111°58′49.42″	5
O. malacophylla	Zabaikalski Krai, Ust-Ilia, steppe	P2	50°23′42.68″	113°42′35.71″	5
O. malacophylla	Primorsky Krai, Khankaiski Distr., Khanka lake sandy dunes	P3	45°11′59.06″	131°59′40.19″	10
O. malacophylla	Primorsky Krai, Nadezdinski Distr., stony hill slope	P4	43°38′58.76″	131°55′1.92″	10
O. malacophylla	Primorsky Krai, Vladivostok, scree slope	P5	43°11′7.23″	131°54′31.46″	10
O. maximowiczii	Primorsky Krai, Khasan Distr., stony beach	P6	42°36′51.65″	131°8′7.88″	10
O. maximowiczii	Primorsky Krai, Khasan Distr., coastal sandy dunes	P7	42°36″8.67″	130°46′24.32″	12
O. cf. *maximowiczii*	Primorsky Krai, Khasan Distr., hill rocky slope	P8	42°32′23.27″	130°41′40.29″	10
O. gorovoii	Primorsky Krai, Shkotovski Distr., limestone outcrops	P9	43°26′14.83″	132°24′36.74″	10
O. gorovoii	Primorsky Krai, Nakhodka town, limestone outcrops	P10	42°49′38.99″	132°59′38.13″	10
O. cf. *malacophylla*	Primorsky Krai, Lazovski Distr., coastal sandy dunes	P11	42°51′55.99″	133°40′23.96″	10
O. cf. *paradoxa*	Primorsky Krai, Lazovski Distr., Unnamed Island, rocky cliff	P12	42°50′13.06″	133°41′26.92″	5
O. cf. *malacophylla*	Primorsky Krai, Lazovski Distr., stony beach	P13	42°49′23.93″	133°42′47.23″	5
O. paradoxa	Primorsky Krai, Dalnegorsk town, mountain stony slope	P14	44°33′3.81″	135°35′44.93″	10
O. cf. *paradoxa*	Primorsky Krai, Terney Distr., stony beach	P15	44°37′6.53″	136°12′57.19″	10
O. paradoxa	Primorsky Krai, Terney Distr., coastal sandy dunes	P16	44°54′25.63″	136°31′57.10″	10
O. paradoxa	Primorsky Krai, Terney Distr., coastal sandy dunes	P17	44°55′59.56″	136°32′40.33″	10

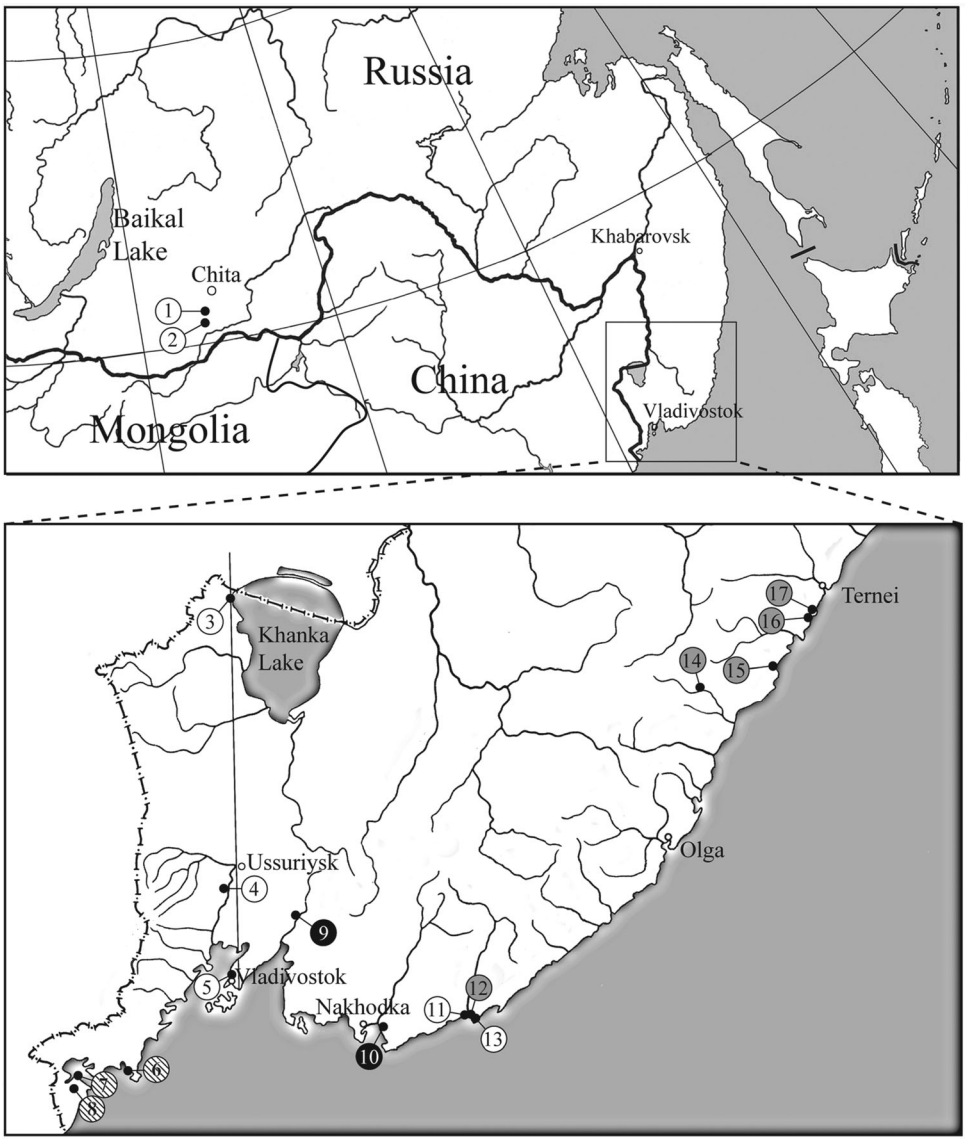

Fig. 2. Map of the sampled populations of *Orostachys* spp. Population codes correspond to those in Table 1. The map was based on images retrieved from Google Earth (http://www.google.com/earth).

1.3 Data analysis

The boundaries and structural elements in ITS rDNA region were determined by comparison with published crassulacean sequences and ITS secondary structure model (Gontcharova & Gontcharov, 2004; Gontcharova et al., 2006). Fold predictions were made at the M. Zuker's web server (Zuker, 2003; http://www.bioinfo.rpi.edu/~zukerm/rna/).

Because cpDNA does not recombine and is, therefore, equivalent to a single locus, sequences for the four fragments were combined into a single concatenated dataset for all analyses. To determine relationships among cpDNA haplotypes we used the TCS software package (version 1.18; Clement et al., 2000). To represent all possible alternative pathways between haplotypes within a single figure, a statistical parsimony analysis with a 95% confidence limit and indels coded as a fifth state was carried out (Clement et al., 2000). Numbers of haplotypes (n_H), values of haplotype diversity (h) and nucleotide diversity (π) were calculated in Arlequin v. 3.11 (Excofflier et al., 2005) software program.

An analysis of molecular variance (AMOVA; implemented in Arlequin) was performed to estimate the distribution of genetic variation within and between populations and the values of pairwise genetic distances

Table 2 Primers, fragment sizes and DDBJ/EMBL/GenBank accession numbers for sequences of the ITS rDNA and four chloroplast regions sequenced for this study

Region	Nucleotide sequence (5′ → 3′)	Fragment size (bp)	Accession numbers
ITS rDNA	[a]CTGCCCTTTGTACACACCGCCCGTC[g]	611	HF565574–HF565584
	[b]TCCTCCGCTTATTGATATGC[g]		
	[c]AGGAGAAGTCGTAACAAGG[h]		
trnH–psbA	[d]CGCGCATGGTGGATTCACAATCC[g]	354 or 367	HF565191–HF565218
	[d]GTTATGCATGAACGTAATGCTC[g]		
trnS–trnG	[d]AGATAGGGATTCGAACCCTCGGT[g]	1326–1332	HF565219–HF565246
	[d]GTAGCGGGAATCGAACCCGCATC[g]		
	[d]GCGGGTATAGTTTAGTGGTAAAA[h]		
	[d]TTTTACCACTAAACTATACCCGC[h]		
trnT–trnL	[e]CATTACAAATGCGATGCTCT[g]	989	HF565247–HF565274
	[e]GGGGATAGAGGGACTTGAAC[g]		
	[e]TCTACCGATTTCGCCATATC[h]		
	[e]CGAAATCGGTAGACGCTACG[h]		
rpl32–trnL	[f]CAGTTCCAAAAAAACGTACTTC[g]	582–601	HF565275–HF565302
	[f]CTGCTTCCTAAGAGCAGCGT[g]		

[a] Elwood et al. (1985); [b] White et al. (1990); [c] Modified from Wen & Zimmer (1996); [d] Shaw et al. (2005); [e] Taberlet et al. (1991); [f] Shaw et al. (2007); [g] Primers used for PCR and cycle sequencing; [h] Primers used for cycle sequencing only.

(F_{ST}) between populations. The significance of the variance components was determined with a permutation test (10 000 replicates).

Phylogenetic trees were inferred using maximum parsimony method (MP) implemented in PAUP* ver. 4.0b10 (Swofford, 2002) by a heuristic search with tree-bisection-reconnection (TBR) branch swapping and 10 random sequence addition replicates. Bootstrap support (Felsenstein, 1985) was estimated with 1000 replications of heuristic search. Both the consistency and retention indices (CI and RI, respectively) were used to assess the amount of homoplasy present in the dataset.

2 Results

2.1 ITS secondary structure and analysis

The ITS region was sequenced from 86 plants (1–7 plants per population). All electrophoretograms produced clear reads with no intragenomic polymorphic sites. To ensure that there was no intrapopulation polymorphism in the ITS, we sequenced all 12 individuals in the population P7. The ITS rDNA (611 bp) revealed 10 polymorphic sites (9 sites are parsimony informative) located in ITS1 (five substitutions), ITS2 (four substitutions) and 5.8S (one substitution; Fig. 3: A–F). Only one substitution (A → G) change in specimens from Zabaikal'e (two accessions from P1 and five from P2) was found in the single-stranded region (ITS1, spacer between the stems 2 and 3; Fig. 3: B). Other nucleotide changes were in the stem regions but mostly involved non-pairing nucleotides in the terminal loops and side bulges (Fig. 3: A–F). Only two substitutions found in these structures represented hemi-compensatory base changes (ITS1, stem 4, C–G → U–G; ITS2, stem 4, U–G → C–G; Fig. 3: C, F). In two instances, the substitutions caused alterations in the architecture of distal parts in stems 1 and 2 of ITS1 (Fig. 3: A, B). Only in case of stem 2 did this alteration cause a noticeable ($\Delta G = -10.40 \rightarrow -14.00$) increase in folding free energy.

Seven ITS ribotypes (R1–7) differing from each other in three substitutions were revealed in the 86 specimens studied (Fig. 3: G). The most common ribotype R1 was found in 33 accessions representing ten populations (*O. malacophylla*—four populations, *O. paradoxa*—four populations and *O. gorovoii*—two populations). Four ribotypes (R4–7) occurred in two (mostly geographically adjacent) populations and the remaining two most similar ribotypes (R2 and R3; 1 bp difference in 5.8S rDNA) were endemic. It should be noted that ribotypes R2–7 shared two synapomorphic substitutions (Fig. 3: B, C) distinguishing them from the most frequent ribotype R1. In the populations P1, P3 (*O. malacophylla*) and P16 (*O. paradoxa*), two ribotypes were revealed (Fig. 3: G). One of these ribotypes was the most frequent in our dataset type R1, however, in these populations, it was found only in one to two individuals.

The grouping of sequences to respective ribotype' clades generally attained weak-to-moderate support in MP phylogenetic analysis (66–90%; Fig. 3: H). Sequences belonging to the ribotypes R5, R6, and R7 as well as highly similar accessions from the ribotypes R2 and R3 formed weakly supported clades (66–90%); however, the branching pattern between R1, R2 + 3, R4 and R5–7 clades remained unresolved.

2.2 cpDNA analysis and phylogenetic relationships

The *trnH–psbA*, *trnS–trnG*, the *trnT–trnL*, and *rpl32–trnL* intergenic spacer regions of cpDNA were successfully sequenced for all 152 plants. The *trnH–*

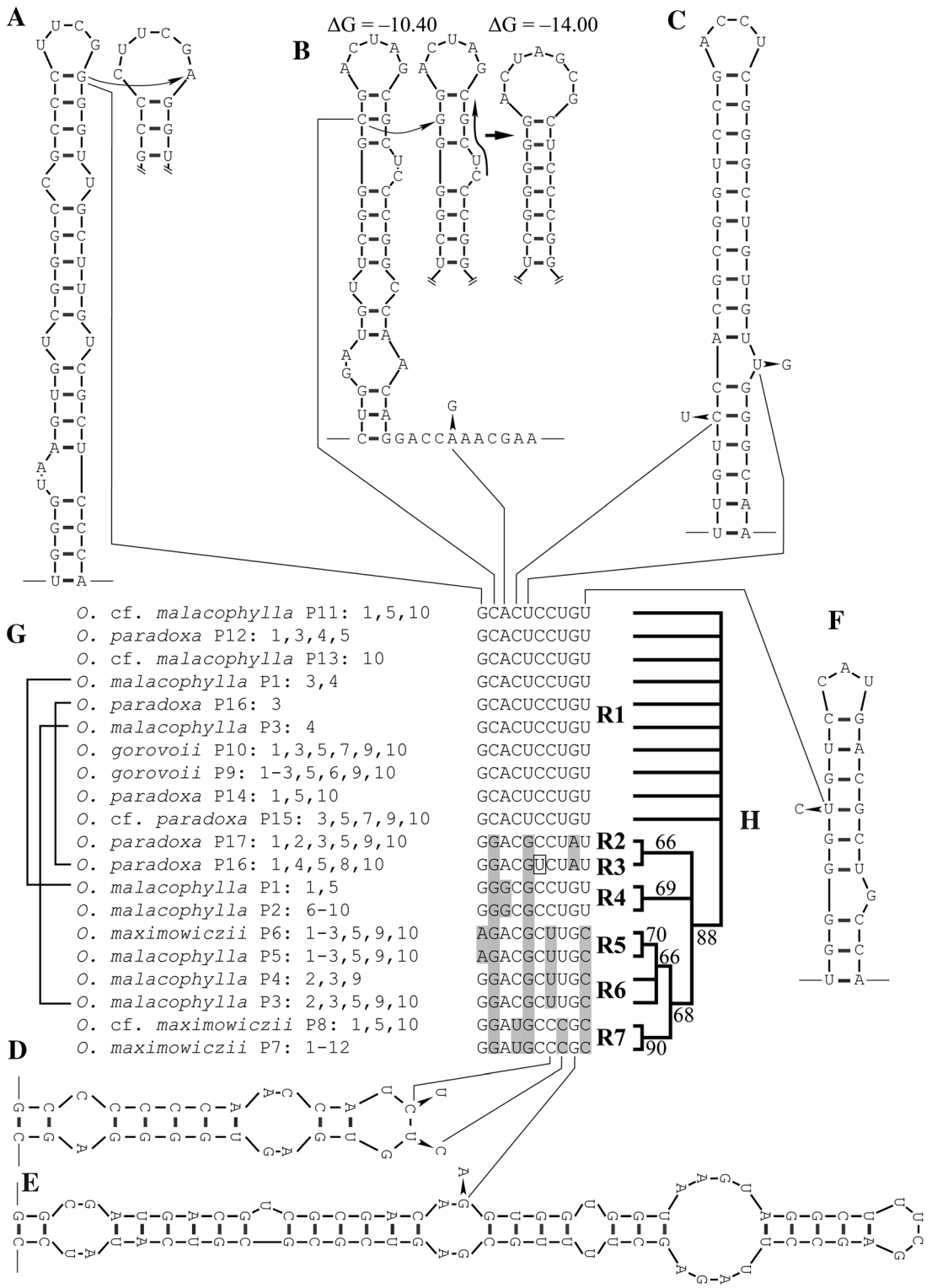

Fig. 3. Elements of ITS1 (**A**, stem 1; **B**, stem 2; **C**, stem 4) and ITS2 rDNA (**D**, stem 1; **E**, stem 3; **F**, stem 4) secondary structure for *Orostachys* subsect. *Orostachys* members based on the model proposed for Crassulaceae (Gontcharova & Gontcharov, 2004), showing substitutions/ribotypes found in the dataset of 86 sequences (**G**) and MP bootstrap tree based on this dataset (**H**). Substitution in the 5.8S rDNA gene distinguishing R3 ribotype is marked by square in the alignment.

psbA sequence was 346 bp in most populations and 367 bp in population P8 as a result of the duplication of a 13 bp motif, which was a marker for this population. Two substitutions provided markers for P14 (A in position 265) and P15 (C in position 305). The length of the *trnS–trnG* spacer ranged from 1326 to 1332 bp. This variability was due to three mononucleotide repeats, a poly-A motif, repeated 9 or 11 times and two poly-T repeats, one varied in length between 9 and 10 bp and the other repeated 12 or 19 times. The shortest *trnS–trnG* spacer characterized population P5. One substitution (T in position 709) in this region provided a marker for population P8. The length of the *trnT–trnL* region was 989 bp in all 152 individuals studied and the sequence variation was due to point mutations in nine positions. One substitution provided markers for population P14 (C in position 843). The presence of two 5 bp indels (SSRs) in the *rpl32–trnL* spacer resulted in its rather uniform length, either 601 or 591 bp in the majority of accessions and an additional 9 bp indel characterized most plants in population P17 (582 bp). In this spacer, a 49 bp inversion shared by all plants from populations P15 and P16 and four plants from populations P12 and P17 (two plants from each) was revealed. This inversion was manually reverse-complemented and retained in the data matrix. The occurrence of the inversion as well as indels in all spacers was coded as a single binary character and appended to the data matrix (Quandt et al., 2003). In the combined dataset of four non-coding regions, 44 polymorphic sites were detected, 10 within the *trnH–psbA*, 19 within the *trnS–trnG*, 9 within the *trnT–trnL*, and 6 within the *rpl32–trnL* region.

Nucleotide substitutions and indel variations revealed 28 haplotypes (Fig. 4). Of these, six haplotypes were found in only single specimens (private haplotypes) and only three haplotypes were found in more than one population (P1 and P2 shared haplotype H7, haplotype H25 was shared by P3 and P10, and haplotype H4 was shared by P11 and P13; Table 3). The haplotype network consisted of two divergent clades separated by more than 30 mutation steps (Fig. 4). Clade A comprised the majority of haplotypes found in populations of *O. malacophylla*, *O. maximowiczii*, and *O. gorovoii* and clade B included all but one haplotype found in populations of *O. paradoxa*. Alternative links between haplotypes (loop structures in the network) revealed in clade B suggested possible recombination or homoplasy here. Six divergent haplotypes (five of *O. malacophylla* and one of *O. paradoxa*) occupied intermediate positions between these clades and were regarded as clade C.

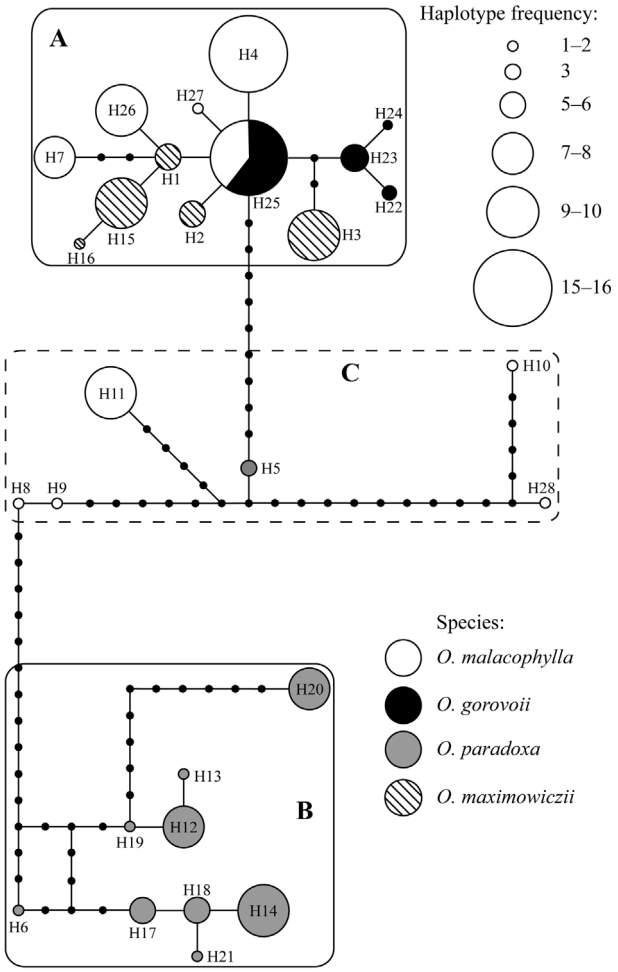

Fig. 4. The resolved cpDNA haplotype network for *Orostachys* subsect. *Orostachys* species. Sampled haplotypes are shown as labeled circles. The size of the circle represents haplotype frequency. Hypothetical, unsampled haplotypes inferred through TCS 1.13 are shown as black dots. For haplotype shared among sites/species, the site and the frequency that the haplotype was sampled for each site are shown as a pie chart. **A**, **B**, and **C** denote haplotype lineages/clades established in this study.

In the populations studied, the levels of haplotype (h) and nucleotide (π) diversity varied in the range from 0.0000 to 0.7000 and from 0.0000 to 0.0045, respectively (Table 3). Seven populations (P4, P5, P8, P10, P11, P13, and P15) revealed no haplotype or nucleotide diversity. In contrast, high haplotype and nucleotide diversity were observed in P1, the geographically westernmost population in our dataset (Table 3). The AMOVA showed that among all populations, the genetic differentiation was high ($F_{ST} = 0.86903$, $P < 0.00001$). For the among-species analysis (*O. malacophylla*, *O. maximowiczii*, *O. paradoxa*, and *O. gorovoii*), only 12% of the total genetic variance resided within populations of one species. The majority of molecular variance was attributable to

Table 3 Estimates of genetic diversity in *Orostachys* populations based on cpDNA sequence data

Population codes	S	p_S	n_H	n_{Hu}	Haplotypes	h (SD)	π (SD)
P1	3274	25	3	2	H7, H8, H9	0.7000 (0.2184)	0.004520 (0.002861)
P2	3278	27	2	1	H7, H10	0.4000 (0.2373)	0.003295 (0.002117)
P3	3274	24	3	0	H25, H27, H28	0.6222 (0.1383)	0.002606 (0.001490)
P4	3273	0	1	0	H26	0.0000 (0.0000)	0.000000 (0.000000)
P5	3260	0	1	0	H11	0.0000 (0.0000)	0.000000 (0.000000)
P6	3273	1	2	1	H15, H16	0.2000 (0.1541)	0.000061 (0.000093)
P7	3273	4	2	0	H1, H2	0.5455 (0.0615)	0.000667 (0.000446)
P8	3286	0	1	0	H3	0.0000 (0.0000)	0.000000 (0.000000)
P9	3274	2	3	1	H22, H23, H24	0.6000 (0.1305)	0.000204 (0.000192)
P10	3273	0	1	0	H25	0.0000 (0.0000)	0.000000 (0.000000)
P11	3286	0	1	0	H4	0.0000 (0.0000)	0.000000 (0.000000)
P12	3264	20	2	0	H5, H6	0.6000 (0.1753)	0.003676 (0.002349)
P13	3273	0	1	0	H4	0.0000 (0.0000)	0.000000 (0.000000)
P14	3264	1	2	0	H12, H13	0.3556 (0.1591)	0.000109 (0.000130)
P15	3264	0	1	0	H14	0.0000 (0.0000)	0.000000 (0.000000)
P16	3263	47	2	0	H17, H18	0.5111 (0.1643)	0.002064 (0.001202)
P17	3264	3	3	1	H19, H20, H21	0.5556 (0.0745)	0.000511 (0.000369)
Total	3292	85	28	6	H1–H28	0.9595 (0.0037)	0.005520 (0.002725)

S, number of observed nucleotide sites; p_S, number of polymorphic sites; n_H, number of haplotypes; n_{Hu}, number of unique haplotypes; h, haplotype diversity; π, nucleotide diversity; SD, standard deviations. For population code, see Table 1.

differences between species (45.76% of the total) and among populations within species (42.24% of the total), and those differences were significant (Table 4).

The phylogenetic reconstruction was based on the dataset consisting of 3262 characters (ca. 2.1–2.5% of the plastid genome) that displayed only a low variation with 46 parsimony informative (including multi-base-pair indels and inversions coded as binary characters) and one variable but uninformative character. In the MP analysis, a heuristic search found 30 equally parsimonious trees of 57 steps having high consistency (CI 0.8246) and retention indices (RI 0.9886), indicating little homoplasy within the dataset.

The MP bootstrap tree (Fig. 5) was consistent with the topology of the genealogy generated with TCS (Fig. 4). The 152 samples were distributed between the three clades A, B, and C. The largest robust clade A, distinct in synapomorphic substitutions in the *trnT–trnL* (T → G; position 1882) and *trnS–trnG* (A → T; position 2235) spacers and in two indels in the *rpl32–trnL* spacer, comprised 92 accessions from 10 populations, representing three species, *O. malacophylla*, *O. gorovoii*, and *O. maximowiczii*. These accessions were arranged into six moderately supported subclades and a large unresolved polytomy of 41 accessions from five populations of the three species. Only populations P4 and P8 were resolved as distinct monophyletic lineages characterized by a synapomorphic substitution C → A in *trnH–psbA* (P4) and A → T in *trnS–trnG* spacers as well as by the duplication event in *trnH–psbA* (P8, see above). Three additional subclades comprised only a portion of the respective population representatives (P1, P2, P7, and P9), with the rest of the population found in a group of highly similar accessions (P7 and P9) or in another clade (P1 and P2). One subclade (65% support) comprised representatives of two adjacent (approximately 4 km) populations, P11 and P13, having identical cpDNA sequences.

The clade B [99% BP, synapomorphic substitutions in *trnH–psbA* (C → T, position 87), *trnT–trnL* (G → A and A → C, positions 1139 and 1257) and *trnS–trnG* (T → G, position 2883)] included 42 accessions of *O. paradoxa* from five populations originating from the northern cost of the Sea of Japan and approaching the Sikhote-Alin Mountains. Only two

Table 4 Analyses of molecular variance (AMOVA) of cpDNA sequence data for *Orostachys* populations

Source of variance	*d.f.*	*VC*	*PV*	Fixation index
Among populations (total)	16	8.30187	86.90	$F_{ST} = 0.86903^{*}$
Within populations	135	1.25111	13.10	
Among species: *O. malacophylla* vs. *O. maximowiczii* vs. *O. paradoxa* vs. *O. gorovoii*	3	5.08435	45.76	$F_{CT} = 0.45762^{**}$
Among populations within species	12	4.69270	42.24	$F_{SC} = 0.77874^{*}$
Within populations	126	1.33333	12.00	$F_{ST} = 0.87999^{*}$

d.f., degrees of freedom; *VC*, variance-component estimates; *PV*, percentage of variation; F_{CT}, correlation of individuals within groups relative to the total; F_{SC}, correlation within populations relative to groups; F_{ST}, correlation within populations relative to the total. Significance levels are based on 10 000 permutations. $^{*}P < 0.00001$; $^{**}P < 0.001$.

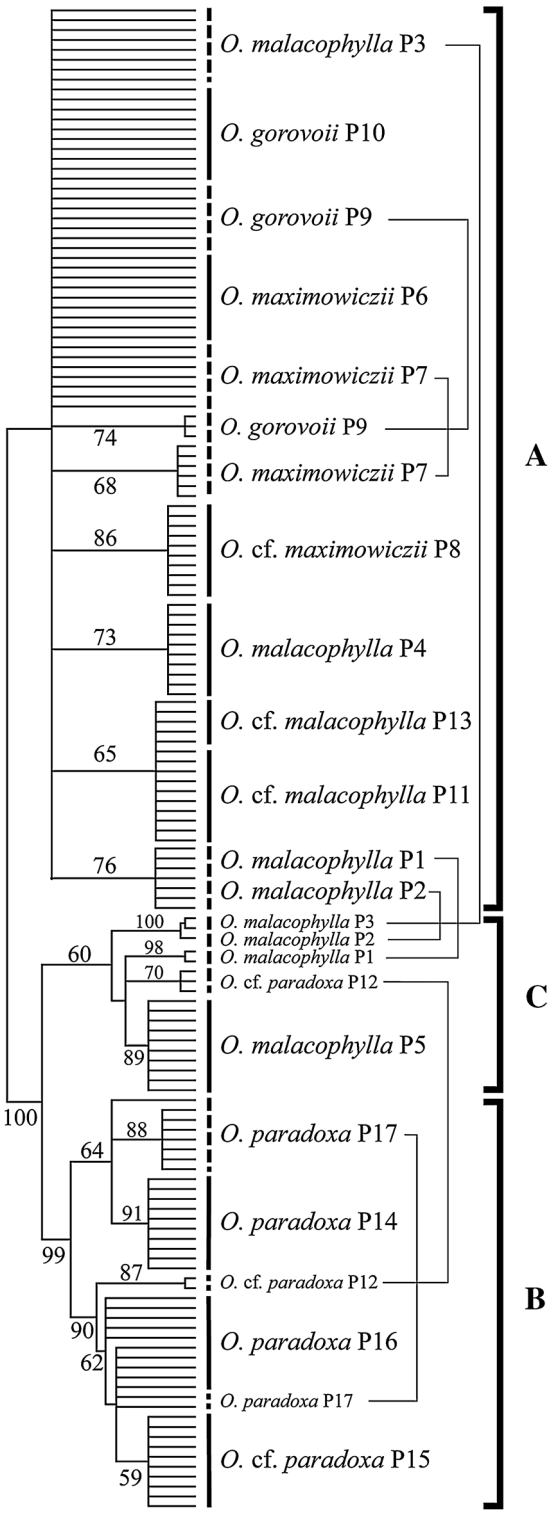

Fig. 5. MP bootstrap tree based on the combined data from *trnH–psbA*, *trnS–trnG*, *trnT–trnL*, and *rpl32–trnL* intergenic spacers (3256 aligned nucleotide positions and 6 indels and inversions coded as binary characters, 46 are parsimony informative) for *Orostachys* subsect. *Orostachys* species. Bootstrap values >50% are given above/below the branches. **A**, **B**, and **C** denote haplotype lineages/clades established in this study.

accessions in this clade represented more the southern coastal population P12. The clade B was split into two subclades (90 and 64% BP; Fig. 5). The better-supported subclade comprised 24 accessions from four coastal populations distinct in a synapomorphic 49 bp inversion in the *rpl32–trnL* spacer (see above) and two substitutions in *trnT–trnL* (A → C, position 1110) and *trnS–trnG* (T → A, position 2425). The second subclade (64% BP, synapomorphic A → C substitution in *trnT–trnL* at position 1041) included 19 accessions of this species from two populations having no inversion (P14 and P17).

The smallest clade C (60% BP, 18 samples from four populations of two species, *O. malacophylla* and *O. paradoxa*) accommodated one monomorphic population P5 and a few distinct samples from populations P1, P2, P3, and P12 generally accommodated in clades A or B.

To test an effect of indels and inversions coded as binary characters on the tree topology and support, we excluded them from the analyses. From the reduced dataset (3256 bp), parsimony recovered 6 identical trees 44 steps long (result not shown). A strict consensus of these trees had topology and support nearly identical to those obtained with the complete dataset.

3 Discussion

Data obtained in this study indicate a lack of congruence between plastid and nuclear phylogenies as well as between them and morphology-based taxonomy in the *Orostachys* subsect. *Orostachys*. Two major cpDNA lineages were identified in a set of *Orostachys* populations, lineage A (cluster/clade in our analyses), comprising 13 haplotypes found in 11 populations of three species (*O. malacophylla*, *O. maximowiczii*, and *O. gorovoii*) and lineage B that included all but one haplotype found in five populations of *O. paradoxa*. These lineages were linked by a group of divergent haplotypes (cluster/clade C) found in the populations generally allied with either A or B lineages (Figs. 4, 5). The pattern of relationships between the taxa revealed by the phylogenetic reconstruction indicates that the current concepts of *O. malacophylla*, *O. maximowiczii*, and *O. gorovoii* are incompatible with the differentiation at the cpDNA level. Neither of the species could be allied to a particular haplotype or uniquely characterized by a haplotype clade. The genetic divergence between haplotypes/populations of these species was generally low; these species differed from each other mostly by one to two steps. Moreover, one of a few

cases of haplotype sharing between populations revealed in this study (Fig. 4) relates to the geographically distant (approximately 300 km) populations of *O. malacophylla* (P3) and *O. gorovoii* (P10) occupying ecologically different habitats, for example, sandy beach on Khanka Lake and limestone at the sea cost, respectively. The lack of clear genetic distinctness between *O. malacophylla*, *O. maximowiczii*, and *O. gorovoii* suggests their relatively recent and rapid divergence from a common ancestor or; less likely due to their generally allopatric distribution, a high level of interspecific hybridization. Although in most cases morphology-based discrimination of *O. gorovoii*, having cartilaginous leaf edges with numerous unequal thickenings at the upper part (Fig. 1: F), and *O. maximowiczii*, distinct in green narrow acute leaves (Fig. 1: A, B), acute petals and trapezoid nectar scales, from *O. malacophylla* is not problematic, these taxa still largely share the same gene pool.

Although we detected a high level of genetic variation across 17 *Orostachys* populations, with 28 cpDNA haplotypes in four intergenic spacers, these haplotypes revealed only a weak phylogeographical structure. The majority of the haplotypes were endemic or private and only three cases of haplotype sharing between populations was observed. The populations studied exhibited high levels of genetic differentiation and had relatively low intrapopulation divergence (Table 4), typical for plants with fragmented ranges. Population isolation leads to a low gene flow, increasing the effects of genetic drift in small populations and possibly resulting in the reduction of its genetic diversity (Reed & Frankham, 2003; Jump & Peñuelas, 2006). For many *Orostachys* populations, a considerable level of haplotype diversity along with a low level of nucleotide diversity was observed (Table 3). A large number of haplotypes with low nucleotide diversity may be a consequence of homoplasy or may result from the rapid population growth of an ancestral population with a low effective population size (Abramson, 2007). The overall low sequence divergence within populations or its absence again points to the recent origin of these populations.

Analyses of ITS rDNA sequences provide poor information regarding the relationships between *Orostachys* subsect. *Orostachys* populations and species. Apart from a generally low ITS rDNA sequences divergence observed in the dataset, seven ribotypes were found in 17 populations (86 specimens sequenced). The pattern of relationships between these ribotypes (Fig. 3: G, H) supported neither the morphology-based taxonomic subdivision of *Orostachys* species nor their grouping according to geographical origin of the populations or lineages recovered with cpDNA data. Instead, we recovered two sometimes sympatric pools of nuclear sequences in the subsection, ribotype R1 found in 10 out of 17 populations (three species) and a group of six ribotypes (R2–7; 1–3 substitutions different) distinct in two synapomorphic substitutions in ITS1 (Fig. 3: G). Screening of *Orostachys* subsect. *Orostachys* sequences in GenBank revealed the presence of additional ribotypes in Eastern Russia (AB480580) and Japan (AB480579, AB088572, AB088573) sharing these synapomorphies as well as the occurrence of the ribotype R1 in *O. malacophylla* var. *aggregata* from Hokkaido (AB088574). It is unlikely that the taxonomic and geographic distribution of the ITS rDNA diversity revealed in the subsection is stochastic. However, our sampling provides insufficient data for recovering any clear pattern.

The nature of relatively high ribotype diversity and sympatry in *Orostachys* populations also remains unclear. Although no intragenomic variations were detected in the ITS region in this study, the preferential amplification of only one repeat type from several present in the genome is still possible (Hřibová et al., 2012). Thus, the incomplete concerted evolution of paralogous ITS copies or hybridization events could contribute to the observed ribotype diversity. A low divergence between the ribotypes, the general stability of their ITS secondary structures and consistency in recovery of these ribotypes rules out the presence of non-functional pseudogenes in our rDNA sequences.

The pattern of genealogical and phylogenetic relationships in the subsection generally supported distinctness of *O. paradoxa* by placing 9 out of 10 haplotypes revealed in five populations into the divergent and strongly supported cluster B (Fig. 4). Although at least one population (P12) of the species was geographically intermediate (2–3 km distance to each) between two populations of *O. malacophylla* (P11 and P13; Fig. 2), there was no habitat overlap between the species (cliff on a small island and sandy/stony beach terraces, respectively), and no interspecific haplotype sharing was observed.

It appears that populations of *O. paradoxa* differ from those in the *O. malacophylla* alliance (clade A) in somewhat higher levels of haplotype and nucleotide diversity (Table 3). Moreover, our analyses revealed two rather divergent lineages in *O. paradoxa* supported by confidence threshold values as well as by molecular synapomorphies (inversion in the *rpl32–trnL* spacer and several substitutions; see Results Section; Figs. 4, 5). This intraspecific polymorphism may have

originated from hybridization followed by subsequent cpDNA introgression and, more likely, from an ancestral polymorphism and lineage-sorting process. Our results favor the latter hypothesis linking *O. paradoxa* (clade/cluster B) to a pool of rare highly divergent haplotypes (clade/cluster C) still maintained in some *O. malacophylla* populations.

Populations comprising clade B differed from each other in plant size and appearance, leaf arrangement in the rosettes, shoot and leaf color, etc. (Fig. 1: D, G) that hampered their confident identification. Hence, based on a presence of glaucous and stoloniferous rosettes, plants from population P15 were initially designated as *O. malacophylla* var. *aggregata* (*O. aggregata* (Makino) Hara; Gontcharova, 2006), occurring in Japan. However, in our analyses this population was allied unambiguously with the populations of *O. paradoxa*, including those assigned to this species by Voroschilov (P16 and P17), one of the authors of the taxon (Voroschilov & Khokhrjakov, 1970).

The perennial habit and presence of vegetative shoots (stolones) terminating with juvenile rosettes (Fig. 1: H) clearly differentiated members of clade B from generally monocarpic representatives of clade A. The perennial stoloniferous *Orostachys* plants seem to be rather common along the northern shores of the Sea of Japan and high altitudes of the Sikhote-Alin and Changbaishan Mountains (Dudkin, 2011, unpublished data). In addition to *O. malacophylla* var. *aggregata* and *O. paradoxa*, two taxa were reported from this area, *O. vyschinii* Bezd. (northern part of the Sikhote-Alin Mts. only; Bezdeleva, 1995) and *O. furusei* Ohwi (Sakhalin and Hokkaido; Abankina & Gontcharova, 1995; Byalt, 1999; Ohba, 2003). According to Byalt (1999), *O. vyschinii* is hardly distinguishable from *O. furusei*; therefore, he referred the former taxon as a synonym of the latter. Ohba (2003) went further and reduced *O. furusei* to a synonym of *O. boehmeri* (Makino) H. Hara, although to our mind, this taxonomic rearrangement is questionable. Thus, based on the literature data and personal observation, we can conclude that *O. paradoxa* and *O. malacophylla* var. *aggregata* are restricted to the western and eastern shores of the Sea of Japan, respectively, but their distribution areas are bridged by *O. boehmeri* s.l. (or *O. furusei*) occurring on both sides of the sea in coastal areas as well as in the northern part of the Sikhote-Alin Mts. Taking into account that these three taxa share important feature, for example, perennial stoloniferous habit differentiating them from *O. malacophylla*, and display extensive morphological plasticity, additional study is necessary to define their concepts and delineate them morphologically.

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