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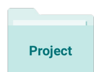

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The Identification of Araliaceae Species by ITS2 Genetic Barcoding and Pollen Morphology

Authors

Arkadiy Reunov[1], Galina Reunova[2], Dmitry Atopkin[2], Yulia Reunova[3], Tamara Muzarok[2], Evgeny Zakharov[4], Yury Zhuravlev[2]

Affiliations

1 University of Ottawa Heart Institute, Ottawa, Canada
2 Federal Biodiversity Scientific Center, Far Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia
3 National Scientific Centre of Marine Biology, Far Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia
4 Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, Guelph, Canada

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Correspondence

Arkadiy Reunov
University of Ottawa Heart Institute, Electron microscopy laboratory
40 Ruskin Street, Ottawa ON K1Y 4W7, Canada
Phone: + 1 613 761 52 82, Fax: + 1 613 761 49 98
areunov@ottawaheart.ca

ABSTRACT

The genetic barcode ITS2 (ITS: internal transcribed spacer) and pollen morphology were used for the identification of the pharmacologically valuable wild Araliaceae species *Panax ginseng, Oplopanax elatus, Aralia elata, Aralia continentalis, Eleutherococcus senticosus*, and *Eleutherococcus sessiliflorus* inhabiting the natural forests of Primorye, Russia. The ITS2 locus successfully identified all six species, which supports the use of ITS2 as a standard barcode for medicinal plants. However, the ITS2 locus was insufficient for intra-specific discrimination in these species, neither within Primorye nor from other world representatives within GenBank. Araliaceae pollen was confirmed to undergo size-reducing metamorphosis. The final morphotypes were species-specific for each of the six species but could not discriminate intra-species geographic localities within Primorye. The morphologies of the final pollen morphotypes from homologous species inhabiting other parts of the world are not yet known. Therefore, whether pollen is applicable for Araliaceae intra-species discrimination between Primorye and other world localities could not be established. Based on these findings, we propose that the ITS2 genetic barcode and the final pollen morphotypes are suitable for the identification of Araliaceae species. However, further studies will be needed to determine the suitability of genetic and pollen traits for Araliaceae geographic authentication.

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Introduction

The Araliaceae family comprises several medicinal plants that are widely used for enhancement of immunity, as analgesic, anti-inflammatory, and anti-diabetic herbs and for relief of fatigue and stress [1–11]. The far eastern region of Russia (Primorye) is one of the few remaining areas that continue to be inhabited by wild ancestral populations of Araliaceae plants that are a high-value natural resource [12–14]. However, the identification of these species was previously done solely based on general morphology [12]. Little is known about the genetic relatedness of these species with those found in other parts of the world.

In recent years, DNA barcoding based on polymerase chain reaction (PCR) has been applied to species identification [15]. The most common genome regions used as plant barcodes are *matK, rbcL*, ITS, ITS2, *psbA-trnH, atpF-atpH, ycf5, psbK-I, psbM, trnD, coxI, nad1, trnL-F, rpoB, rpoC1*, and *rps16* [16]. The ITS2 region of nuclear ribosomal DNA is considered one of the most suitable barcoding regions for plant identification [17–20]. The China Plant Working Group of the Consortium for the Barcode of Life has recommended incorporating ITS2 into the core barcode for seed plants [21]. Chen et al. [22] have proposed that ITS2 is a universal genetic barcode for medicinal plants. Yao et al. [23] have reported on the high universality of ITS2 for both plants and animals. Based

▶ **Table 1** Samples of Araliaceae species examined regarding to ITS2 gene. Asterisks show samples used for pollen study.

Species	Locality	Code	Sample size	Voucher
*A. elata**	Shkotovskiy region, Tumannaya Mountain	ARE 1	3	PR1-PR3
A. elata	Stenin Island	ARE 2	3	PR4-PR6
*A. elata**	Bolshoi Pelis Island	ARE 3	2	PR7-PR8
*A. continentalis**	Khasanskiy region, Gamov peninsula, Astaphyev cape	ARC 1	1	PR9
*A. continentalis**	Khasanskiy region, Gamov peninsula, Astaphyev cape	ARC 2	1	PR10
*A. continentalis**	Khasanskiy region, Gamov peninsula, Astaphyev cape	ARC 3	1	PR11
*E senticosus**	Shkotovskiy region, Tumannaya Mountain	EST 1	3	PR12-PR14
*E senticosus**	Matveev Island	EST 2	3	PR15-PR17
E senticosus	Stenin Island	EST 3	1	PR18
E sessiliflorus	Stenin Island	ESL 1	3	PR19-PR21
*E sessiliflorus**	Bolshoi Pelis Island	ESL 2	3	PR22-PR24
*E sessiliflorus**	Khasanskiy region, Posjet bay	ESL 3	1	PR25
*O. elatus**	Shkotovskiy region, Tumannaya Mountain	OPE 1	1	PR26
*O. elatus**	Khasanskiy region, Kedrovaya Pad Reserve	OPE 2	5	PR27-PR31
O. elatus	Shkotovskiy region, Lozovaya Mountain	OPE 3	5	PRD32-PR36
O. elatus	Terneiskiy region, Sikhote-Alin Reserve	OPE 4	5	PR37-PR41
*P. ginseng**	Dalnegorskiy region	PAN 1	2	PR42-PR43
*P. ginseng**	Nadezdinskiy region	PAN 2	5	PR44-PR48
P. ginseng	Ussuriyskiy region	PAN 3	5	PR49-PR53
P. ginseng	Partizanskiy region	PAN 4	5	PR54-PR58
P. ginseng	Chuguevskiy region	PAN 5	5	PR59-PR63
P. ginseng	Yakovlevskiy region	PAN 6	5	PR64-PR68
P. ginseng	Pozharskiy region	PAN 7	5	PR69-PR73
*P. ginseng**	Spasskiy region	PAN 8	5	PR74-PR78
P. ginseng	Olginskiy region	PAN 9	5	PR79-PR83

on the above-cited literature, ITS2 is likely a suitable DNA barcode for the identification of Araliaceae species found in Primorye.

According to Will et al. [24], the combination of genetic data and classical taxonomy is the most valid approach to plant species identification. However, in addition to gross morphology (that cannot be applied to cut, powdered, or extracted materials), some microscopic methods are required for the successful authentication of botanical ingredients to prevent possible inter-species crude adulteration [16, 25]. Pollen morphology has been proposed as a species-specific trait that could be used for the identification of Araliaceae species [26, 27]. However, as noted by Reunov et al. [27] in their study of three Araliaceae species, taxonomic assignments based on pollen morphology should be established carefully due to the highly expressed heteromorphism that occurs during "size-reducing pollen metamorphosis"; only the most mature grains that appear the smallest are taxonomical reliable. Also, further study involving more species is needed to confirm if the morphology of the smallest pollen grains could be used as a microscopic trait applicable to species identification in Araliaceae.

Here, we tested the suitability of the ITS2 genetic barcode and pollen morphology for the identification of six medicinal Araliaceae species, such as *Panax ginseng* C. A. Mey., *Oplopanax elatus* (Nakai) Nakai, *Aralia elata* (Miq.) Seem., *Aralia continentalis* Kitag., *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim., and *Eleutherococcus sessiliflorus* (Rupr. & Maxim.) S. Y. Hu, found in the forests of Primorye. This study was conducted as a joint project between the Federal Biodiversity Scientific Center of Russian Academy of Sciences (Vladivostok, Russia) and the Canadian Centre for DNA Barcoding (Guelph, Canada).

Results and Discussion

The ITS2 region was successfully amplified for all studied samples (▶ **Table 1**). The sequence efficiency was 92.8 %. The ITS2 sequence lengths in the studied taxa ranged from 454 to 455 base pairs (bp). For the ITS2 matrix, the aligned sequence length was 455 bp, with no indels; 42 informative sites and 43 variable sites were dispersed in the matrix. The mean sequence divergence was 4.9 %. The intra-specific distance was 0.2 % (▶ **Table 2**). The discriminating power at the species level was 100 % (▶ **Table 2**). The ITS2 nucleotide sequences of the studied species were deposited in GenBank (accessions KX271052–KX271137).

Analysis of species-specific variation in the ITS2 region found that the ITS2 locus contains 23 species-specific variable sites (▶ **Table 3**). In the ITS2 dataset, every species had unique character states that differentiated them from the other studied species.

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For example, *A. continentalis* and *E. senticosus* each had one unique diagnostic site; *A. elata* and *E. sessiliflorus* each had two diagnostic sites, and the *O. elatus* sequence contained seven unique diagnostic sites. Of the studied species, *P. ginseng* had the most species-specific sites (10) in its ITS2 fragment (▶ **Table 3**).

Phylogenetic analysis using the ITS2 regions was also conducted. We found that each species formed a monophyletic group, including samples collected from different sites. The resulting neighbor-joining (NJ) tree clearly discriminated the studied Araliaceae species (▶ **Fig. 1**). These species were combined into two main clusters. One cluster contained the species *P. ginseng, A. continentalis*, and *A. elata*. The second cluster was formed by the species of the genus *Eleutherococcus* and *O. elatus*. The same clustering was obtained in our study of the Araliaceae species using sequences of the ITS region of nuclear DNA [28]. Thus, the character-based results and phylogenetic analysis demonstrate that the ITS2 region can discriminate between the tested Araliaceae species. The high ability of ITS2 for inter-specific discrimination of Araliaceae was shown previously by other researchers [29, 30]. The present study adds more evidence to both the applicability of ITS2 for plant identification [21], as well as the conclusion of Chen et al. [22], who suggested that ITS2 could be considered a standard barcode for medicinal plants.

We also tested whether the ITS2 barcoding region is suited to intra-species discrimination. Such an authentication appears necessary because of the popularity of Araliaceae on world trade markets. The quality of wild forms is typically higher than that in cultivated forms, which have lost many of their original traits due to genetic bottleneck-induced losses in genetic variation [31–33]. Hereupon, wild plants are of special interest to traders [34]. The limited availability of wild Araliaceae might also encourage illegal trade and intentional mislabelling [16]. Thus, there is a need for their reliable control. Therefore, we compared the ITS2 sequences of six Araliaceae species collected for Primorye against the sequences of the same species deposited in GenBank. We found that the ITS2 nucleotide sequences of *A. continentalis, O. elatus*, and *E. sessiliflorus* were identical to those deposited in the GenBank database (▶ **Table 4**). Comparing our Primorye *P. ginseng* ITS2 sequences against 16 *P. ginseng* ITS2 sequences deposited in GenBank, the sequences were identical in 15 cases, differing only for the accession KF727975 *P. ginseng* specimen. However, the KF727975 *P. ginseng* sample differed by just two singleton substitutions (0.04% of the sequence) (▶ **Table 4**). Our Primorye *A. elata* sample was distinct from the *A. elata* var. *mandshurica* sample deposited in GenBank (0.27% genetic differentiation) (▶ **Table 4**). This intra-species divergence value is lower than those between the species tested in our study (▶ **Table 4**). For example, the ITS2 divergence between *A. elata* and *A. continentalis* was 2.74% (▶ **Table 4**). Thus, the ITS2 locus did not discern the representatives of studied species collected in different geographic localities of Primorye nor discriminate between Primorye and the other regions of southeastern Asia, from which samples are represented in GenBank. These findings are in accordance with other data showing quite low intra-specific genetic distances for ITS2, as well as for other typical DNA barcodes such as *matK* and *psbA-trnH* [30].

However, the geographic authentication of Araliaceae species seems potentially possible in Primorye. Indeed, Mahankov et al. [14] showed that ginseng plants from various locations within Primorye have distinct ginsenoside profiles. Our previous studies of the genetic diversity of *P. ginseng* and *O. elatus* using DNA

▶ **Table 2** Evaluation of DNA markers.

DNA region	ITS2
No. species (individuals)	6 (83)
Percentage PCR success	100%
Percentage sequencing success	92.8
Aligned sequence length (bp)	455
Indels length (bp)	0
No. information sites/variable sites	42/43
Interspecific distance mean (range), %	4.9 (0.9–7.5)
Intraspecific distance mean (range), %	0.2 (0–0.8)
Ability to discriminate, %	6/6, 100

▶ **Table 3** Character states (nucleotides) at 26 selected positions for Araliaceae species from ITS2 region. Species-specific sites are bold. Abbreviations of taxa are according to ▶ **Table 1**.

Position	Taxon					
	ARE	ARC	EST	ESL	OPE	PAN
26	C	C	C	C	**T**	C
105	C	C	C	C	C	C
108	C	C	T	T	C	**A**
109	G	G	G	G	G	**T**
118	C	C	C	C	C	**T**
119	A	A	A	A	A	**G**
120	T	T	T	T	**G**	**C**
127	C	C	C	C	C	**T**
146	C	C	C	C	C	**A**
150	C	C	C	C	**T**	C
175	G	G	G	G	**A**	G
197	A	A	A	**G**	A	A
198	C	C	C	C	C	**T**
228	A	A	A	A	**G**	A
232	**T**	C	C	C	C	C
249	G	G	G	**T**	G	G
252	G	G	G	G	G	G
253	A	A	**G**	A	A	A
264	G	G	G	G	G	G
265	C	C	C	C	**T**	C
269	A	**G**	A	A	A	A
274	T	T	A	A	T	T
276	A	A	G	G	**T**	A
302	C	C	A	A	C	**T**
394	**T**	C	C	C	C	C
445	T	T	T	T	T	**C**

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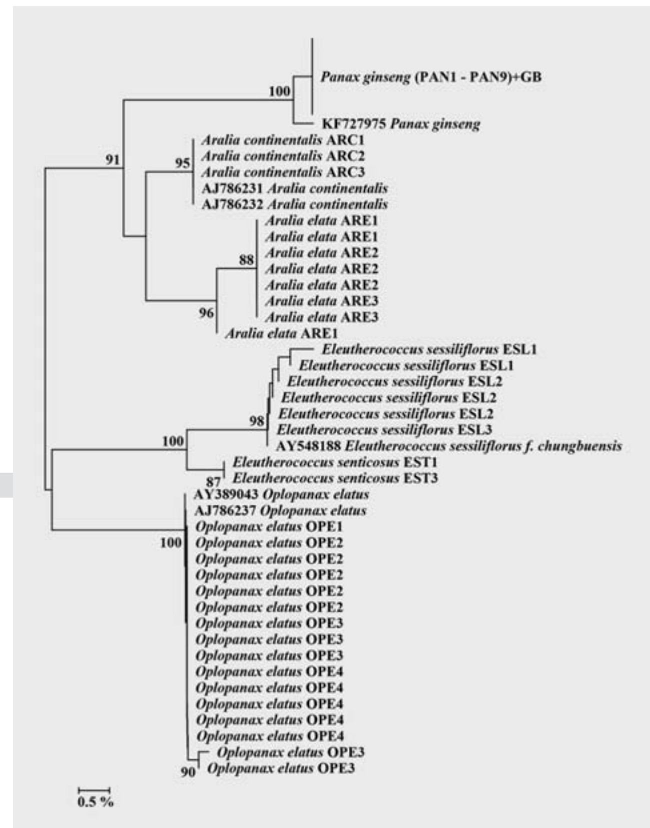
markers other than ITS2 found these populations to be highly differentiated [35, 36]. In studies with AFLPs, gene flow between *P. ginseng* populations was less than 1 ($N_m = 0.45$) [35]. Such a level of migration would be unable to prevent continued divergence among populations [37] and might result in species divergence. Moreover, some Araliaceae species (e.g., *A. elata*, *E. senticosus*, and *E. sessiliflorus*) have been found growing both in continental Primorye and on the islands of Peter the Great Bay in the Sea of Japan (▶ **Table 1**). Hence, it seems possible that the genetic isolation of these island plants has been accompanied by sequence divergence. It should be stressed that all intra-specific clusters of *Panax bipinnatifidus* were distinguished when Zuo et al. [38] used a combination of the *psbA-trnH* and ITS loci. Thus, it could be supposed that using some barcode combinations might make the techniques sensitive enough to discriminate intra-specific locations within Primorye and distinguish Primorye Araliaceae species from those growing elsewhere.

We also tested whether pollen traits could be useful for the identification of Araliaceae species. Previous work from our lab has investigated the pollen differentiation of *P. ginseng, O. elatus, A. elata*, and *A. continentalis* [27, 39]. In these studies, we found that pollen grains undergo size-reducing metamorphosis and that the final morphotype has the smallest diameter (▶ **Fig. 2**). Using this final morphotype, we could distinguish between *P. ginseng, O. elatus, A. elata*, and *A. continentalis* (▶ **Fig. 3 a–d**). Here, we show that pollen of *E. senticosus* and *E. sessiliflorus* undergo the same size-reducing metamorphosis typical of Araliaceae (data not shown) and that the final pollen morphotypes of *E. senticosus* and *E. sessiliflorus* have species-specific features (▶ **Fig. 3 e, f**). Thus, it seems evident that the smallest pollen morphotypes identify Araliaceae species inhabiting Primorye. Therefore, the supplement of dry flowers having anthers with pollen might be recommended for potential providers offering Primorye species.

Additionally, we tested whether intra-species geographic authentication is possible for Araliaceae in Primorye based on pollen morphology. For this we made intra-specific comparisons of pollen for each of the six studied Araliaceae species according to their geographic localities (▶ **Table 1**). However, we found complete intra-species similarity in pollen morphology (of the final morphotype; data not shown). It would be interesting to test whether pollen could be used to establish the geographic authenticity of Primorye Araliaceae versus the same species inhabiting remote geographic locations. Unfortunately, we were unable to find data on the final pollen morphotypes of Araliaceae species collected from other parts of the world and were, therefore, unable to test this possibility.



▶ **Fig. 1** NJ tree inferred from analysis of sequence data of ITS2 regions of nuclear DNA of Araliaceae species. Bootstrap values (%) are shown on each branch. The indicated scale represents 0.5 % of genetic divergence.

Conclusion

Primorye is a natural reservation inhabited by wild, high-value medicinal Araliaceae plants. The identification of these plants was previously done based on gross morphology. Here, we tested whether genetic barcode ITS2 could be used to identify Primorye Araliaceae species and distinguish them from homologous species growing in remote areas of the world. We found that the ITS2 sequence could reliably identify the Primorye Araliaceae species but was insufficient for geographic authentication of these species both in Primorye and regarding to discrimination between Primorye species and species from other world localities (data from GenBank). We also tested if morphology of the smallest pollen might be introduced as a suitable microscopic trait for the identification of Araliaceae species. We found that the appearance of the smallest pollen grains successfully discriminate Primorye Araliaceae species. However, intra-species discrimination in Primorye was impossible based on pollen because of inter-population pollen similarity. Because of an absence of data about the final pollen morphs for Araliaceae from other parts of the world, we were unable to test if pollen of Primorye species might differ from pollen of homologous species from remote world areas. Thus, the ITS2 and pollen were confirmed as an effective combination of genetic and microscopy traits for the identification of Araliaceae medicinal plants. However, further work will be needed to determine whether genetic and pollen traits can be used to establish their geographic authenticity.

Materials and Methods

Plant material

The leaves and flowers of *P. ginseng, O. elatus, A. elata, A. continentalis, E. senticosus*, and *E. sessiliflorus* plants were collected from

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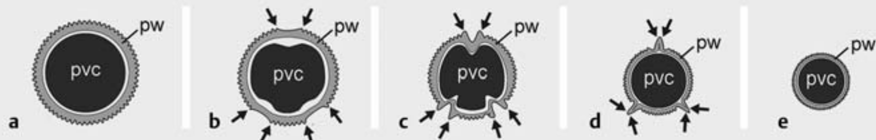
▶ **Table 4** Genetic p-distances (%) calculated for species of the family Araliaceae by the ITS2 sequence data. Asterisks show samples from GenBank.

		1	2	3	4	5	6	7	8	9	10	11
1	*P. ginseng**		1.16	1.27	1.40	1.53	1.16	1.28	1.43	1.53	1.40	0.03
2	*A. continentalis**	4.24		0.91	1.17	1.32	0.00	0.92	1.21	1.32	1.17	1.17
3	*A. elata var. man-dshurica**	5.12	2.65		1.34	1.38	0.91	0.18	1.25	1.38	1.34	1.27
4	*O. elatus**	6.71	4.59	6.18		1.37	1.17	1.34	1.25	1.37	0.00	1.40
5	*E. sessiliflorus*. f. chu.*	8.13	5.65	6.54	5.65		1.32	1.40	0.82	0.00	1.37	1.54
6	*A. continentalis*	4.24	0.00	2.65	4.59	5.65		0.92	1.21	1.32	1.17	1.17
7	*A. elata*	5.21	2.74	0.27	6.27	6.71	2.74		1.27	1.40	1.34	1.28
8	*E. senticosus*	7.42	4.95	5.83	4.95	2.12	4.95	6.01		0.82	1.25	1.43
9	*E. sessiliflorus*	8.13	5.65	6.54	5.65	0.00	5.65	6.71	2.12		1.37	1.54
10	*O. elatus*	6.71	4.59	6.18	0.00	5.65	4.59	6.27	4.95	5.65		1.40
11	*P. ginseng*	0.04	4.24	5.12	6.71	8.13	4.24	5.21	7.42	8.13	6.71	



▶ **Fig. 2** A schematic drawing of pollen size-reducing metamorphosis in Araliaceae (reproduced from [27] with permission of Cambridge University Press). The large pollen pattern (**a**). The intermediate pollen patterns (**b–d**). The smallest pollen pattern (**e**); pvc: pollen vegetative cytoplasm; pw: pollen wall exine; arrows show arising of pollen wall exine invaginations (**a–c**) and formation of projections (**c, d**) that tends to disappear at final stage of metamorphosis (**e**). The smallest pollen morph lacked any projections (**e**) is typical for *P. ginseng*. Pollen size-reducing metamorphosis was discovered in *P. ginseng, O. elatus, A. elata* [27], *A. continentalis* [39] and confirmed for *E. senticocus* and *E. sessilifloris* during the present study.

continental Primorye and islands of Peter the Great Bay in the Sea of Japan during the summers of 2012–2014. In total, 83 individuals were sampled and used for ITS2 barcoding analyses (▶ **Table 1**). Pollen morphology was investigated in 32 individuals (shown by asterisks in ▶ **Table 1**). The samples were identified based on their gross morphology by an academician of the Russian Academy of Sciences (Yu.N. Zhuravlev) and by the head of the Botany Department at the Federal Biodiversity Scientific Center, Far Eastern Branch of Russian Academy of Sciences (FBSC FEBRAS), in Vladivostok, Russia (V.Yu. Barkalov). All authentic specimens were marked by voucher numbers (▶ **Table 1**) and were deposited at the Plant Molecular Genetics Sector of the FBSC FEBRAS.

Analysis of ITS2 gene

ITS2 sequence analyses were done at the Canadian Centre for DNA Barcoding. Total genomic DNA was extracted from leaves dried in silica gel. Small pieces of leaves were subjected to overnight lysis in CTAB buffer with proteinase K (Invitrogen) followed by DNA extraction on a glass-fiber membrane using an automated protocol. The ITS2 regions of nuclear DNA were amplified using the primers ITS_S2F (5′-ATGCGATACTTGGTGTGAAT-3′) and ITS_S3R (5′-GACGCTTCTCCAGACTACAAT-3′). The PCR mixture included 10% trehalose (6.25 µL), 10 × PCR buffer (1.25 µL), 2.5 mM $MgCl_2$ (0.625 µL), 10 mM forward and reverse primers (0.125 µL each), 10 mM dNTPs (0.625 µL), Platinum Taq Polymerase (0.625 µL), H_2O (3 µL), and DNA template (1 µL). The PCR profile was as follows: 1 min at 94 °C; five cycles of 30 s at 94 °C, 40 s at 55 °C, and 1 min at 72 °C; 35 cycles of 30 s at 94 °C, 40 s at 55 °C, and 1 min at 72 °C; and a final extension cycle of 10 min at 72 °C. The PCR products were sequenced using an ABI 3730 xl DNA Analyzer using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems), primers for amplification, and the manufacturer's recommended protocols. ABI trace files for each specimen were assembled into contigs using CodonCode Aligner v. 2.0.6. The sequences were aligned using MUSCLE software [40] integrated in MEGA 5 [41]. All original specimen metadata, sequences, and trace files were uploaded to the Barcode of Life Data Systems (BOLD) at http://www.boldsystems.org (project code GRAEV, ticket no. 102725). The estimation of variable and phylogenetically informative nucleotide sites and genetic p-distances and the analysis of species-specific variable nucleotide positions were performed with MEGA 5 [41]. In addition, genetic differentiation was estimated between the species from our study and the same species obtained from GenBank. The processed data were analyzed by NJ analysis [42]. Nucleotide sequence divergences were calculated using the Kimura two-parameter (K2P) nucleotide substitution model [43]. The NJ trees of K2P genetic distances showing intra-specific and inter-specific variation were created

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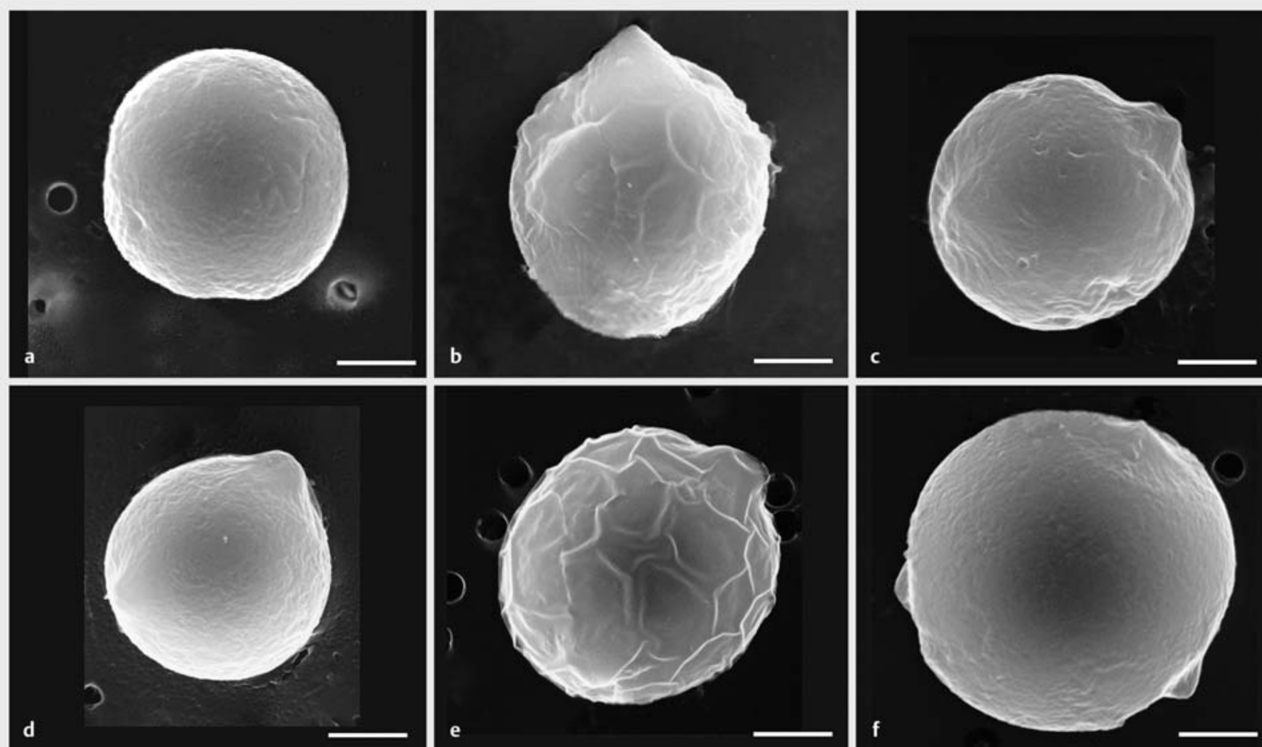

▸ **Fig. 3** A comparative view of the smallest pollen patterns in *P. ginseng* (**a**), *O. elatus* (**b**), *A. elata* (**c**), *A. continentalis* (**d**), *E. senticosus* (**e**), and *E. sessiliflorus* (**f**). Images **a, b** were reproduced from [27] with permission of Cambridge University Press; images **c, d** were reproduced from [39] with permission of Nauka publishing company. The morphologies shown are typical for Araliaceae species collected from various localities of Primorye (▸ **Table 1**). On all images, the bar is 5 µm.

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using BOLD and MEGA 5 [41]. The robustness of NJ phylogenetic trees was tested via the bootstrap method, with 1000 replicates.

Pollen studies

In this study, we investigated the pollen of *P. ginseng, O. elatus, A. elata, A. continentalis, E. senticosus*, and *E. sessiliflorus* collected from several areas of Primorye (shown by asterisks in ▸ **Table 1**). The flower stamens were removed and fixed for up to 24 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and post-fixed in 2% OsO_4 in the same buffer for 2 h. After washing in the buffer, samples were dehydrated in up to 70% ethanol and further underwent subsequent treatment for light microscopy. The anthers (fixed as above) were placed into a large 70% ethanol droplet on an object-plate coverslip and crushed by pincers, and the pollen stock was mechanically withdrawn. Drops of the pollen-ethanol mixture were placed on the slides, allowed to dry at room temperature, and studied using a Polyvar light microscope. The phenomenon of pollen size-reducing metamorphosis previously found for Araliaceae plants [27] was noted. The average diameters of the smallest pollen grains were scored for each sample according to our method [27].

To describe pollen morphology, the grains were processed for scanning electron microscopy. The anthers were fixed as above, washed in a sodium cacodylate buffer, and placed into the large sodium cacodylate buffer droplet on a poly-L-lysine-coated Thermanox coverslip. The anthers then were demolished by pincers. The pollen stock was then mechanically withdrawn. Drops of the pollen buffer mixture were allowed to dry at room temperature. The Thermanox coverslips with the pollen attached were washed several times in a buffer, dehydrated in a graded ethanol series, transferred to acetone, and critical-point dried in carbon dioxide. Dried samples were mounted onto aluminum stubs and gold-coated before examination with a Leo-340 scanning electron microscope.

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Conflict of Interest

The authors declare no conflicts of interest.

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