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PLANT GENETICS

Molecular Phylogenetic Analysis of the Genus *Abies* (Pinaceae) Based on the Nucleotide Sequence of Chloroplast DNA

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Abstract—A phylogenetic study of firs (*Abies* Mill.) was conducted using nucleotide sequences of several chloroplast DNA regions with a total length of 5580 bp. The analysis included 37 taxa, which represented the main evolutionary lineages of the genus, and *Keteleeria davidiana*. According to phylogenetic reconstruction, the *Abies* species were subdivided into six main groups, generally corresponding to their geographic distribution. The phylogenetic tree had three basal clades. All of these clades contained American species, and only one of them contained Eurasian species. The divergence time calibrations, based on paleobotanical data and the chloroplast DNA mutation rate estimates in Pinaceae, produced similar results. The age of diversification among the basal clades of the present-day *Abies* was estimated as the end of the Oligocene–beginning of Miocene. The age of the separation of Mediterranean firs from the Asian–North American branch corresponds to the Miocene. The age of diversification within the young groups of Mediterranean, Asian, and "boreal" American firs (*A. lasiocarpa*, *A. balsamea*, *A. fraseri*) was estimated as the Pliocene—Pleistocene. Based on the phylogenetic reconstruction obtained, the most plausible biogeographic scenarios were suggested. It is noted that the existing systematic classification of the genus *Abies* strongly contradicts with phylogenetic reconstruction and requires revision.

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INTRODUCTION

The fir (*Abies* Mill.) is one of the largest genera of coniferous trees, consisting of about 50 species. The members of this genus are the essential mesophilic elements of boreal and temperate forests of the Northern Hemisphere [1–3]. Firs are demanding of humidity, cannot tolerate high and low temperatures, and avoid habitats with stagnant moisture. These features determine the coenotic position of firs as elements of mainly the upper part of mountain-forest belt, where they form dark coniferous forests, usually together with the species of *Picea*, *Tsuga*, *Pseudotsuga*, *Pinus* subgenus *Strobus*. The fir's economic and environmental importance is related to its forest-forming role. The biogeography of firs, as well as the history of their speciation and dispersal, deserves special interest. The main centers of fir diversity are the west of North America and Mesoamerica (14 species), East Asia (23 species), and the Mediterranean (nine species). Less important in this respect is Siberia with Central Asia (two species) and the eastern part of North America (two species). Monophyly of the genus *Abies* has been repeatedly proven [4–6] and is beyond question with respect to morphology and molecular systematics. At the same time, classification of the genus *Abies* based on morphological characters is difficult because of its high variability and ability to form the hybrid taxa upon secondary contact [1, 2, 7–12]. Up to now, a number of classification systems of the genus, considerably different in the number of species and the number and composition of sections, have been suggested [2, 13, 14].

Although molecular phylogeny of *Abies* was examined in several studies [4–6, 15, 16], no comprehensive investigation encompassing most of the fir species and presenting statistically significant topology, along with age estimates of the main clades, have been conducted so far. In one of the first studies performed by Suyama et al. [15], nucleotide sequences of eight chloroplast DNA regions (>5000 bp) were obtained. The sequence variation of these regions was sufficient for discrimination of most of the 13 *Abies* species examined. The phylogeny of firs was also examined based on the ITS (nuclear ribosomal internal transcribed spacer) sequences [4]. As a result, a number of species groups were identified. According to the authors of the ITS phylogeny, these groups corresponded to most of the sections recognized within the genus [14]. However, statistical support for many clades was low, and the relationships between the main groups were unclear. It could be explained by the variation deficiency, as well as by the ITS properties in the species of the family *Pinaceae*, such as slow concert evolution, intragenomic variation, and the presence of pseudogenes [17–19].

Recently, phylogenetic analysis of the position of Mesoamerican *Abies* species in the genus and their evolution, based on the chloroplast DNA sequences,

was performed [5]. In the phylogeny obtained, 33 *Abies* species were divided into five main groups [5], with higher statistical support than in the analysis with the help of ITS [4]. However, chloroplast phylogeny [5] was characterized by insufficient resolution, especially in the basal part and in some of the main clades. In the study [5], only 12 Asian species were included into the analysis, which was probably the reason for the low resolution in the Asian–American clade. In addition, a partial inconsistency of phylogeny in [5] and the results of Suyama et al. [15] was observed. This inconsistency can be caused by insufficient variation of the chloroplast DNA regions chosen for analysis in the study [5].

The present study was focused on the molecular phylogenetic analysis of the genus *Abies*, based on nucleotide sequences of the chloroplast DNA, with age estimates of the main clades and a suggestion of the most plausible biogeographic scenarios. For these purposes, the chloroplast DNA fragments with a sufficient level of variation were used. Furthermore, the total length of the fragments was considerably enlarged, and the fir species examined represented the main evolutionary lineages of *Abies*.

MATERIALS AND METHOD

Plant material examined. Phylogenetic analysis was conducted using 52 DNA samples representing 37 *Abies* taxa (Table 1). For the nine fir species, the material was collected from natural populations; 28 taxa, including a number of *Abies* subspecies and variations, were represented by the samples obtained from botanical gardens and arboreta (Table 1). In the absence of confirmation from the botanical gardens on the origin of the species accessions from natural populations, the species were examined in several replicates from different botanical gardens. Species assignment of *A. cephalonica* and *A. koreana*, represented in our analysis by single accessions from botanical gardens with no specification of natural origin, was confirmed by the coincidence of the sequences of some of the fragments determined in the present study with those from the GenBank. The taxa examined represented all 15 sections of the genus, distinguished by Liu [2], and nine out of ten sections of the genus, corresponding to the system of Farjon and Rushforth [14], except for the section *Amabilis* (two species). *Keteleeria davidiana* was chosen as outgroup, because a close relatedness between *Keteleeria* and *Abies* based on molecular phylogenetic data [4–6] had been demonstrated earlier. The complete chloroplast genome sequence of *K. davidiana* [20] was taken from the GenBank (accession number AP010820.1).

DNA extraction, PCR amplification and sequencing. Except for the fir DNA samples obtained from the Royal Botanical Gardens, Kew (Table 1), the total DNA was extracted from fresh needles and buds or from silica-gel dried tissues using the CTAB-NaCl method [21]. After initial testing for amplification success and the presence of polymorphism, five chloroplast DNA regions, *atp*F-*atp*I and *rbc*L-*psa*I (Semerikov, unpublished data), *trn*Q-*trn*R [http://www.pierroton.inra.fr/genetics/labo/cpdna. html, unpublished], *rps*18-*rp*120, and *trn*L-*trn*F [15] (Table 20) were chosen for further analysis.

To overcome amplification difficulties within several chloroplast DNA fragments due to their large size and/or the presence of microsatellites, found in *Abies* regions (*atp*F-*atp*I, *rbc*L-*psa*I, and *trn*Q-*trn*R), additional PCR primers were designed based on the obtained sequences and employing the Primer3 software program [22]. In the following, the left and right segments of these regions were amplified and sequenced separately. As a result, from each sample eight fragments of five chloroplast genome regions were amplified. Primer sequences, as well as the position of the primers and the aligned sequences in the chloroplast genome of *K. davidiana*, are demonstrated in Table 2.

The PCR reaction was carried out in a volume of 25 μL containing one-fold PCR buffer (75 mM Tris-HCl (pH 8.8 at 25°C); 20 mM $(NH_4)_2SO_4$; 0.1% Tween 20); 2.5 mM $MgCl_2$; 200 μM of each dNTP; 0.2 μM of forward and reverse primers; 0.8 units of *Taq* DNA polymerase (produced by MBI Fermentas (Lituania) or SibEnzyme (Russia)). PCR conditions consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s; primer annealing for 45 s at 59°C (for fragments *atp*F-F2, F12*atp*I, *rbc*L-L2, LI2-*psa*I), at 52°C (for fragments *trn*Q-Q2, R2-*trn*R), at 50°C (for *rps*18-*rpl*2), and 55°C (for *trn*L-*trn*F); extension at 72°C for 1–3 min, depending on the fragment length; and final extension at 72°C for 10 min. PCR products were purified by means of extraction from 1% agarose gel (in TAE) using the Gel Extraction Kit (QIAGEN).

Sequencing was performed using the BigDye v. 3.1 kit (Applied Biosystems, United States). After purification, the reaction products were examined in the ABI 3130 Genetic Analyzer automatic sequencer (Applied Biosystems). If required, the fragments were sequenced from both PCR primers. The sequences obtained were edited and aligned manually using the BioEdit software program [23].

Phylogenetic analysis. Phylogenetic reconstruction of the genus *Abies* based on the sequence variation of chloroplast DNA was conducted using the method of maximum parsimony (MP) and Bayesian inference (BI). MP trees were constructed in the PAUP*4.0b10 software package [24] with the heuristic search option, MulTrees and MaxTrees = 500, and the tree bisection–reconnection (TBR) branch swapping algorithm. The clade robustness was evaluated using the bootstrap analysis with 1000 replicates. All characters were weighted equally. Gaps were considered as missing data. Indels and inversions were treated as single

Table 1. List of species and accessions used in phylogenetic analysis of Abies

Taxon/*n**/accession identification number/(accession origin**)	Distribution
A. alba Mill./1/Is6-2010/(47°48′/13°02′ E) /2/Is7-2010/(47°48′/13°02′ E)	Europe
A. balsamea (L.) Mill./1/A0712/(A) /2/A0715/(A)	East of North America
A. balsamea (L.) Mill. var. *phanerolepis* Fernald//Is07-2011/(B)	″
A. bracteata (D. Don) A. Poit./1/36527/(K) /2/Is12-2011/(N)	West of North America– California, United States
A. cephalonica Loudon//Is02-2011/(B)	Mediterranean
A. chensiensis Tiegh. ssp. *salouenensis* (B. & G.) Rush.//37131/(K)	China
A. cilicica (Antoine & Kotschy) Carriere ssp. *cilicica*//40673/(K)	Mediterranean
A. concolor (Gordon) Lindl. ex Hildebr./1/Is05-2011/(B) /2/Is13-2012/(37°45′ /119°30′ W)	North America and Mesoamerica
A. delavayi Franch.//37132/(K)	China
A. densa Griff.//37133/(K)	Himalaya, Tibet
A. durangensis Martinez//36849/(K)	Mexico
A. firma Siebold & Zucc./1/Is20-2011/(S) /2/Is25-2011/(P)	Japan
A. forrestii Coltm.-Rog.//37134/(K)	China
A. fraseri (Pursh) Poir.//A0471/(A)	East of North America
A. gracilis Kom./1/Is51-2010/(54°07′/159°59′ E) /2/Is58-2010/(54°07′/159°59′ E)	Kamchatka
A. grandis (Douglas ex D. Don) Lindl./1/40674/(K) /2/Is26-2011/(P)	West of North America
A. guatemalensis Rehder//7720/(K)	Mexico
A. holophylla Maxim.//Is20-2006/(43°10′/132°00′)	Northeast of Asia
A. homolepis Siebold & Zucc./1/A0571/(A) /2/Is27-2011 (P)	Japan
A. koreana E. H. Wilson//A048/(A)	South Korea
A. lasiocarpa (Hook.) Nutt.//A0520/(A)	West of North America
A. lasiocarpa var. *arizonica* (Merriam) Lemmon//Is06-2011/(B)	″
A. magnifica A. Murray/1/Is16-2012/(37°45′/119°30′ W) /2/Is17-2012/(37°45′/119°30′ W)	″
A. nephrolepis (Trautv. ex Maxim.) Maxim./1/Is01-2008/(49°00′/131°05′ E) /2/Is02-2008/(48°15′/134°40′ E)	Northeast of Asia
A. nordmanniana (Steven) Spach//Is04-2011/(B)	Caucasus, Asia Minor
A. equi-trojani (Asch. & Sint. ex Boiss.) Coode &Cullen//Is13-2011/(N)	Mediterranean
A. numidica de Lannoy ex Carriere//40675/(K)	″
A. pinsapo Boiss./1/Is14-2011/(N) /2/Is30-2011/(C)	″
A. recurvata Mast.//36850/(K)	China
A. religiosa (Kunth) Schltdl. & Cham.//37361/(K)	Mexico
A. sachalinensis (F. Schmidt) Mast./1/Is41-2008/(46°40′/141°50′ E) /2/Is42-2008/(46°40′/141°50′ E)	Hokkaido, Sakhalin, and Kurils
A. semenovii B. Fedtsch./1/Is31-2010/(41°52′/71°54′ E) /2/Is33-2010/(41°52′/71°54′ E)	Tien Shan
A. sibirica Ledeb./1/Is03-2007/(49°30′/111°00′ E) /2/Is04-2007/(55°55′/92°30′ E)	Siberia and Eastern Europe

Table 1. (Contd.)

Taxon/*n**/accession identification number (accession origin**)	Distribution
A. spectabilis (D. Don) Spach/37135/(K)	Himalaya, Hindu Kush
A. squamata Mast./1/37136/(K)	China
A. veitchii Lindl./1/A0531/(A) /2/Is10-2011/(B)	Japan
A. vejari Martinez/37359/(K)	Mexico
Keteleeria davidiana//GenBank: number AP010820.1—chloroplast DNA, full nucleotide sequence	

* Taxon replicate numbers.

** Botanical gardens and arboreta: K, Royal Botanical Gardens, Kew (United Kingdom); A, Tsitsin Botanical Garden of the Russian Academy of Sciences; B, Botanical Garden of the Botanical Institute of the Russian Academy of Sciences; N, Nikitsky Botanical Garden, National Research Center (Yalta); P, Kornik Arboretum (Poland); S, Sochi Arboretum; C, Botanical Garden of Charles University in Prague. Coordinates of natural populations (N/longitude).

Table 2. Chloroplast DNA regions, fragments, and primers, used in phylogenetic analysis of *Abies*

Region	Fragment	Primer	Primer sequence (5'–3')	Position****		GenBank accession numbers
				of primer	of aligned nucleotide sequences	
*atp*F-*atp*I*	*atp*F-F2	FI-F	tggtcaaatgctctttgctg	52646	52701–	KC597319–
		FI-F2	tgagttattggccttctgctg	53824	53735	KC597370
	FI2-*atp*I	FI-I2	tctcctcttactaaggttgggaaa	54390	54615–	KC597371–
		FI-I	caggtgctcttcttccttgg	55496	55449	KC597422
*rbc*L-*psa*I*	*rbc*L-L2	LI-L	gacgtgatcttgctcgtgaa	76705	76752–	KC597527–
		LI-L2	tgcctgtttgaatagcatcag	77867	77689	KC597578
	LI2-*psa*I	LI-I2	cgcactcggagttatggatt	77893	78198–	KC597423–
		LI-I	accattgtaattgccggaag	78963	78934	KC597474
*trn*Q-*trn*R**	*trn*Q-Q2	QR-Q	gggacggaaggattcgaacc	48697	48741–	KC597475–
		QR-Q2	ttacctttgcgaactgctga	49484	49435	KC597526
	R2-*trn*R	QR-R2	tccgtttcttccctcatacc	50392	50441–	KC597268–
		QR-R	attacgtccaataggatttgaa	50845	50787	KC597318
*rps*18-*rpl*20***	*rps*18-*rpl*20	*rps*18-1	agtcgatttattagtgagca	87636	87657–	KC597631–
		*rps*18-2	cttcgtcgtttgttgattac	88235	88187	KC597682
*trn*L-*trn*F***	*trn*L-*trn*F	*trn*L-1	ttggctttatagaccgtgag	106808	106342–	KC597579–
		*trn*L-2	ccaggaaccagatttgaact	106321	106787	KC597630

* Semerikov, unpublished.

** http://www.pierroton.inra.fr/genetics/labo/cpdna.html, unpublished.

*** Suyama et al. [15].

**** Position in chloroplast genome of *K. davidiana* [20].

events and were coded as binary characters (1/0), while microsatellite sequences were not taken into account.

The absence of contradictions between certain chloroplast DNA regions (congruence of the corresponding trees) was established using the Partition Homogeneity Test (PHT) in PAUP.

Bayesian inference (BI) of phylogeny was performed as implemented in the MrBayes v. 3.1.2 software program [25] based on the GTR+G+I nucleotide substitution model, chosen using the Modeltest 3.7 software program and Akaike Information Criterion [26]. In the MrBayes program, two independent MCMC chains with a length of 10 million steps were used, saving the parameters every 1000 steps. To monitor the adequacy of the Markov chains' length, the average standard deviation of split frequencies was used. After 2.5 million of generations, the value of this

Table 3. Sequence variation of five chloroplast DNA regions used in phylogenetic analysis of *Abies*

Chloroplast region	Mean sequence length, bp	Aligned sequence length*, bp	Number of informative mutations (point mutations/insertions, deletions, inversions		Total number of mutations, *Abies* + *Keteleeria*	Mutations per 100 bp, *Abies* + *Keteleeria* (informative per 100 bp)
			Abies	*Abies* + *Keteleeria*		
*atp*F-*atp*I	1863	1902	18/5	19/5	101	5.42 (1.29)
*rbc*L-*psa*I	1696	1876	25/8	30/9	184	10.86 (2.3)
*trn*Q-*trn*R	1038	1175	21/3	21/3	110	10.6 (2.31)
*rps*18-*rp*l20	536	558	6/1	7/1	45	8.4 (1.49)
*trn*L-*trn*F	447	563	9/5	10/5	47	10.5 (3.36)
Total	5580	6074	79/22	87/23	487	8.73 (1.97)

* The length of aligned sequences for *Abies* and *Keteleeria* accessions together.

measure was less than 0.01, and due to this, the first 2.5 million generations were discarded as burn-in. The remaining 7.5 million trees were used to construct the consensus tree.

The divergence time estimates were performed using the BEAST v. 1.7.4 software program [27], based on the phylogenetic trees simulation with the use of Markov chains and the GTR+G+I substitution model. It was assumed that the mutation rates in individual tree branches were the same (strict clock model). The length of the Markov chains constituted 20×10^6 trees; every 2000th tree was saved. To ensure that the MCMC process completely investigated the multidimensional space of tree topology and its parameters, the Tracer v. 1.3 software program [27] and ESS (estimates sample size) statistics, which was higher than 200 for all parameters, were used. The consensus tree was constructed in the TreeAnnotator software program (a part of the BEAST software package). Ten percent of the trees were discarded as burn-in. The consensus tree was visualized using the FigTree software program. The divergence time calibration was performed using three approaches. In the first variant, the age of separation of *Abies* from *Keteleeria* was limited to 100.4–113.8 million years ago (MYA) [28, 29]. Following the second variant, the mutation rate was fixed at the value of 0.22×10^{-9} substitution site^{-1} · year^{-1}, according to the existing estimate of silent substitution mutation rates in chloroplast DNA in the species of Pinaceae [30]. In the third variant, the age of separation of *Abies* from *Keteleeria* was constrained to the range of 45.5–55.0 MYA [31, 32].

RESULTS

The summarized length of fir chloroplast DNA fragments obtained in the present study constituted about 5580 bp. The length of aligned sequences for 53 *Abies* and *Keteleeria* accessions constituted 6074 bp (Table 2). The sequences of *Abies* accessions obtained were deposited in the GenBank database under the accession numbers shown in Table 2.

Fragment R2-trnR from the *trnQ-trnR* region was not amplified in *A. densa* because of possible mutation at the QR-R primer annealing site in this species. In *A. densa*, this fragment was scored as missing data. The *rbcL-psaI* and *trnQ-trnR* regions were found to contain one poly-T microsatellite each, which were not scored in phylogenetic analysis.

The data on the variation of the examined chloroplast DNA regions are demonstrated in Table 3. In total, in *Abies* and *Keteleeria*, 487 variable characters were identified, of which 110 (without replicates) were parsimony-informative between the taxa. Among the 101 characters informative between the *Abies* taxa, 79 were point mutations and the remaining 22 were indels and inversions. In the five regions examined, the mean number of mutations per 100 bp was 8.73. In *Abies*, the most variable regions were *rbc*L-*psa*I, *trn*Q-*trn*R, and *trn*L–*trn*F (Table 3).

Of the *Abies* taxa for which more than one accession was examined, eight species were characterized by intraspecific variation. In these species, a total of 22 variable characters, represented by 14 point mutations and eight indels, were identified. Of these characters, 16 were unique and six characters were probably the homoplasies, since their derived traits were found also in some other species. This means that it should be assumed that these mutations repeatedly appeared or

disappeared in the course of the phyletic evolution of the chloroplast DNA of *Abies*. The highest intraspecific variation was observed in *A. concolor* (eight mutations, including three homoplasies), *A. grandis* (four mutations, including one homoplasy), *A. firma* (four mutations), and *A. homolepis* (two mutations). *A. sibirica*, *A. sachalinensis*, *A. alba*, and *A. veitchii* contained one intraspecific mutation each. In general, the intraspecific variation was substantially lower than the variation between the species. With the exception of *A. alba*, accessions of one species always formed monophyletic groups.

Phylogenetic Reconstructions

The PHT test did not reject the null hypothesis on the absence of phylogenetic conflict among the data set ($P = 0.8$), providing grouping of the sequence data for individual chloroplast regions into one dataset. The MP and BI trees had similar topology, which consisted of six main groups of the *Abies* species. In both topologies, statistical support of all main clades and most of subclades was rather high. However, the Bayesian tree (Fig. 1) had higher support of all clades, compared to the MP phylogeny. The distinguishing feature of the BI tree was the grouping of *A. magnifica* and the species from group III into one clade, and the resolution of a greater number of subclades. The Bayesian tree, as the more completely topologically resolved, is demonstrated in Fig. 1 with the indication of the clade statistical support values for both of the trees.

The Californian endemic *A. bracteata*, assigned in the present study to group I, was characterized by a high number of ancestral (grouping it with *Keteleeria* and separating from the other *Abies* species) and species-specific mutations. In our study, group II was represented by a single species of *A. magnifica* (west of North America). Similarly to *A. bracteata, A. magnifica* was characterized by the presence of a great number of ancestral characters and specific mutations. In the MP tree, *A. magnifica*, similarly to *A. bracteata*, formed a separate basal clade. At the same time, unlike *A. bracteata*, *A. magnifica* had characters in common with the species from group III (Mesoamerican and western North American species), which was reflected in grouping of the branches of *A. magnifica* and those of the species from group III into a single clade in the BI phylogeny (posterior BI support, 75%) (Fig. 1).

Group III was comprised by Mesoamerican and western North American species, which formed a common clade (100/100 BI- and MP-support, respectively) that, with solid statistical support, was divided into two subclades. One of these subclades was formed by the *A. grandis* individuals (100/100). In the second subclade (100/98), a branch of *A. concolor* accessions separated from the branch, uniting all Mexican fir species (98/75), which split into pairs of *A. religiosa–A. vejari*, *A. durangensis–A. guatemalensis*.

Groups IV–VI formed a clade with high statistical support (100/100). This large clade was composed of two branches. One branch consisted of the European-Mediterranean species (group IV) (100/100) and second one—an alliance of sister groups V and VI (100/100). The group IV of European-Mediterranean species is characterized by the presence of a great number of specific mutations, distinguishing it from the other *Abies*. However, the phylogenetic topology within the group remains unresolved.

Group V of "boreal" or northern North American fir species (100/100) clearly splits into two relative subclades, including the subclade of northwestern North American firs (*A. lasiocarpa–A. lasiocarpa* var. *arizonica*) (100/97) and that of northeastern North American firs (*A. balsamea*, *A. fraseri*, and *A. balsamea* var. *phanerolepis*) (100/97).

Group VI (100/100) is the main and most abundant group, which includes all Asian fir species examined. Within group VI, a subclade of Far Eastern species (*A. nephrolepis*, *A. koreana*, *A. sachalinensis*, *A. gracilis*, *A. veitchii*, and a Chinese fir species *A. chensiensis*) is distinguished (100/89). This subclade with low statistical support (97/-) is joined by a subclade of Himalayan firs, *A. spectabilis–A. densa* (99/66). In general, group VI is weakly differentiated. Some species within this group form some more subclades with different statistical support levels. These are the branches of *A. sibirica–A. semenovii* (99/65), *A. holophylla–A. homolepis* (88/63), and *A. forrestii–A. squamata–A. delavayi* (100/63). Within the subclade of Far Eastern species, a branch of *A. sachalinensis–A. gracilis* is distinguished (100/61).

Relationships Among the Basal Groups, Homoplasies, and Recombination of Abies Chloroplast DNA

The tree obtained reveals strongly statistically supported relationships between the groups of species. However, in the basal part the tree is weakly resolved. Many characters supported alternative topologies and determined different groups as basal (*A. bracteata* (I) or *A. magnifica* (II)). In other words, these characters behave themselves as homoplasies. In total, 24 characters, which were probably the homolpasies, were identified. The highest number of such characters (13) was found in the basal part of the tree. Ancestral characters in common with *Keteleeria* were typical of either *A. bracteata*, or of *A. magnifica*, or, to a lower extent, of the group of either Mexican or Mediterranean fir species. In the case that *A. magnifica* is excluded from the MP analysis, the clade of *A. bracteata* becomes the basal one, with statistical support of the clade uniting the remaining fir species (constituting 84). On the contrary, with exclusion of *A. bracteata* (I) from the analysis, *A. magnifica* (II) becomes the basal group relative to all other *Abies*, with statistical support of the remaining clade of 91 (data not shown). Thus, unambiguous topology resolution at the basement of the

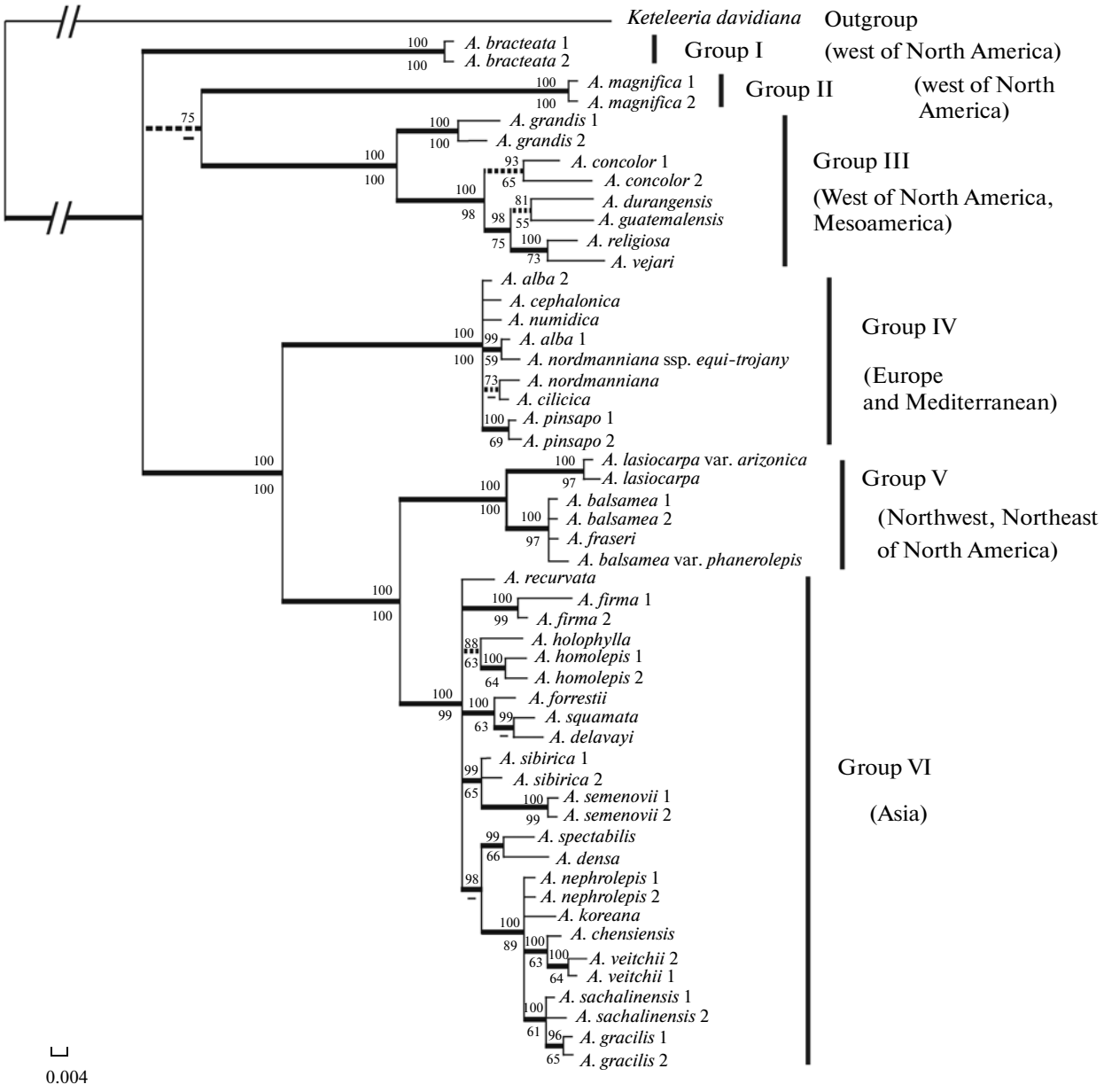

Fig. 1. Fig. 1. Phylogenetic tree of *Abies* constructed by means of Bayesian inference (BI, 50% majority rule consensus) for 53 *Abies* and *Keteleeria* accessions based on nucleotide sequences of the chloroplast DNA fragments with the total length of 5580 bp. Numbers above the branches indicate the Bayesian statistical support values (posterior probabilities multiplied by 100); below the branches are the bootstrap support values (MP, 1000 replicates). Solid horizontal lines, support above 95%; hatching line, support values between 50 and 95%; dash means that reproducibility of this clade is below 50% of bootstrap replicates. The scale reflects the number of substitutions per nucleotide position. The species groups defined are discussed in the text. Numbers after the species names correspond to the taxon replicate numbers in Table 1.

Abies tree, based only of the chloroplast data, seems to be impossible, even increasing the number of fragments included into the analysis. Such resolutions require additional data on nuclear and mitochondrial genomes.

The four-gamete test for recombination events of Hudson and Kaplan [33] was performed and it confirmed the presence of recombination for a number of characters (plausible homoplasies). The recombinations were characteristic of all chloroplast fragments examined, especially of highly variable fragments.

Divergence Time Estimates of the Main Clades

The tree constructed in the BEAST program (Fig. 2) almost completely repeated the topology of the MrBayes BI tree (Fig. 1), differing in lower resolution, probably as a result of ignoring the indel information

by the BEAST algorithm. Calibration of phylogenetic tree using fossil records (variant 1) based on the divergence time between *Abies* and *Keteleeria* at 100 to 113 million years (MY) [28, 29] was basically similar to the calibration based on the rate of silent substitutions in chloroplast genome of the *Pinaceae* species [30] (variant 2), estimating the divergence time of the most basal clades at the Late Oligocene–Early Miocene (Fig. 2). At the same time, the calibration based on the estimate of the divergence time between *Abies* and *Keteleeria* at 45.5 to 55 MY [31, 32] (variant 3) reduces the age of all clades by about two times. According to the first two calibration variants, the divergence time between Mediterranean (IV) and Asian–North American species (groups V and VI) was estimated at the Miocene. At the same time, according to the third, the so-called young variant, the divergence time between these species groups was estimated at the border between the Miocene and Pliocene. The occurrence of *Abies* fossil records, dated back to the Miocene and even to the Oligocene, in Europe and Asia makes the first two calibration variants more likely, compared to the third variant. The first two calibrations are also favored by the similarity of the results obtained upon the independent nature of the two approaches. Hereinafter, time estimates obtained with the help of the first two calibrations will be discussed (Fig. 2, variants 1 and 2).

The divergence time between groups II and III corresponds to the Early–Middle Miocene. The divergence time within group III (Mexican species–*A. grandis*) corresponds to the Late Miocene. The separation of "boreal" North American species (*A. lasiocarpa*, *A. balsamea*, *A. fraseri*) (V) from Asian species (VI) occurred in the Late Miocene. At the same time, differentiation within the largest fir species group (most of Asian *Abies* species) (VI) occurred in the Late Miocene through the Pliocene and Pleistocene. The divergence of the "boreal" American species (V) into the western (*A. lasiocarpa*) and eastern (*A. balsamea*) groups corresponded to the border between the Pliocene and Pleistocene. The divergence within the group of European-Mediterranean species (IV) took place in the Late Pliocene–Early Pleistocene.

DISCUSSION

Comparison of Chloroplast Phylogeny with Other Phylogenetic Studies and the Existing Systematic Schemes

The phylogenetic resolution obtained in the present study surpasses the earlier ones, which were obtained based on the analysis of three chloroplast DNA fragments [5] and ITS phylogeny [4]. In total (excluding species replicates), the BI tree contained 23 clades with statistical support values higher than 50%. For comparison, in the previous studies, there were only 15 and 13 statistically supported clades [4, 5]. The phylogenetic tree obtained in the study [5] showed the split of *Abies* into five main groups. Four of these groups, taking into account a somewhat differing set of species, were congruent with our groups I—IV. Similar groups, however, without the resolution of the relationships between them, were also present in the phylogeny based on the ITS data [4].

The study [5] was conducted involving a great number of western North American and Mesoamerican species and accessions, as well as the species of section *Amabilis* (*A. mariesii* Mast. and *A. amabilis* Doug. ex Forb.) The similarity in the evolutionary position of the groups corresponding to groups II and III from our study could be seen. Specifically, *A. magnifica* formed a clade together with the relative species *A. mariesii* and *A. amabilis*. This clade was sister to the clade of Mesoamerican and western American species. Furthermore, the combined clade also occupied the position at the basement of *Abies* tree [5].

Monophyly and a deep differentiation of Mediterranean species from the rest of firs was demonstrated earlier [4, 16], along with the fact that the group consisted of closely related species. However, the phylogenetic position of the group was variable depending on the method of investigation and the degree of representation of the other groups. The sister position of Mediterranean group (IV), relative to the combined clade of Asian and "boreal" American firs, revealed in the present study, was congruent with the topology of the Mediterranean clade reported in [5].

One of the main results of the phylogenetic reconstruction performed was the newly described deep separation of the sister groups VI and V—Asian and "boreal" North American species, respectively. This result was in good agreement with one of the conclusions of Suyama et al. [15]. In the latter study, based on sequence data for eight chloroplast DNA regions, a phylogenetic tree for 13 fir species was constructed. The tree showed a clear separation of American firs *A. lasiocarpa* and *A. fraseri* from the Asian species. The absence of such separation in the study [5] can be explained by a number of reasons. First, that could be due to generally low variation of the three fragments examined (*rbc*L, *rps*18-*rpl*20, *trn*L-*trn*F, 2378 bp, in total). Fragments *rbc*L and *rps*18-*rpl*20 were characterized by low variation in groups V and VI. Moreover, there was no variation supporting the separation of these groups. However, according to our data, fragment *trn*L-*trn*F contained two such characters (deletion and point mutation in the tandem repeat). Hence, the second explanation for the absence of separation could be the sequencing errors in the study [5]. In particular, comparison of the sequences reported in [5] with those obtained in our study led to the conclusion that in the analysis of fragment *trn*L-*trn*F, the samples of *A. sibirica* (GenBank no. JN935672), *A. fraseri* (JN935660), and *A. numidica* (JN935668) could be mixed up, which finally resulted in the

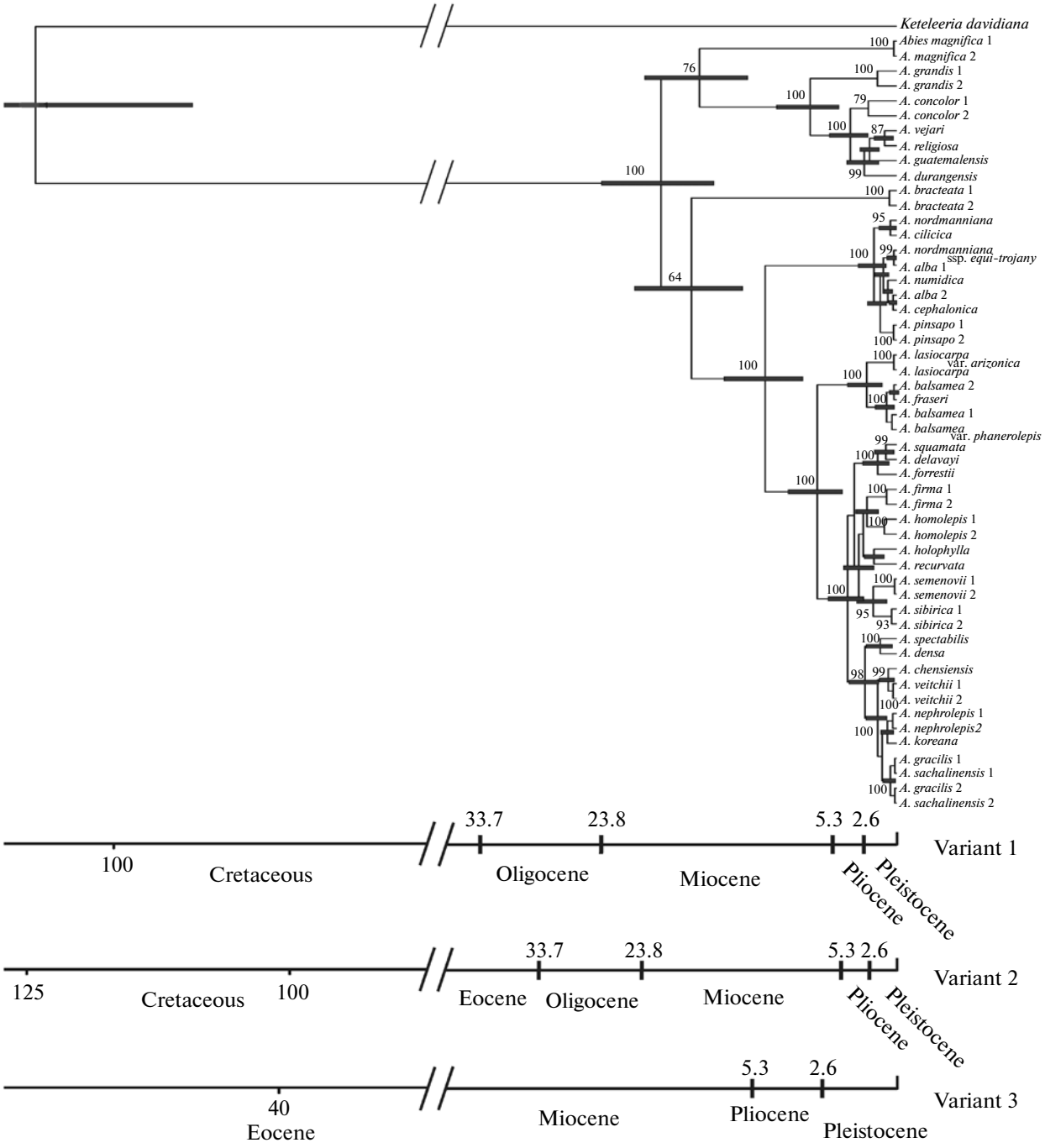

Fig. 2. Bayesian chronogram of the fir species phylogeny. The divergence time estimates were performed using three calibration variants (see the text). Boxes at the nodes designate the 95% confidence intervals of the divergence time corresponding to the first calibration variant (HPD95%, highest posterior density).

absence of separation of the clades, corresponding to groups V and VI. Furthermore, we suggested that in the study [5], the sequences of fragment *rps*18-*rpl*20 were mixed between *A. pinsapo* (JN935725), *A. recurvata* (JN935726), and *A. magnifica* (JN935721). There were also the errors in the *trn*L-*trn*F sequence editing and alignment, typical to the sequence start and end. Furthermore, some of the deletions were missed, etc. Taken together, the low variation level along with data errors resulted in low statistical support (or the absence of support) of some of the clades and in the shift in the divergence time estimates of some of the groups. For instance, the confusion with *A. numidica* determined its separation from the other species of

Mediterranean clade, which in turn, resulted in an overestimation of the divergence time of Mediterranean species. The confusion with *A. recurvata* resulted in an overestimation of the divergence time of the Asian species. In our opinion, this situation could lead to the generally overestimated species divergence time in all clades of *Abies* phylogeny, presented in [5].

The subdivision of firs into sections in the classification of *Abies* based on morphological characters [2, 14] was only partly congruent with the obtained phylogenetic reconstruction. It should be noted in this respect that the most basal groups were more consistent with the morphological classification than the young groups. For instance, the separation of *A. bracteata* into an individual section was confirmed [4]. Undoubtedly, the species was isolated at the early stages. It remained a Miocene relict [34], having no contacts with the other extant *Abies* lineages, and is currently characterized by the highest morphological and molecular divergence from the other fir species. Based on the chloroplast data available, it is impossible to make a definite conclusion on the basal position of *A. bracteata* relative to other firs, and consequently, to validate the isolation of this species into monotypic subgenus [2].

The data obtained do not contradict the recognition of the section *Nobilis* [14], which includes *A. magnifica* and its close relative *A. procera* Rend., which was, however, not included in the analysis. The species of the section *Amabilis* (*A. amabilis* and *A. mariesii*) are not yet included in our analysis. At the same time, sequences of fragments *rps*18-*rpl*20 and *trn*L-*trn*F from *A. magnifica* determined in the present study were to a great degree congruent with the sequences of *A. mariesii* (GenBank database [15]), supporting their evolutionary closeness.

Group III is formed by the species of sections *Grandis* and *Oiamel* [14]. Although these species are clearly separated from the species of other sections, the separation among them is not consistent with classification into the sections. Group IV includes the species of sections *Abies* and *Piceaster* [2, 14]. However, within the group, these species do not form clades corresponding to the sections recognized.

In younger groups, the discrepancy between phylogeny and morphological classification is more pronounced. The phylogenetic tree constructed in the present study shows a deep separation of the species assigned by Farjon and Rushforth to the section *Balsamea* [14]. American species from this section (*A. lasiocarpa*, *A. balsamea*, and *A. fraseri*) form group V. These species are considerably different from the Asian species of the section, which are included in group VI. Moreover, within group V, a separation into the subclades of northwestern (*A. lasiocarpa*, *A. lasiocarpa* var. *arizonica*) and northeastern (*A. balsamea*, *A. fraseri*) American taxa was obtained. This topology does not support grouping *A. balsamea*, *A. lasiocarpa*, and *A. sibirica* into the subsection *Laterales*, section *Balsamea* [14], and *A. fraseri*, along with *A. nephrolepis* and other Far Eastern species, to the subsection *Mediana*e. The chloroplast phylogeny obtained in many respects is congruent with the earlier classification of Liu [2], which to a large extent takes into account the geographic distribution of the species. In this classification, the North American firs *A. lasiocarpa*, *A. balsamea*, and *A. fraseri* are grouped into the separate section *Balsamea*; Far Eastern species, *A. nephrolepis*, *A. sachalinensis*, *A. koreana*, *A. veitchii*, into the section *Elate*; and *A. sibirica* and *A. semenovii*, into the section *Pichta*. This grouping is consistent with the position of these species in the individual subclades of the Asian clade. The species from other sections, recognized in different classifications as *Momi* and *Pseudopicea* [14], or *Homolepides, Chensienses, Elateopsis*, and *Pindrau* [2], though included in the same group, do not form monophyletic clades. Obviously, the existing classifications, based only on morphological characters, should be revised taking into account molecular data.

Divergence Time Estimates and the History of Abies

The earliest macrofossils of *Abies* are known from the Eocene in western North America and East Asia [32, 35, reviews in 29, 36]. However, pollen data indicate the fir appearance much earlier, in Late Cretaceous deposits from Siberia and China [29, review], which served as the calibration point for the *Abies*–*Keteleeria* divergence. The molecular dating obtained suggest that the basal clades of extant *Abies* species separated at the border between the Oligocene and Miocene (about 19 MYA, according to the first calibration variant, or about 22 MYA, with a range starting from the Middle Oligocene, according to the second, more conservative variant (HPD95% = 18–27 MYA)). These findings are in good agreement with estimates in the recent analysis of the evolutionary dynamics of conifers, conducted with use of the literature data on nucleotide sequences of two chloroplast fragments (*mat*K and *rbc*L) and partial sequences of the nuclear gene from 489 coniferous species, including 25 *Abies* species and two *Keteleeria* species [6]. The divergence between *Abies* and *Keteleeria* was estimated as having occurred about 80 MYA. The separation of the basal clades of *Abies* was dated to the border between the Palaeogene and Neogene (about 25 MYA) [6]. The start of *Abies* diversification roughly coincides with the start of diversification of the genera *Picea* and *Tsuga*, and the subgenera *Pinus* and *Strobus*.

The existing paleontological data are not enough for a robust determination of the region of origin and the dispersal pathways of different *Abies* lineages. At the same time, the phylogeny obtained enables some suggestions on the history of genus *Abies*. For the extant *Abies* lineages, the Bering Land Bridge can be considered as the only migration rout between the continents. This is because the North Atlantic Land

Bridges existed until the Eocene, while according to data obtained the age of divergence of the Eurasian species from the basal American species is estimated at the Late Oligocene—Middle Miocene. The extant fir species are thought to originate from North America, since there the highest differentiation of certain *Abies* species groups is observed. This is also the place where basal species groups are found. Among the latter, the earliest diverged are *A. bracteata*, as well as the group of *A. magnifica* and its relative species. This means that Eurasian centers of fir diversity are obviously secondary ones relative to the American centers. The first migration wave occurred from America to Asia, and further on, to the Mediterranean. According to the estimates obtained, the separation of Mediterranean and Asian firs occurred in the Miocene, which does not contradict with the Miocene age of fir fossil records from Europe and Asia Minor [10, references]. Then, during the latter part of Miocene, the reverse migration from Asia to America, which gave rise to "boreal" lineage of American fir species, took place.

In a recent review on the biogeography and evolution of Mediterranean firs, Linares [10] underlined that major differentiation and formation of extant Mediterranean fir species took place in the Pliocene to Pleistocene, at the same time, it was suggested [10] that isolation of some lineages (for instance, *A. pinsapo*) occurred as early as in the Miocene. These conclusion contradict the results of the present study, according to which the differentiation of extant Mediterranean species took place later, in the Late Pliocene to Pleistocene. Thus, although Mediterranean firs are considerably isolated from other groups, the differentiation among the species themselves is low. In our view, the evolutionary closeness of all Mediterranean species can be explained in terms of a dramatic bottleneck associated with the Late Miocene Messinian Salinity Crisis (5.6 MYA) occurring in the Mediterranean Sea basin and resulting in considerable aridization of the surrounding territories. Alternatively, the bottleneck could be associated with some other unfavorable climatic events during the Pliocene [10, references].

Almost all extant Asian fir species are thought to be rather young. This suggestion follows from rather low differentiation among these species, corresponding to the age of the Pliocene—Pleistocene. The exception is *A. mariesii* (Japan)—species related to *A. magnifica*, which was not included in our study. It can be suggested that *A. mariesii* is the product of a rather recent migration of the *A. magnifica* relative firs to Asia.

The center of the fir's diversity is the west of North America, where a number of extant, highly differentiated *Abies* taxa are found. This is not surprising, taking into account the unusual abundance of other gymnosperm genera characteristic of this region. The regions of North America that are distant from the diversity center were characterized by groups of species that were more evolutionarily close to each other [8]. For instance, in our analysis, the divergence in the group of the Mexican species was estimated at the Pliocene to Pleistocene, which corresponded to the age of the earliest fir fossil records in Mexico [5] and reflected the relatively recent history of fir penetration into the local mountain systems. The differentiation of "boreal" firs into western (*A. lasiocarpa*) and eastern (*A. balsamea* and other species of the eastern coast of North America) clades occurred during the Pleistocene. Supposedly this differentiation was associated with the beginning of the glacial period and the biogeographic break between the Pacific and Atlantic parts of North American boreal biota. Monophyly and close relationships between the species of eastern clade are congruent with the data of the population study of this species complex, based on the analysis of nuclear microsatellite loci [37].

Recombination of Chloroplast DNA and Hybridization in Abies

Usually, chloroplast DNA is thought to be non-recombining. However, there is some evidence pointing to its possible recombination in the species of the Pinaceae [38, 39] and Geraniaceae [40] families, as well as in *Dactylorhiza maculata* [41], that can restrict the use of chloroplast DNA in phylogeny. It should be noted in this respect that, in our study, homoplasy was found mostly in basal branches of the *Abies* tree, pointing to possible ancient recombination. It was also detected in the species growing or having grown on the same territory, e.g., in North America in *A. concolor* and a clade of Mexican species; in *A. grandis* and Mexican species; in *A. lasiocarpa* and *A. balsamea*; in *A. grandis*, *A. lasiocarpa*, and *A. magnifica*. Such examples were also found in Asia between subclade of *A. squamata–A. delavayi* and the Far Eastern species. These findings support the hypothesis on the hybrid-recombination origin of homoplasies discovered in *Abies*. This is because common territory is a necessary prerequisite for hybridization, which in turn is quite usual for relative species of conifers inhabiting the same territory [for example, 11]. The identified signs of recombination between the distant lineages of chloroplast DNA suppose the presence of the historical gene flow between the *Abies* species, as well as the hybrid origin of some of these species. It is clear that a comprehensive investigation of this event cannot be limited to the analysis of only chloroplast markers. Since chloroplast DNA is usually inherited as a single locus, the chloroplast DNA genealogy can be not congruent to the genealogy of a species. From here it seems reasonable to supplement the analysis of chloroplast DNA with the analysis of multilocus nuclear data and mitochondrial DNA. Previous biogeographic studies of coniferous species pointed to the great potential of the simultaneous sequence analysis of maternally inherited mitochondrial DNA, paternally

inherited chloroplast DNA, and the nuclear genome [for example, 8, 11, 12, 39, 42–45] in phylogenetic analysis at the low taxonomic level.

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