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Original Article

Genetic variation and DNA barcoding of the endangered agarwood-producing *Aquilaria beccariana* (Thymelaeaceae) populations from the Malesia Region

Malezya Bölgesinde nesli tükenmekte olan *Aquilaria beccariana* (Thymelaeaceae) popülasyonlarının genetik varyasyonu ve DNA barkodu

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ABSTRACT

The endangered agarwood-producing *Aquilaria beccariana* is reportedly secluded in the southern region of the Malay Peninsula (MPen) and more dispersed in the northern and central regions of the Borneo Island (Bor). The two are geographically separated by the South China Sea. Fresh leaf samples from 47 individuals were collected from six natural populations, including Mersing (MERS) of MPen; Upper Baram (BARA1), Marudi (BARA2), and Lawas (LAWA) of Sarawak; Tongod (TONG) of Sabah, and Kalimantan (KALI) of Indonesia, to study their phylogenetic relationship. Seven non-coding chloroplast DNA (cpDNA) regions and the nuclear internal transcribed spacer (ITS) region were amplified and sequenced using polymerase chain reaction (PCR). Several closely related *Aquilaria* species were included to demonstrate the molecular position of *A. beccariana*. Phylogenetic analysis, median-joining (MJ) network, and principal coordinate analysis (PCoA) assembled the six populations into two major clusters, MPen and Bor, when using the combined cpDNA dataset, whereas the Bor populations were further clustered into northern and central populations. DNA barcoding analysis using the combined *trnL–trn*F+ITS2 loci of 12 *Aquilaria* species revealed that species discrimination is possible for *A. beccariana* at both species and population levels. In conclusion, this work provides useful genetic information that may help in the management and conservation of agarwood resources.

Keywords: Chloroplast DNA, conservation genetics, gene fragment region

ÖZ

Nesli tükenmekte olan *Aquilaria beccariana*'nın Malay Yarımadası'nda (MPen) güney bölgesinde az rastlandığı ve Borneo Adası'nın (Bor) kuzey ve merkezi bölgelerinde de daha dağınık olarak bulunduğu bildirilmektedir. Güney Çin Denizi, coğrafi olarak ikiye ayrılmış bulunmaktadır. Filogenetik ilişkilerin incelenmesi için, altı doğal popülasyonda 47 bireyden yaprak örnekleri alınmıştır, bu gruplar Saravak'ın Yukarı Baram (BARA1), Marudi (BARA2) ve Lawas (LAWA); Sabah'dan Tongod (TONG) ve Endonezya'dan Kalimantan (KALI)'dır. Yedi kodlayıcı olmayan kloroplast DNA (cpDNA) bölgesi ve nükleer dahili kopyalanmış aralayıcı (ITS) bölgesi, polimeraz zincir reaksiyonu (PCR) kullanılarak güçlendirilmiş ve sıralanmıştır. *A. beccariana*'nın moleküler pozisyonunu göstermek için yakından ilişkili birkaç *Aquilaria* türü çalışmaya dahil edilmiştir. Filogenetik analiz, medyan birleştirme (MJ) ağı ve ana koordinat analizi (PCoA) altı popülasyonu iki ana kümede (MPen ve Bor) birleştirilmiştir, kombine cpDNA veri setini kullanılarak Bor popülasyonları ayrıca kuzey ve merkezi popülasyonlara kümelenmiştir. 12 *Aquilaria* türünün birleşik trnL – trnF + ITS2 lokusları kullanılarak yapılan DNA barkod analizi, hem tür hem de popülasyon düzeyinde *A. beccariana* için türleri ayırt etmenin mümkün olduğunu ortaya koymuştur. Sonuç olarak, bu çalışma *Aquilaria beccariana* kaynaklarının yönetimi ve korunmasında yardımcı olabilecek yararlı genetik bilgiler sağlamaktadır.

Anahtar Kelimeler: Kloroplast DNA, koruma genetiği, gen parçalanma bölgesi

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INTRODUCTION

Aquilaria (Thymelaeaceae), a tropical tree genus comprising 21 species, is widely distributed in the Indo-Malesian region (Lee and Mohamed, 2016a). *Aquilaria* species are generally known for their ability to produce the highly valued fragrant resin, agarwood, which is extensively used as a raw ingredient in perfumes, incense, and traditional medicine. Agarwood is traded in several assortments

and derivatives, such as wood pieces, wood chips, powder, and most importantly, essential oil (TRAFFIC East Asia-Taipei and TRAFFIC Southeast Asia, 2005; Mohamed and Lee, 2016). The market value of agarwood products is determined by the agarwood grading system, which is collectively dependent upon fragrance strength and longevity, resin content, geographic origin, and purity (Mohamed and Lee, 2016). However, scarcity of agarwood natural resources became intensified due to critical standard evaluation and increasingly demanding market, yielding a reduced supply in agarwood (Azren et al., 2019; Jensen and Meilby, 2008). The genus *Aquilaria* is presently listed under Appendix II of the Convention on International Trade in Endangered Species (CITES) over scrutinized international trades (UNEP-WCMC, 2019).

Studies on *A. beccariana* are relatively more limited compared with researches on its close relative *Aquilaria malaccensis*. The species is generally found in the Malesia region. Its natural populations in Malaysia are isolated in the southern region of the Malay Peninsula, although it is more widely spread in Borneo Island (Faridah-Hanum et al., 2009). *Aquilaria beccariana* was first discovered in Sarawak in 1893 by Odoardo Beccari, an Italian botanist, resulting in the epithet name "beccariana" (Tawan, 2004). Official herbarium records of the species were last reported in 1971 in Mersing, Johor; 1992 in Sabah; and 2005 in Sarawak (Faridah-Hanum et al. 2009). It is known to produce agarwood that is a livelihood source for the aboriginal people of Sarawak (Kanazawa, 2017). Several names are attributed to this species, such as *gaharu tanduk* (horned agarwood) or *engkaras* in Sarawak, whereas the Penan tribe identifies agarwood as *gaharu ba* (mountain agarwood) (Dawend et al., 2005; Kanazawa, 2017). Agarwood poachers were reported to be conducting destructive harvesting, such as indiscriminate tree felling, in search of agarwood, although agarwood from natural *A. beccariana* populations are sustainably being harvested by aboriginal people (Kanazawa, 2008; Newton and Soehartono, 2001). Not all *Aquilaria* trees in the wild produce agarwood (Barden et al., 2000). Agarwood formation involves a specific defense mechanism from the tree, protecting itself against pathogen infestation in its exposed tree stem wounds (Rasool and Mohamed, 2016). The regeneration rate of wild *Aquilaria* trees is low and inconsistent (Devi et al., 2019; Soehartono and Newton, 2001) due to the over-exploitation of this species, and *A. beccarriana* population is drastically declining over the years (Soehartono and Newton, 2001). Conservation efforts are deemed requisite to ensure the continued survival of this tree species in the wild, although cultivation attempts for sustainable agarwood production using *A. beccariana* have been reported in East Kalimantan, Indonesia (Soehartono and Newton, 2000; Turjaman and Hidayat, 2017).

Interspecific genetic variations of *Aquilaria* and *Gyrinops* species, including *A. beccariana*, using the amplified fragment-length polymorphism method indicated a high genetic variation among the *Aquilaria* species (Toruan-mathius et al., 2009). This is further supported when analyzed using several chloroplast DNA (cpDNA) regions with additional species from the Aquilarieae tribe (Farah et al., 2018). Other studies have reported on molecular-based identification of *A. beccariana,* such as from using sequence-characterized amplified region markers (Roslan et al., 2017) and the barcode DNA-high resolution melting (Bar-HRM) technique (Lee et al., 2019), but not the sequence-based DNA barcoding technique. The latter is useful for species discrimination because agarwood is commonly traded in wood or product forms, whereas *Aquilaria* species identification is normally facilitated by its flower and fruit (Tawan, 2004; Lee et al., 2016a). CITES has suggested improvements in the identification method for effective control of international agarwood trade (Barden, et al. 2000). Conventional identification methods, such as through wood anatomy, unfortunately have not identified agarwood at the species level (Gasson, 2011), whereas the presence of counterfeits with similar wood anatomy features and texture to the *Aquilaria* spp. has made agarwood identification a challenging task (Yin et al., 2016). Therefore, using effective molecular markers, such as from DNA barcoding techniques, could provide a rapid, accurate, and automatable agarwood species identification method at the species level (Hebert and Gregory, 2005). On another note, further investigation on their intraspecific genetic diversity can provide supporting information toward promoting its genetic conservation.

Natural populations of *A. beccariana* are confined to limited areas in the Malesia region, and information on their population genetics is scant. In this study, we used sequence data for seven non-coding cpDNA regions (*psb*B-*psb*H, *psb*C-*trn*S, *rps*16-*trn*K, *trn*L-*trn*F, *rps16-trn*Q, *trn*F-*ndh*J, and *trn*L intron) and the internal transcribed spacer (ITS) region to estimate the level of intraspecific variations of natural *A. beccariana* within and among the populations from the Malay Peninsula (MPen) and several parts of Borneo Island (Bor). In addition, we barcoded *A. beccariana*. The addition of *A. beccariana* in the DNA barcoding database serves as a useful agarwood identification reference, specifically as a tool in monitoring illegal agarwood trade and adulteration.

MATERIALS AND METHODS

Fresh leaf samples were collected from 47 individuals out of the six different *A. beccariana* natural populations. Among them, 12 individuals were from Mersing (MERS), Johor in the MPen; 12 individuals each were from both Upper Baram (BARA1) and Marudi (BARA2), and six individuals were from Lawas (LAWA), Sarawak; three individuals were from Tongod (TONG), Sabah; and two individuals were from Kalimantan (KALI) of Indonesia (Figure 1). The Kalimantan samples were donated by Dr. Maman Turjaman from the Forestry and Environmental Research Development and Innovation Agency, Bogor, Indonesia. The species grows well by the river banks, hills, and rocky areas. *Aquilaria beccariana* can grow up to 20 m in height and has a whitish pale, smooth bark. The leaf is generally large in size, oblong to elliptic in shape, curving and ascending toward the margin, raised above, and prominent below. The flower is 5-merous, produced in umbel, sparsely hairy, and green-to-yellowish in color. The fruit is a loculicidal capsule that narrows toward the base into an elongated stalk and is protruding from top of the calyx tube (Forestry Department Peninsular Malaysia, 2015; Kanazawa,

2017). *A. beccariana* produces a higher number of flowers per inflorescence per receptacle when compared with other *Aquilaria* species (Soehartono and Newton, 2000). The leaf samples were kept in aluminum Ziplock bags and stored at a temperature of -80°C prior to DNA extraction. The voucher specimens were kept in the Forest Biotechnology Laboratory, Faculty of Forestry, Universiti Putra Malaysia, for future references.

A total of 5 g fresh leaf sample was pulverized in liquid nitrogen using a mortar and pestle. The total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, USA), following the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications for the seven cpDNA loci and ITS (Table S1) were carried out using a MyCycler™ thermal cycler (BioRad, USA). Each PCR reaction had a total volume of 25 µL, containing 12.5 µL of 2X PCRBIO Taq Mix Red (PCR Biosystems, UK), 0.4 µM each of forward and reverse primers, and 25 ng of DNA template. The PCR condition was programmed as follows: initial denaturation step at 95°C for 1 min, followed by 40 cycles at 94°C for 15 s, T_a, depending on the primer used, then at 72°C for 1 min, and the final extension step at 72°C for 3 min (Table S1). Other than primer *rps16-trnK*, the annealing time (t_a) was determined, following the manufacturer's instructions. The PCR products were separated on 1.0% agarose gel, stained with ethidium bromide, viewed under UV light, and then sent for direct sequencing for both strands. Sequencing was performed in a commercial service facility (1st BASE Laboratory Sdn. Bhd., Seri Kembangan, Malaysia) using an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystem, USA).

The DNA sequences were manually edited, visually inspected, and adjusted using MEGA 7 (Kumar et al., 2016). All sequences obtained from this study were deposited into the NCBI GenBank (Table 1). The seven cpDNA sequences were concatenated to form a combined dataset in the order of *psb*B-*psb*H, *psb*C-*trn*S, *rps*16-*trn*K, *trn*L-*trn*F, *rps*16-*trn*Q, *trn*F-*ndh*J, and *trn*L intron. The combined cpDNA datasets were later aligned through the Multiple Alignment using Fast Fourier Transform online program (MAFFT) (Katoh et al., 2017) and saved in FASTA format, and the indel and substitution number was calculated using MEGA 7 (Kumar et al., 2016).

For genetic distance analysis, the inter- and intraspecific pairwise distances were calculated based on the Kimura two-parameter (K2P) model (Kimura, 1980) across all *A. beccariana* individuals of the same population and among populations using MEGA 7, and the gaps and missing data were excluded from the analysis. For the phylogenetic tree analysis, the suitable DNA substitution models were separately analyzed using the "find best DNA/Protein model (ML)" function embedded in MEGA 7 for both the combined cpDNA dataset and ITS. The best model that fitted the combined cpDNA dataset and ITS was based on the Tamura three-parameter (T92) model and gamma-distributed (+G) (=T92+G) according to the estimated values of all parameters for each model. The phylogenetic trees were constructed based on the maximum likelihood (ML) criterion implemented in MEGA 7. A total of 1,000 bootstrap replicates were conducted to assess the relative support for the branches, and the gaps and missing data were treated as complete deletions in the analysis. For the combined cpDNA dataset, the gene sequences of the respective loci were extracted from the chloroplast genomes of *A. crassna* (MK779998), *A. malaccensis* (MH286934), *A. sinensis* (KT148967), *A. yunnanensis* (MG656407), and *Gonystylus bancanus* (EU849490) by fishing out sequences complement to *A. beccariana* sequences. The sequences were cut, manually inspected, and trimmed accordingly. For the ITS, the sequences from 10 *Aquilaria* species, namely *A. agallocha* (MH134137), *A crassna* (MH134149), *A. cumingiana* (MH134140), *A. malaccensis* (MH134142), *A. microcarpa* (MH134143), *A. rostrata* (MH134144),

Table 1. Voucher details, localities, and GenBank accession numbers of the Aquilaria beccariana specimens generated and used in this study

Collector's name	Collection number	Region of specimen origin (number of specimens sequenced)	Genbank accession number							
			*psb*B–*psb*H	*psb*C–*trn*S	*trn*K–*rps16*	*trn*L–*trn*F	*trn*Q–*rps16*	*trn*F–*ndh*J	*trn*L intron	ITS
Lee & Mohamed[c]	FBL04006-FBL04007	Mersing-MERS, Johor (12)	MK603075	MK603081	MK603100	MK603093	MK603106	MK787457	MK787462	MK603111
Pern & Mohamed[c]	FBL04008-FBL04010	Upper Baram-BARA1, Sarawak (12)	MK603076	MK603082	MK603101	MK603094	MK603107	MK787458	MK787463	MK603112
Pern & Mohamed[c]	FBL04011-FBL04013	Marudi-BARA2, Sarawak (12)	MK603076	MK603082	MK603101	MK603095	MK603107	MK787458	MK787463	MK603112
Pern & Mohamed[c]	FBL04014	Lawas-LAWA, Sarawak (6)	MK603077	MK603083	MK603102	MK603096	MK603108	MK787459	MK787464	MK603112
Pern & Mohamed[c]	FBL04015-FBL04017	Tongod-TONG, Sabah (3)	MK603078	MK603084	MK603103	MK603097	MK603109	MK787460	MK787465	MK603112
Lee & Mohamed	FBL04001	Kalimantan-KALI, Indonesia (2)	MK603079	MK603085	MK603104	MK603098	MK603110	MK787461	MK787466	MK603112

Figure 1. Distribution of the five *Aquilaria beccariana* populations (indicated with black circles) from Malaysia used in this study. The five *Aquilaria beccariana* populations were BARA1 (Upper Baram), BARA2 (Marudi, Baram), LAWA (Lawas), MERS (Mersing), and TONG (Tongod)

Table 2. Interspecific pairwise distances of sequences among different Aquilaria beccariana populations used in this study

combined cpDNA dataset	MERS	BARA1	BARA2	LAWA	TONG
BARA1	0.0011	-	-	-	-
BARA2	0.0011	0.000	-	-	-
LAWA	0.0011	0.000	0.000	-	-
TONG	0.0011	0.000	0.000	0.000	-
KALI	0.0031	0.0024	0.0024	0.0024	0.0024
ITS	**MERS**	**BARA1**	**BARA2**	**LAWA**	**TONG**
BARA1	0.0180	-	-	-	-
BARA2	0.0180	0.0000	-	-	-
LAWA	0.0180	0.0000	0.0000	-	-
TONG	0.0180	0.0000	0.0000	0.0000	-
KALI	0.0180	0.0000	0.0000	0.0000	0.0000

MERS: Mersing, Johor; BARA1: Upper Baram, Sarawak; BARA2: Marudi, Baram, Sarawak; LAWA: Lawas, Sarawak; TONG: Tongod, Sabah; KALI: Kalimantan, Indonesia

A. rugosa (MH134145), *A. sinensis* (MH134146), *A. subintegra* (MH134147), *A. yunnanensis* (MH134148), and the outgroup *G. bancanus* (MH134152) were extracted from the Genbank.

For population genetic analyses, median-joining (MJ) network was constructed using the Network V5.0 program (Fluxus Technology, UK) and default settings. Nucleotide sequence data were converted to a compatible format using DnaSP V5 (Librado and Rozas, 2009) and then manually rearranged. *Aquilaria malaccensis* served as the outgroup for both MJ network analyses. Principal coordinate analysis (PCoA) was performed using the GeneAlEx V6.5 software (Peakall and Smouse, 2006). Both the cpDNA combined dataset and ITS sequences were numerically coded as follows: A=1, T=2, C=3, G=4, and gap/missing data were coded as 0. Separate calculations were carried out for both datasets using the Eigenvector method. Sequences of *A. malaccensis* were included as the outgroup for both PCoA analyses.

The combined loci, *trn*L-*trn*F+ITS2, was applied in DNA barcoding analysis as it is the proposed optimal DNA barcode for *Aquilaria* (Lee et al., 2016a). For DNA barcoding analysis, the DNA sequences from *A. beccariana* and from 11 *Aquilaria* species (*A. agallocha*, *A. crassna*, *A. cumingiana*, *A. hirta*, *A. malaccensis*, *A. microcarpa*, *A. rostrata*, *A. rugosa*, *A. sinensis*, *A. subintegra*, and *A. yunnanensis*), as well as from the two outgroup species (*G. bancanus* and *Gyrinops versteegii*), were downloaded from the NCBI GenBank (Lee et al., 2016a; Farah et al. 2018). The sequences were prepared in a concatenate form of *trn*L-*trn*F+ITS2, then aligned using ClustalW embedded in MEGA 7. A neighbor-joining (NJ) tree with 1,000 bootstrap replicates was constructed using the Kimura two-parameter (K2P) substitution model, and all positions containing gaps and missing data were removed from the analysis (complete deletion).

RESULTS AND DISCUSSION

Amplification, Sequencing, and Distance Threshold Analyses

A total of 376 sequences were generated, and 44 of them were deposited in the GenBank (Table 1). In the order of *psb*B-*psb*H, *psb*C-*trn*S, *rps*16-*trn*K, *trn*L-*trn*F, *rps*16-*trn*Q, *trn*F-*ndh*J, *trn*L intron and ITS, the fragment lengths for *A. beccariana* (without primer sequences) prior to alignment were 585, 775-782, 519, 460-469, 1,143-1144, 534, 575, and 750 bp, respectively, whereas the aligned lengths were 585, 784, 461, 519, 1,145, 534, 575, and 880 bp, respectively. Furthermore, 23 polymorphic sites were detected in the concatenated length of the cpDNA fragments with a final length of 4,606 bp, and 13 polymorphic sites were observed in the ITS fragment. The interspecific pairwise distance among the six *A. beccariana* populations from the combined cpDNA dataset ranged from 0.000 to 0.031; whereas the ITS ranged from 0.000 to 0.180 (Table 2). There was no intraspecific pairwise distance vari-

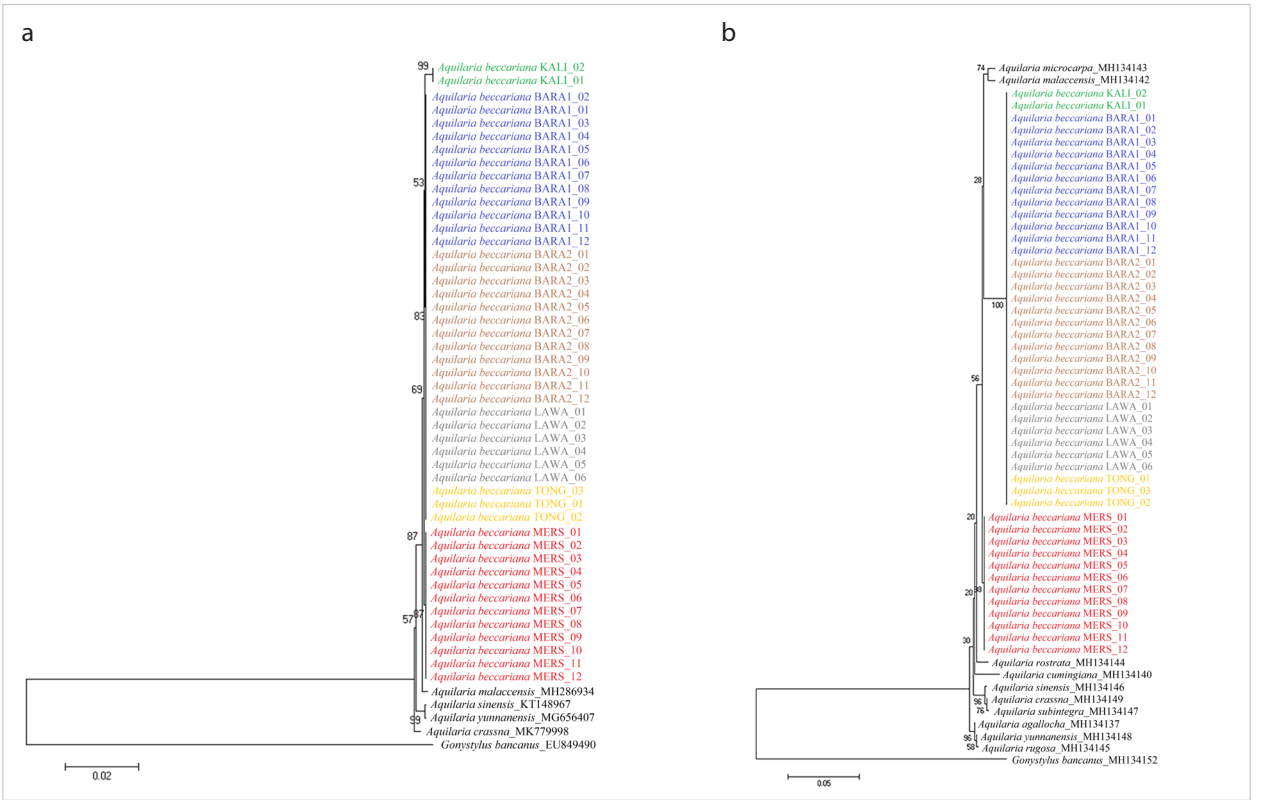

Figure 2. a, b. Maximum likelihood (ML) tree of six *Aquilaria beccariana* natural populations based on (a) a combined dataset of seven non-coding chloroplast DNA (cpDNA) regions (*psb*B–*psb*H, *psb*C–*trn*S, *rps16*–*trn*K, *trn*L–*trn*F, *rps16*–*trn*Q, *trn*F–*ndh*J, and *trn*L intron); and (b) the nuclear DNA internal transcribed spacer (ITS) region. *Aquilaria beccariana* populations were: BARA1 (Upper Baram), BARA2 (Marudi, Baram), KALI (Kalimantan), LAWA (Lawas), MERS (Mersing), and TONG (Tongod). The sequencing data obtained from this study have been deposited in the Genbank (accession number in Table 1), whereas those downloaded from the Genbank were indicated at the end of each species name. ML calculation was based on Tamura three-parameter (T92) model and gamma distributed (+G) (=T92+G). The gaps and missing data were exempted from the analysis (complete deletion). *Gonystylus bancanus* serves as the outgroup. Bootstrap values (1,000 replicates) are indicated on the corresponding nodes

ation detected within the *A. beccariana* populations in both the combined cpDNA and ITS fragments (data not shown).

Phylogenetic Relationship

The ML tree constructed from the combined cpDNA dataset (Figure 2a) revealed that *A. beccariana* populations BARA1, BARA2, LAWA, and TONG formed a cluster (bootstrap support=85%), which is related to KALI (53%), whereas the MERS population appeared to branch out, although only with moderate bootstrap support (57%). In addition, it shows that the *A. beccariana* clade is closely related to *A. malaccensis* (85%). Interestingly, the ITS ML tree (Figure 2b) showed all five *A. beccariana* populations (BARA1, BARA2, LAWA, TONG, and KALI) clustered in the same clade (100%). This clade became a sister to the *A. malaccensis*+*A. microcarpa* clade. Moreover, the MERS population branched from the rest of the *A. beccariana* populations (50%).

Population Grouping and Haplotype Aggregation

The MJ network revealed the genealogical relationships among haplotypes in *A. beccariana* and *A. malaccensis*. There were three *A. beccariana* haplotypes that were distributed into two divergent lineages, which were separated by four mutation steps when using the combined cpDNA (Figure 3a), or by 14 mutation steps when using ITS (Figure 3b). The nucleotide substitutions and indel variations disclosed three different haplotypes when using the combined cpDNA dataset. Hap1 consisted of a MERS-specific haplotype, whereas Hap2 is a haplotype commonly found in BARA1, BARA2, LAWA, and TONG. Hap3 consisted of a KALI-specific haplotype. For the ITS, Hap1 consisted of MERS-specific haplotype (MPen), and Hap2 is a haplotype commonly found in BARA1, BARA2, KALI, LAWA, and TONG (Bor). The MJ analyses were supported by PCoA, which clearly showed that *A. beccariana* populations were clustered into three and two distinct groups, respectively. For cpDNA, BARA1, BARA2, LAWA, and TONG were grouped together, whereas MERS and KALI each formed a group of their own (Figure 4a). For ITS, BARA1, BARA2, KALI, LAWA, and TONG were grouped together and separated from MERS (Figure 4b).

Species Identification through DNA Barcoding

The NJ tree showed *A. beccariana* as a distinct clade from oth-

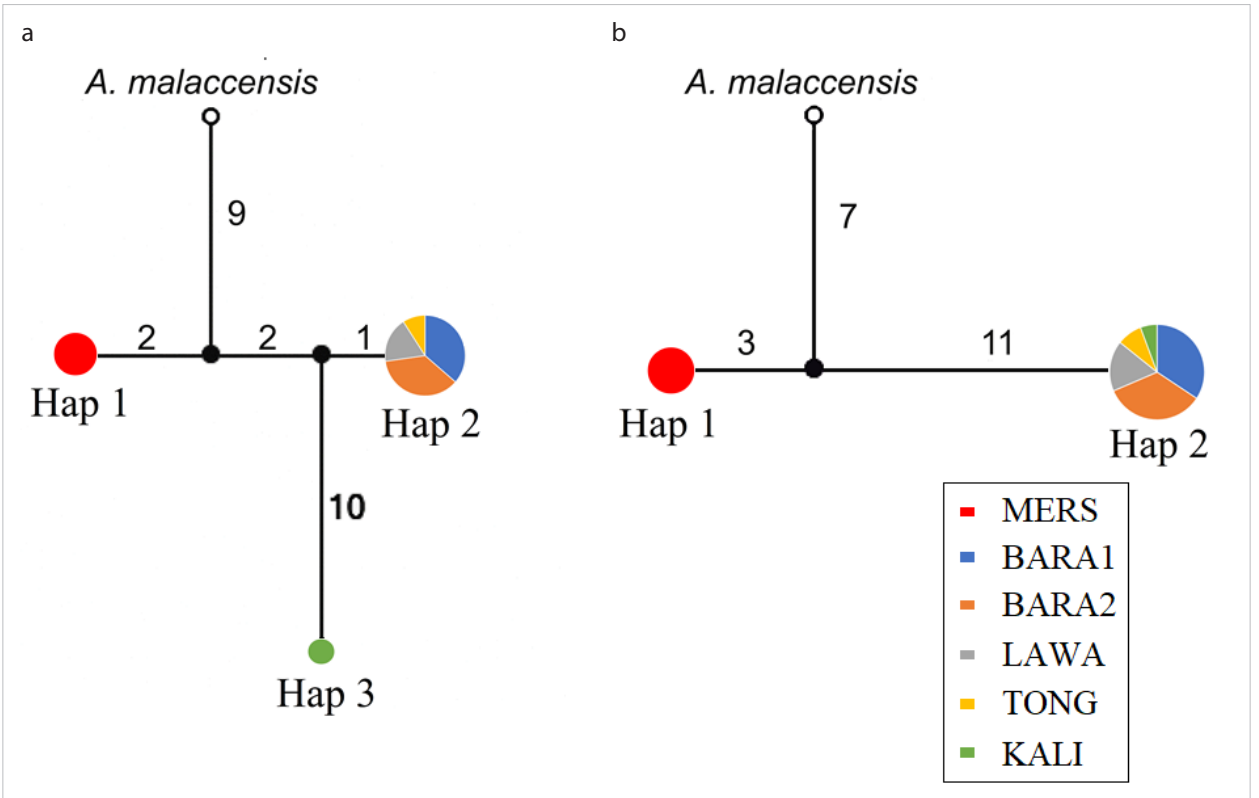

Figure 3. a, b. Median-joining (MJ) network constructed for six *Aquilaria beccariana* populations using (a) the combined cpDNA dataset and (b) ITS sequences. The circles represent haplotype samples of *A. beccariana* (Hap1–Hap3), and the size of the circle is proportionate to the frequency of each sampled haplotype. The black node on branches indicates the median vector. Values above the lines indicate the mutation steps separating adjacent haplotypes and outgroup

er *Aquilaria* species (with a bootstrap support of 60%) at the branch node of *A. malaccensis*+*A. microcarpa* clade (Figure 5). At the population level, *A. beccariana* appeared to segregate into three clades, namely Clade I (MERS), Clade II (KALI), and Clade III (BARA1+BARA2+LAWA+TONG). Clades II and III were separated under a strong bootstrap support (99%).

Depleting forest genetic resources is a global issue because of rapid urbanization and ecosystem encroachment by humans. A conservation strategy is indispensable for maintaining the genetic diversity of forest trees. This is especially true for the case of the valuable agarwood-producing tree species, such as *A. beccariana*. To establish a working conservation plan, prior information about each potential population that needs to be conserved has to be analyzed and documented properly for priority setting, which is a pre-requisite for sustainable resource supply mechanism (Newton et al., 2013). The species distribution for the five *Aquilaria* species native to Malaysia, namely *A. beccariana, A. hirta, A. malaccensis, A. microcarpa,* and *A. rostrata*, has been recorded elsewhere (Faridah-Hanum et al., 2009). The voucher records for *A. malaccensis* are abundant in the MPen, whereas *A. microcarpa* has greater specimen records in Sarawak and Sabah. The records for *A. hirta* is confined to regions on the east coast of the MPen. Moreover, *A. beccariana* has greater records in the northern region of Sarawak, whereas the endemic species with the least record, *A. rostrata*, has only three records in the MPen region (Faridah-Hanum et al., 2009; Lee and Mohamed, 2016b).

To our knowledge, this is the first report on the genetic variations of *A. beccariana* using gene-based markers at the population level. A total of 3 haplotypes and 2 haplotypes were identified from 15 and 14 variable sites in the combined cpDNA dataset and ITS sequences, respectively. The ML tree findings were congruent to those in the MJ network and PCoA. In the MJ network for the combined cpDNA dataset, the most common haplotype, Hap2, was found solely in the East Malaysia region (Sabah and Sarawak), whereas the remaining haplotypes, Hap1 and Hap3, were populations specific to the MPen and Kalimantan, respectively. In the MJ network for ITS sequences, the two haplotypes were grouped in a manner where Hap1 was from the MPen while Hap2 was from Bor. The PCoA genetic structural analysis revealed three and two genetically diverse pools where the clustering patterns are similar to what was reported in the MJ networks. A comparable study on *A. malaccensis* using the combined seven cpDNA datasets (*trn*K-*rps*16, *ycf*3-3-2, *psb*B-*psb*H, *rpl*16-2-1, *psb*J-*pet*A, *ndh*J-*trn*F, and *trn*Q-*rps*16) revealed a total of 29 haplotypes from 35 natural populations in MPen (Lee et al., 2016b). Several populations were detected with multiple haplotypes; however, intraspecific variation was not detected when using the ITS data (Lee et al., 2018a).

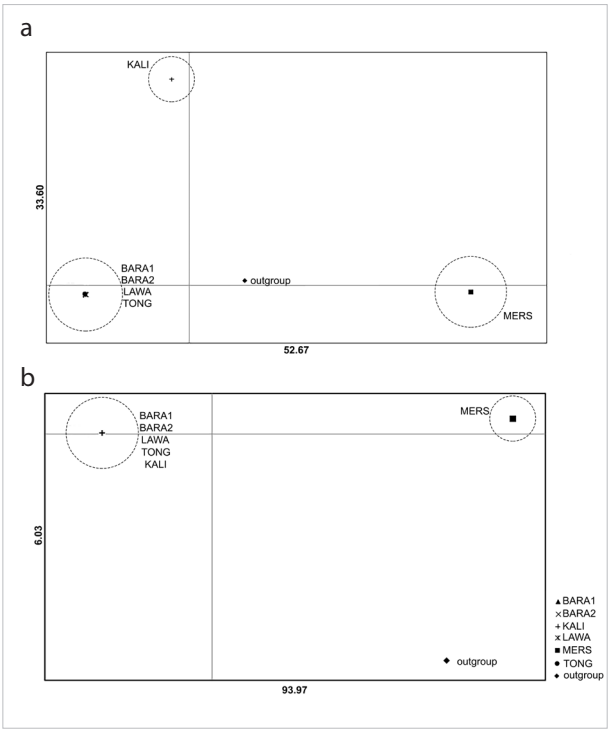

Figure 4. a, b. Principal coordinate analysis (PCoA) of six *Aquilaria beccariana* populations based on the (a) combined cpDNA dataset and (b) ITS sequences. The distinct clusters are in circles

The predominantly uniparentally inherited cpDNA in most angiosperms is more conserved and has a slower evolution rate compared with the biparentally inherited nuclear ribosomal DNA (Wolfe et al., 1987). The cp genome often displays evolutionary conservatism that result in little or no intraspecific cpDNA variability in most plants, although the interspecific cpDNA variation is common among closely related species (Lee and Wen, 2004; Rajora and Dancik, 1995). Interspecific variations are expected when the cp gene loci have evolved independently, which have diverged from a common ancestor (Kress et al., 2005). In this study, low cpDNA intraspecific divergence was observed among the *A. beccariana* populations (Table 2, Figure 2a), suggesting that the low within-population diversity may be caused by the limited gene flow among populations in *A. beccariana*. The genetic differentiation in *A. beccariana* may be due to geographic barriers that are known factors affecting the population structure of trees (Salvador-Figueroa et al., 2015). Good examples of *Aquilaria* species with affected population structures due to geographic barriers are the *A. malaccensis* from MPen and *A. sinensis* from China (Jia et al., 2010; Lee et al., 2016b; Lee et al., 2018a; Lee et al., 2018b; Zou et al., 2012). These geographic limits can be in the form of great distance separated by water or presence of highly elevated mountain ranges that occur in earlier time, which caused geographic separation. *A. beccariana* is naturally confined to lower altitudes and rarely at higher altitudes (200-1000 m) as seed dispersal in *Aquilaria* relies heavily on wind and small insects (Faridah-Hanum et al., 2009; Tawan, 2004). Poor seed dispersal, genetic drift, and local

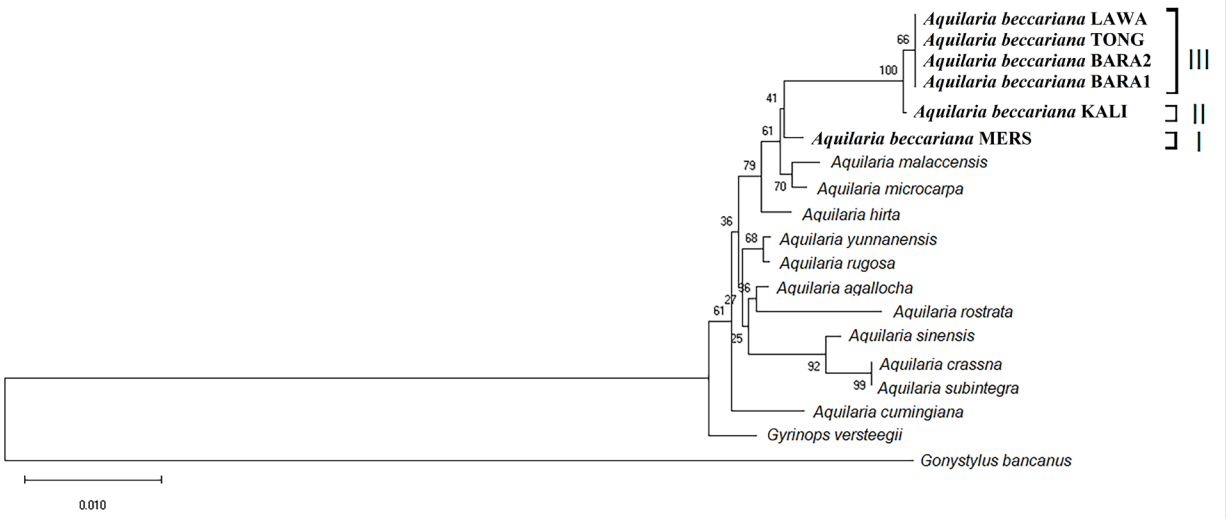

Figure 5. Neighbor-joining (NJ) tree constructed from the proposed DNA barcode for *Aquilaria*, *trn*L–*trn*F+ITS2. Calculation was based on the Kimura two-paramater (K2P) DNA substitution model using 1,000 bootstrap replicates, and the gaps and missing data were removed from the analysis (complete deletion). Bootstrap values are indicated on each corresponding nodes. Gyrinops versteegii and Gonystylus bancanus serve as the outgroups. Reference species and Genbank accession numbers of the *trn*L–*trn*F and ITS2 sequences that were used are as follows, respectively: *A. agallocha* (MF443428, MH134137), *A. crassna* (KU244030, KU244108), *A. cumingiana* (KT726320, MH134140), *A. hirta* (KU244034, KU244112), *A. malaccensis* (KU244037, KU244115), *A. microcarpa* (KU244040, KU244118), *A. rostrata* (KT364475, MH134144), *A. rugosa* (MF443430, MH134144), *A. sinensis* (KU244046, KU244124), *A. subintegra* (KU244049, KU244127), *A. yunnanensis* (KU244051, KU244129), *G. versteegii* (KU244054, KU244132), and *G. bancanus* (KU244055, KU244133)

adaptation via genotype through environmental interactions can be the contributing factors toward highly conserved intraspecific genetic variations among *A. beccariana* species (Zou et al., 2012).

Habitat fragmentation was presumably responsible for the decreased local population, resulting in regional extinction of most endangered species that exists in small population sizes or in confined areas (Szczecińska et al., 2016). Agricultural development has increased the conversion of land use to crop planting. Based on our own field experience and personal communication with several natural agarwood collectors and local residents living nearby forest edges, the decrease in *A. beccariana* natural populations in easily accessible regions is likely caused by land conversion to agricultural plantations, whereas the population decrement in remote regions is likely due to destructive harvesting methods by irresponsible agarwood poachers rather than habitat fragmentation. As agarwood trade provides a lucrative income for several underprivileged communities, especially the Penan tribe in Sarawak, agarwood hunters from the Penan tribe practice sustainable harvesting by only collecting the tree parts that contain agarwood and at the same time preserving the habitat for the next generation (Kanazawa, 2017), whereas agarwood poachers often practice indiscriminate felling of mother trees in search of agarwood (Wyn and Awang Anak, 2010).

In recent years, the DNA barcoding database for *Aquilaria* has been expanded to include nearly a dozen candidate barcode loci and up to seven species (Jiao et al., 2014; Lee et al., 2016a). However, the existing database has not expanded species-wise although there are new updates using different candidate barcode loci (Feng et al., 2019; Li et al., 2018; Thitikornpong et al., 2018; Tanaka and Ito, 2019). We asserted that the new DNA barcoding analysis for *Aquilaria* species presented in this study has brought it to a new height, whereby a total of 12 *Aquilaria* species were included. Using *trn*L-*trn*F+ITS2 to construct the NJ tree, we successfully distinguished *A. beccariana* into its own clade. However, the inclusion of five *Aquilaria* species (*A. agallocha*, *A. beccariana*, *A. cumingiana*, *A. rostrata*, and *A. rugosa*) lowered the overall bootstrap values when compared with a previous report (Lee et al., 2016a). The variable site reduction across these closely related *Aquilaria* species led to a high percentage of conserved region in the DNA barcode loci. Species discrimination in *Aquilaria* was possible at a lower bootstrap support value at its current state; however, we suggest to reconduct DNA barcoding analysis with new candidate DNA barcode loci and to include more taxa. There is no guarantee that the increased number of gene sequences or taxa will promise an accurate phylogenetic reconstruction (Hedtke et al., 2006). Alternatively, highly variable sites can be identified from the chloroplast genomes of various *Aquilaria* species with the advent of sequencing technologies. Such approach was proven effective in developing DNA barcodes powerful enough to discriminate *Pterocarpus* species with high morphological and anatomical similarities from the genus (Jiao et al., 2019).

CONCLUSION

Genetic information on *A. beccariana* is generally lacking. This study has improved our understanding on the genetic variation across *A. beccariana* populations in the Malesia region. Conservation efforts should be carried out with haste, because the depletion of *A. beccariana* natural stands has threatened its survival in the wild. Furthermore, their genetic differences exist although they are confined to limited areas in the Malesia region, leading to great challenges in conserving their natural habitat. Further genetic studies, such as this, should be a priority for *Aquilaria* conservation.

Ethics Committee Approval: This study does not contain any studies performed on human or animal participants by any of the authors. Therefore, ethics committee approval was not necessary.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - S.Y.L., R.M.; Design - S.Y.L., R.M.; Supervision - N.K., R.M.; Resources - R.M.; Data Collection and/or Processing - Y.C.P.; Analysis and/or Interpretation - Y.C.P., S.Y.L.; Literature Search - Y.C.P.; Writing Manuscript - Y.C.P.; Critical Review - N.K., R.M.

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Table S1. Details on the primers used in this study

Gene Locus	Primer name	Sequence direction	Primer sequence (5′-3′)	Annealing temperature, Ta (°C)	Annealing time, ta (s)	Expected size (bp)	References
*psb*B–*psb*H	*psb*B	Forward	GTTTACTTTTGGGCATGCTTCG	58	15	850	Hamilton, 1999
	*psb*H	Reverse	CGCAGTTCGTCTTGGACCAG				Hamilton, 1999
*psb*C–*trn*S	CS2_F	Forward	GTTTTACGGCCCACTGGAC	55	15	900	Farah et al. 2018
	*trn*S	Reverse	GGTTCGAATCCCTCTCTCTC				Demesure et al. 1995
*rps*16-*trn*K	*rpS*16×2F2	Forward	AAAGTGGGTTTTTATGATCC	58	75	850	Shaw et al. 2007
	*trn*K(UUU)×1	Reverse	TTAAAAGCCGAGTACTCTACC				Shaw et al. 2007
*trn*L–*trn*F	e	Forward	GGTTCAAGTCCCTCTATCCC	55	15	500	Taberlet et al. 1991
	f	Reverse	ATTTGAACTGGTGACACGAG				Taberlet et al. 1991
*rps*16-*trn*Q	*rpS*16×1	Forward	GTTGCTTTYTACCACATCGTTT	55	15	1400	Shaw et al. 2007
	*trn*Q(UUG)	Reverse	GCGTGGCCAAGYGGTAAGGC				Shaw et al. 2007
*trn*F–*ndh*J	*trn*F	Forward	CTCGTGTCACCAGTTCAAAT	52	15	600	Dumolin-Lapegue et al. 1997
	*ndh*J-r	Reverse	TTTTYATGAAATACAAGATGCTC				Heinze, 2007
*trn*L intron	c	Forward	CGAATCGGTAGACGCTACG	50	15	600	Taberlet et al. 1991
	d	Reverse	GGGGATAGAGGGACTTGAAC				Taberlet et al. 1991
ITS	ITS-p5F	Forward	CCTTATCAYTTAGAGGAAGGAG	55	15	750	Cheng et al. 2016
	S3R	Reverse	GACGCTTCTCCAGACTACAAT				Chen et al. 2010

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Short Communication

Shoot structure variation in latitudinal and longitudinal ecotypes of *Pinus sibirica* in a common garden experiment

Bir bahçe deneyiminde *Pinus sibirica*'nın enlem ve boylam ekotiplerinde aşı kalemi yapısı değişimleri

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ABSTRACT

Pinus sibirica Du Tour is one of the primary forest species in Russia. This work aimed to determine the variation in growth and elementary structure of shoots in latitudinal and longitudinal ecotypes using a common garden experiment, with emphasis on the ratio between shoot metamere types. The study was conducted in the southern taiga ecoregion in the south-east of the West Siberian Plain in Russia. We investigated 23-year-old grafts from eight *Pinus sibirica* ecotypes grown under uniform conditions. Ten-year-old branches were selected from the crowns of the grafted trees, and a retrospective analysis of shoot structure was conducted. The length and metameric composition of the branches varied significantly between different latitudinal and longitudinal ecotypes. Growth rates in the southern and eastern ecotypes were higher than those in the northern and western ecotypes. However, the proportions of auxiblasts did not differ between ecotypes. The southern ecotype showed the largest shoot sterile zone and the longest internodes and needles. The eastern ecotype showed the largest number of brachyblasts and relatively long internodes. Thus, the proportions of metameres involved in shoot elongation and internode length depend on the geographic origin of ecotypes when growing their vegetative progeny *ex situ*

Keywords: Adaptive traits, boreal conifers, grafts, intraspecies variation, shoot metameres

ÖZ

Pinus sibirica Du Tour, Rusya'daki başlıca orman ağacı türlerinden bir tanesidir. Bu çalışma, ortak bir bahçe deneyi kullanarak enlem ve boylam ekotiplerinde sürgünlerin büyüme ve temel yapısındaki değişimlerin, sürgün metamere türleri oranına vurgu yaparak belirlenmesi amaçlanmıştır. Çalışma, Rusya'daki Batı Sibirya Ovası'nın güney doğusundaki güney tayga ekolojik bölgesinde gerçekleştirilmiştir. Eşit şartlar altında yetiştirilen sekiz *Pinus sibirica* ekotipinden 23 yaşındaki aşı kalemleri araştırılmıştır. Aşılanmış ağaçların taçlarından on yaşındaki dallar seçilerek ve sürgün yapısında geriye dönük analizler yapılmıştır. Dalların uzunluğu ve metamerik bileşimleri, farklı enlem ve boylam ekotipleri arasında önemli farklılıklar gösterdiği saptanmıştır. Güney ve doğu ekotiplerindeki büyüme oranlarının, kuzey ve batı ekotiplerindeki büyüme oranlarından daha yüksek olduğu tespit edilmiştir. Bununla birlikte, oksiblast oranlarında ekotipler arasında farklılık bulunmamıştır. Güney bölgesi ekotipi en büyük sürgün steril bölgesini ve en uzun internod ve iğne yapraklarına sahip olduğunu göstermiştir. Doğu bölgesi ekotipi ise, en fazla sayıda brakilast ve nispeten daha uzun internodlara sahip olduğu görülmüştür. Bu nedenle, sürgün uzaması ve internod uzunluğuna dahil olan metamerlerin oranları vejetatif sürgünleri doğal gelişmenin üzerinde büyüdüğü görülürken, bu durumun ekotiplerin coğrafi kökenine bağlı bulunduğu sonucuna varılmıştır.

Anahtar Kelimeler: Uyarlanabilir özellikler, kuzey bölgesi iğne yapraklı ağaçları, aşı kalemi, tür içi değişim, sürgün metamerleri

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INTRODUCTION

Widely distributed tree species show intraspecific variation associated with their adaptation to specific environments in different parts of their range. Intraspecific variation in forest trees most often manifests itself in significant variation, between populations, in growth rate and duration, flowering time, and crown size and structure.

Table 1. Location and geographic characteristics of the eight Pinus sibirica ecotypes and study area in Russia

Region of ecotype origin	Latitude, N	Longitude, E	Elevation(m, asl)	Vegetation zone (according to Kurnaev, 1973)	Parental tree age (years)
Latitudinal ecotypes					
1. Abaza	52°30′	90°05′	350	Southern taiga	100
2. Strezhevoy	60°45′	77°30′	40	Northern part of middle taiga	140
3. Noyabrsk	63°10′	75°20′	110	Southern part of northern taiga	160–210
4. Urengoy	65°50′	78°10′	40	Forest and tundra	100–170
Longitudinal ecotypes					
5. Nevyansk	57°15′	60°10′	300	Subtaiga	120
6. Tayshet	55°50′	98°00′	350	Southern taiga	160
7. Sludyanka	51°30′	103°40′	900	Low part of forest belt	200
8. Severobaikalsk	55°40′	109°25′	700	Low part of forest belt	180
Study area					
Tomsk region	56°13'	84°51'	80	Southern taiga	–

Common garden studies have confirmed the inheritance of geographical variation in conifers (Sáenz-Romero et al., 2019). These studies distinguish genetic variation and modification in trees from different provenances, thereby revealing general patterns in growth variation for different coniferous species. Common garden experiments on individuals from environments with harsh climatic conditions have shown that they have a shorter period of seasonal growth (Andersson Gull et al., 2018), fewer frost injuries (Malmqvist et al., 2017), a slower growth rate (Vitasse et al., 2009), and a lower trunk height (Nagamitsu et al., 2018) than trees from a relatively mild climate. The level of species variation depends on the distance between the origin of the species and the location of the experiment, the climatic heterogeneity of the species range (Ye and Jayawickrama, 2014), and genetic variation in the species (Martínez-Berdeja et al., 2019). Notably, adaptation to climatic conditions requires more than adaptation to seasonal temperatures. Therefore, the effect of heat on transferred trees during the growing season can be influenced by other environmental factors (Liepe et al., 2016).

The Siberian stone pine, *Pinus sibirica* Du Tour, is one of the primary forest species in Russia. Patterns of intraspecific variation in Siberian stone pine largely correspond to those of other boreal conifers (Khutornoy et al., 2018; Zhuk and Goroshkevich, 2018). Most investigations on Siberian stone pine and other conifers deal with geographical variation in traits characterizing growth rate and resistance to external factors. However, limited research has been conducted on elementary shoot structure and ratios of metamere types. Shoot structure in natural populations of Siberian stone pine varies significantly, with different ratios of metamere types involved in shoot growth and branching (Goroshkevich and Popov, 2004). However, it is unclear whether this ratio is retained when trees are grown *ex situ*.

This study aimed to assess variation in growth and elementary structure of shoots in 10-year-old branches in different latitudinal and longitudinal ecotypes of Siberian stone pine, with emphasis on the ratio between shoot metamere types, using a common garden experiment.

MATERIALS AND METHODS

Study Area

The study was conducted in the southern taiga ecoregion on the West Siberian plain, Russia (56°13′ N 84°51′ E). The investigation was conducted using eight ecotypes of Siberian stone pine (Table 1) from different parts of the species range (Figure 1), which were grown in a common garden. Each latitudinal ecotype represented one of the vegetation zones on the West Siberian plain, which has a transect length of ~1600 km. The longitudinal ecotypes represented two vegetation zones and the same part of different mountain forest belts. The longitudinal transect length was ~3000 km, but it had a gap due to the lack of *Pinus sibirica* in some southern vegetation zones of Russia. Only natural stands that were typical for each region were included in the investigation.

Sample Collection and Measurement

Scions were cut from 9 to 19 randomly selected trees in a natural stand and grafted onto local 4-year-old Siberian stone pine seedlings in the spring of 1996. The grafts were grown with a spacing of 3 × 6 m in a common garden experiment at the "Kedr" Scientific Station, which is managed by the Institute of Monitoring of Climatic and Ecological Systems, Russian Academy of Sciences, Tomsk, Russia.

In 2018, 10-year-old branches were chosen on the southern side of the crown in the 23-year-old grafts, and retrospective analysis of the shoot structure along the branches was performed (Vo-

Figure 1. Geographical distribution of *Pinus sibirica*. Each point represents an ecotype or common garden plantation. Latitudinal ecotypes: 1, Abaza, 2, Strezhevoy, 3, Noyabrsk, and 4, Urengoy. Longitudinal ecotypes: 5, Nevyansk, 6, Tayshet, 7, Sludyanka, 8, Severobaikalsk. A, common garden plantation. All the points are located in Russia

robjev et al., 1992). The length of all 10 shoots and needles on the three youngest shoots was measured to the nearest 1 mm using a measuring tape, and the number of sterile cataphylls, brachyblasts, lateral auxiblasts, and sleeping buds was counted on each shoot. The number of fallen axillary structures was estimated using traces on the shoot bark.

Statistical Analysis

Raw data consisted of trait means for each clone, and the value for needle length was averaged over three years. The variances of these values for the different ecotypes were homogeneous. According to the Kolmogorov–Smirnov test, all trait means were normally distributed. Differences between the ecotypes were determined using analysis of variance (ANOVA). When significant differences ($p<0.05$) were detected, comparisons between ecotypes were conducted using Duncan's test.

RESULTS AND DISCUSSION

After the first year of branch appearance, differences between ecotypes were small, but they gradually increased with age. By the tenth year of branch life, differences between the latitudinal ecotypes reached 1.4 times, while that between the longitudinal ecotypes was 1.2 times. Based on ANOVA and Duncan's test, significant differences were found in branch length. Among the latitudinal ecotypes, the southern ecotype Abaza showed the highest growth rate, and the northern ecotypes Urengoy and Noyabrsk showed the weakest growth (Figure 2a). The intermediate ecotype Strezhevoy had a moderate growth rate.

Among the longitudinal ecotypes, the south-eastern ecotype Sludyanka had the highest growth rate. All other ecotypes, the western ecotype Nevyansk, the intermediate ecotype Tayshet and the north-eastern ecotype Severobaikalsk, showed relatively low growth (Figure 2b).

Shoot structure also varied significantly between different latitudinal and longitudinal ecotypes. Among the latitudinal ecotypes, the results of the ANOVA and Duncan's test showed that there were differences in the number of sterile cataphylls and brachyblasts, which are elongation structures, but no differences in the number of auxiblasts, which are branching structures (Table 2). From the southern to northern ecotypes, the number of sterile cataphylls and brachyblasts decreased by 1.6

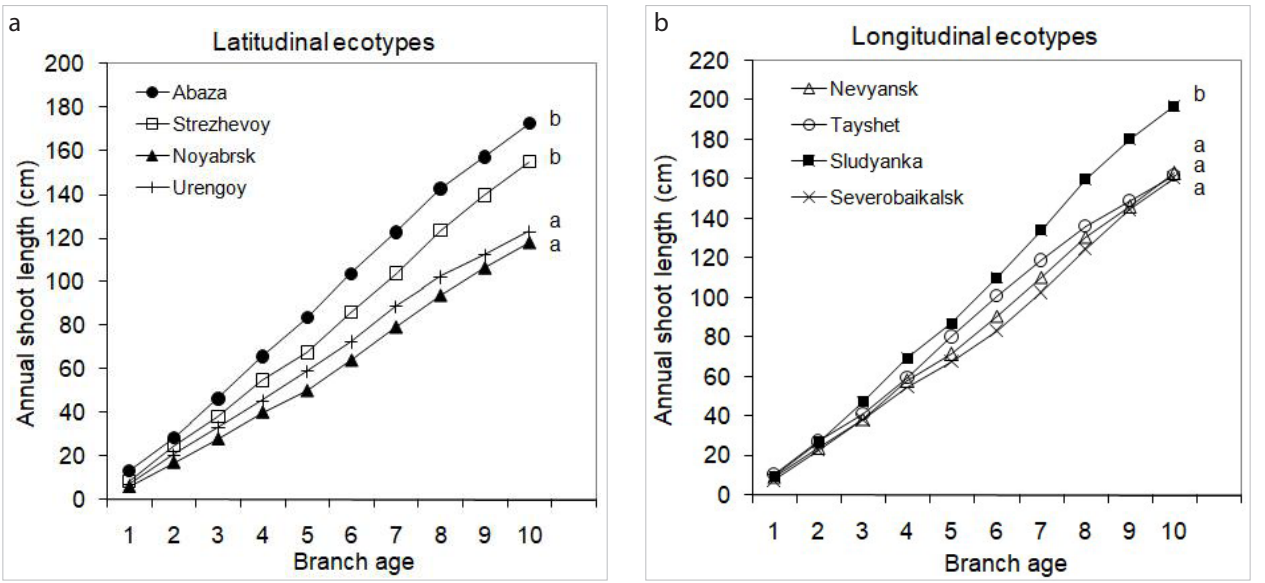

Figure 2. a, b. Growth rate of 10-year-old branches in *Pinus sibirica* ecotypes: (a), latitudinal ecotypes and (b), longitudinal ecotypes. Means associated with a different letter are statistically different ($p<0.05$), according to Duncan's test

Table 2. Total number of different axillary structures, and internode and needle length on the 10-year old branches of *Pinus sibirica*

Ecotype	Number of sterile cataphylls on the branch	Number of brachyblasts on the branch	Number of auxiblasts on the branch	Internode length in the annual shoot, mm	Needle length, cm
Latitudinal ecotypes					
1. Abaza	106.3±13.9^{b}	694.8±81.5^{b}	28.6±8.5^{a}	2.1±0.3^{b}	11.2±0.8^{b}
2. Strezhevoy	79.0±18.9^{a}	667.3±97.8^{b}	34.1±4.7^{a}	2.0±0.2^{ab}	10.6±0.6^{b}
3. Noyabrsk	77.4±20.4^{a}	582.6±109.3^{a}	29.7±6.8^{a}	1.7±0.2^{a}	9.0±1.0^{a}
4. Urengoy	66.7±16.2^{a}	577.8±109.3^{a}	27.8±5.2^{a}	1.8±0.2^{ab}	8.5±0.6^{a}
Longitudinal ecotypes					
5. Nevyansk	84.3±17.4^{a}	718.1±116.1^{a}	31.3±5.2^{a}	1.9±0.2^{a}	9.9±0.9^{a}
6. Tayshet	79.2±14.4^{a}	668.8±76.8^{a}	29.5±2.4^{a}	2.1±0.1^{b}	10.9±0.9^{b}
7. Sludyanka	90.6±26.2^{a}	903.3±200.5^{b}	34.1±7.5^{a}	1.9±0.2^{a}	10.4±0.9^{ab}
8. Severobaikalsk	80.1±18.7^{a}	725.1±126.9^{a}	33.1±4.9^{a}	1.9±0.2^{a}	10.3±0.5^{ab}

Comparisons were made between latitudinal and longitudinal ecotypes separately. Means associated with a different letter are statistically different ($p<0.05$), according to Duncan's test.
Means±standard deviations are indicated

and 1.2 times, respectively. In addition, the southern ecotype had the longest internodes, which made shoot elongation even more effective. This ecotype, together with the intermediate one, also had the longest needles, probably to support the intensive growth.

The tendency in the variation among the longitudinal ecotypes was unambiguous. There were differences in the number of brachyblasts, but no differences in the number of sterile cataphylls and auxiblasts. The south-eastern ecotype had the highest number of brachyblasts. Despite their origins, the other ecotypes did not show any differences. However, an intermediate ecotype had the longest internodes and needles.

Thus, significant variation among latitudinal and longitudinal ecotypes *ex situ* was observed both in branch growth and shoot structure, but the ecotypes from the profiles showed different patterns of variation. There was no variation among all ecotypes in the number of branching structures. The number of elongation structures and the length of internodes were maximal in the southernmost ecotypes in both profiles. From southern to northern ecotypes, the tendency to decrease structures was

pronounced, but there was no pronounced tendency from western to eastern ecotypes.

Variation in the growth rates of Siberian stone pine ecotypes was comparable with the variation in other boreal conifers both in direction and magnitude. The variation in growth between different species, when growing under uniform conditions, depends on the experimental region and the observation period (Nord-Larsen and Pretzsch, 2017). However, in most cases, ecotypes from regions with relatively high growing-season temperatures showed high growth rates *ex situ*. In previous experiments, this pattern was found for *Pinus sylvestris* L. (Govindarajulu, 2014), *P. pinea* L. (Loewe Muñoz et al., 2017) and *Larix gmelinii* Rupr. (Lukkarinen et al., 2010). Therefore, there seems to be an adaptive convergence of latitudinal ecotypes of most coniferous species, although the degree of intraspecific variation may vary between species and depend on the origin of ecotypes.

Branch length varied significantly between the ecotypes of Siberian stone pine. Further, ecotypes originating from the most favorable conditions had the longest branches. Over the 10 years of branch growth, the annual growth rate, with the exception of several years, showed similar differences between ecotypes. The age-related growth curves of tree species usually demonstrate that annual growth is relatively fast at first and decreases throughout life. This is associated with tree aging and the accompanying changes in root-leaf relationships (Bond et al., 2007). The growth curves of branches in the Siberian stone pine ecotypes showed that there was no apparent age-related growth decrease in any ecotype over the 10-year period. The only intermediate ecotype, Tayshet, showed a remotely similar trend over the last four years. This differentiated the lateral branches from the tree trunk. This finding is explained by a previous study, which showed that stem growth in 23-year-old ecotypes did not decrease with age in the southern ecotype, while the northern ecotype showed an annual growth that halved over the last five years (Zhuk and Goroshkevich, 2018).

Needle length varied significantly among the Siberian stone pine ecotypes. Again, ecotypes from favorable conditions had the longest needles. Together with physiological characteristics, needle length is an important indicator of the growth energy of a tree and its sensitivity to external impacts (Grulke, 2010). Thus, ecotypes with long needles have the highest growth rates of branches.

The number and proportion of metameres responsible for elongation of the shoot, as well as internode length, varied significantly among the ecotypes. Two ecotypes contributed most to the variation: the eastern ecotype had the largest number of brachyblasts, and the southern ecotype had the largest shoot sterile zone and the longest internodes. A large number of metameres and long internodes are adaptive traits that allow trees to compete sufficiently for the first layer of the forest stand. This is especially relevant in relatively mild environments where intraspecific and interspecific competition is most pronounced.

Both the number of metameres responsible for branching and their proportion of the total number of metameres were similar for all ecotypes. In contrast, among altitudinal Siberian stone pine ecotypes *ex situ*, significant differentiation was found, and high-altitudinal ecotypes had a larger number of latent buds, which are part of the branching metameres (Zhuk, 2010). Having a large number of latent buds allows adaptiveness, as apical growth can resume after frost injuries of shoots. Perhaps, unlike for mountain ecotypes, latitudinal and longitudinal ecotypes form metameres in mild conditions. Therefore, in the geographical ecotypes of Siberian stone pine, the number of metameres responsible for shoot elongation as well as internode and needle length are the primary adaptive traits.

CONCLUSION

The length and metameric composition of branches varied significantly among both latitudinal and longitudinal ecotypes of *Pinus sibirica* in a common garden experiment. The growth rates in the southern and eastern ecotypes were higher than those in the northern and western ecotypes. However, the proportions of auxiblasts involved in branching did not differ among ecotypes. The proportions of metameres involved in shoot elongation and internode length depended on the geographic origin of ecotypes when growing their vegetative progeny *ex situ*. Both latitudinal and longitudinal ecotypes were less morphologically differentiated than the altitudinal ecotypes of *Pinus sibirica*, despite their much longer transect lengths.

Ethics Committee Approval: Ethics committee approval is not applicable for this type of research in Russia because this article does not contain any studies with human or animal subjects.

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