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Elucidating time divergence and biogeography of *Euonymus hamiltonianus* complex using complete plastome analysis

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The evolutionary history of *Euonymus* L., within the Celastraceae family has been understudied. Within this genus *E. hamiltonianus* Wall., belonging to *E.* sect. *Biloculares* have slight variations in morphology due to their high ecological range, leading to different varieties and subspecies. This has caused debate on the species status of many taxa. This study we investigate the plastome of *E. hamiltonianus* complex and related species in *E.* sect. *Biloculares*. It was aimed to observe the similarities and differences in the plastome, to clarify the ambiguities and evolutionary relationships within this complex. Plastome showed high similarities in coding regions and conserved quadripartite structure (156,881–158,434 bp). *E. hamiltonianus* complex had *rps*19 located in the LSC region. *ndh*F was reportedly a pseudogene in *E. hamiltonianus* (Mugabsan, South Korea). In all the investigated species, *rps*16 lacked introns. A resolved relation was observed among the taxa of *E. hamiltonianus* complex, and the species closely related to these (100% Bootstrap & 1.0 PP). *E. sieboldianus* Blume was observed at a position between the two taxa of *E. hamiltonianus*, making it a possible synonymy. *E. europaeus* L., and *E. velutinus* Fisch. & C.A.Mey., formed separate lineages. Molecular dating suggests that *E. hamiltonianus* complex originated in East Asia during the Middle Miocene Transition, which is a significant phase within the Neogene era, supporting the hypothesis that *Euonymus* originated in East Asia and *E. hamiltonianus* complex is estimated to develop in East Asia and diversified across the Far East during this period.

Keywords Disjunction, *Euonymus hamiltonianus* complex, *Euonymus* sect. *Biloculares*, East Asia, West Asia, North America, Plastid genome, Vicariance

Abbreviations

BI	Bayesian inference
BBM	Bayesian Binary Method
Bp	Base pairs
Cp	Chloroplast
HPD	Highest Posterior Density
IR	Inverted repeat
Kash	Kashmir
LSC	Large single copy
MCMC	Markov chain Monte Carlo
Mya	Million years ago
ML	Maximum likelihood
MP	Maximum Parsimony
NCBI	National Centre for Biotechnology Information
PP	Posterior Probability
Sect	Section
St. dev	Standard deviation
Sp	Species
SSC	Small Single copy

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Background

Euonymus is one of the largest genus in Celastraceae which includes 129–200 species. Leonova in (1974) and J. S. Ma (2001) reported 129–130 species. Savinov and Baikov (2007) suggested up to 200 species[1–3,5]. This count reflects various taxonomic interpretations in this genus with time. Gavrilova et al., 2018 described that the genus comprises both deciduous and evergreen shrubs and small trees[4]. Beck in 1892, classified genus *Euonymus* into two subgenera, namely *E.* subg. *Euonymus* and *E.* subg. *Kalonymus*. This classification was based on the flower merosity, filament length, the number of anther sacs, and ovule structure. Different sections of the genus have been recognized till date. Due to the classification of the genus into various sections, two primary taxonomic systems for the genus are accepted and used around the world. One is R. A. Blakelock system which was proposed in 1951[6] and the second classification system was J. S. Ma 2001 system[1,4]. R. A. Blakelock classification system contained the adjustments made by T. Leonova (1974)[3], T. Nakai[7] and Th. Loesener (1942)[8]. R. A. Blakelock in synapsis of the genus *Euonymus* has mentioned about two subgenera similar to Beck (1892) and 12 sections of the two subgenera. R. A. Blakelock further divided the subgenus *Euonymus* Beck. into six sections. This was based on the shape of the fruit capsules. The sections include (I) *E.* sect. *Melanocarya*, (II) *E.* sect. *Biloculares*, (III) *E.* sect. *Multiovulatus*, (IV) *E.* sect. *Stenocarpus*, (V) *E.* sect. *Ilicifolia* and (VI) *E.* sect. *Echinococcus*[6]. In 2008 J. S. Ma and Funston revised the R. A. Blakelock classification. They combined the three sections, namely *E.* sect. *Biloculares*, *E.* sect. *Multiovulatus* and *E.* sect. *Stenocarpus* into one section called *E.* sect. *Euonymus*[2]. Flora of China follows the classification of J. S. Ma, dividing the genus *Euonymus* into two subgenera: *E.* subg. *Kalonymus* and *E.* subg. *Euonymus*. This organization is based on winter buds, stamens, anthers, and capsules[1,4]. *E.* subg. *Kalonymus* is represented by one section: *Uniloculares*. In contrast, *E.* subg. *Euonymus* is represented by four sections, namely *E.* sect. *Melanocarya*, *E.* sect. *Echinococcus*, *E.* sect. *Ilicifolia* and *E.* sect. *Euonymus* based on the fruit structure[2]. Ruoy and Faucaud identified section *Biloculares*, distinguishing this section based on the presence of two locules in the anthers. Blakelock provided additional character definition for the section *Biloculares*, based on lobed or unlobed capsules, deciduous or evergreen leaves, and types of inflorescences. These traits served as the basis for characterizing them into *E.* sect. *Biloculares*[6].

Euonymus is widely distributed in both tropical and temperate climates[1,6,9]. The hotspots of the species are present mainly from Eastern Asia to the Himalayan region, as well as South Asia and Southeast Asia[1,10]. It is now considered a global genus according to Blakelock's (1951) report of its presence in Madagascar, North Africa as well as Australia[6]. *E.* sect. *Uniloculares* (subgenus *Kalonymus*) has 16 species that are limited to East Asia and the Far East, and only one species, *E. latifolius* (L.) Mill. It is distributed in West Asia, Europe, and North Africa[2]. *E.* sect. *Biloculares*, has 39 species with a broad geographic range that includes Asia, Europe, North America, and Australia[2,6]. Blakelock described morphologically distinctive features of this section, including capsuled fruit which are smooth or somewhat rough, unlobed or lobed to the middle of the chambers[6]. Species of this section are widespread in Asia and Europe in comparison to other section in genus *Euonymus*[6,11]. Although the species are identified through their morphological descriptions, describing these features (fruit, flower, and inflorescence) are difficult to clearly define the species[12]. Closely related taxa specially share the problem of ambiguous species delimitation[13]. In *E.* sect. *Biloculares* this problem is evident, where species are renamed and synonymized due to the character overlap.

Within the section *Biloculares*, *E. hamiltonianus* is the most widely distributed and common species[2]. The species is distributed in East Asia, also it grows in India, Pakistan, Russia, Nepal, Burma, and in the mixed broad leaved forests of East Himalaya[14,15]. *E. hamiltonianus* resembles *E. europaeus* clearly, although their geographic distributions are different[2]. *E. hamiltonianus* exhibits the interesting character of heterostyly[16]. Heterostyly is a type of genetic variation that influences the floral appearance of plants and is present in populations[17]. Two unique manifestations of this polymorphism, known as distyly and tristyly, which involve two or three different types of mating, respectively, are the two ways it shows itself[18]. The variation in height between the stigma and anthers is what distinguishes these variants. Those with short filament stamens have long styles, and vice versa. The self and intramorph incompatibility mechanism that results from this structural variation in floral morphology restricts plant mating to those with organs that are the same height[17]. By ensuring that only plants with the same morph can mate, this physiological incompatibility prevents hybridization between various morphs[19].The taxa of *E. maackii* Rupr. and *E. bungeanus* Maxim., which are distributed in East Asia and the Far East, also share this common characteristic[11]. *E. europaeus*, which is considered as a European relative of *E. hamiltonianus*, also exhibit heterostyly[20,21]. By observing the morphological similarities and geographical differences, we can infer that both the species may form an *E. hamiltonianus* complex. A similar hypothesis applies to *E. bungeanus*, *E. maackii* and *E. sieboldianus* which are also closely related morphologically. So, the main questions to be addressed are the possible origin of these species and whether they share common ancestors. To focus on the analysis of chloroplast genomes would be crucial.

Particularly, the generic status, taxonomic classification, infrageneric division, and species delimitation of the characterization of *Euonymus* are still under debate. This could be the result of intricate morphological patterns. Researchers utilized internal transcribed spacer (ITS) of nuclear ribosomal DNA, external transcribed spacer (ETS)[22,25], and selected genes from the plastid genomes, including *psb*A, *rpl*36, *inf*A, and *rps*8[26] were used as molecular markers for phylogenetic study of *Euonymus*[27]. However, these analyses resulted in polytomy and unresolved relationships.

The goal of the current study is to use plastid genome analysis to evaluate the phylogenetic position of *E. hamiltonianus* and similar species within the genus *Euonymus*. This study aims to investigate the monophyly of *E. hamiltonianus* complex and *E.* sect. *Biloculares*, as well as to determine the age of this species. We used plastid genome analysis to build a phylogenetic tree that can reveal information about the evolutionary relationships among the target species to accomplish these goals. Overall, 25 taxa of *Euonymus* were used, within 25 taxa, 12 were analysed in this study (Table 1) and thirteen species' plastome data were downloaded from NCBI GenBank (Table 3). The species were gathered from a variety of geographic areas, including North America, East Asia, the

Taxon	Herbarium accession number/collector & identifier/collection date	Collected country/region	Botanical garden/arboretum
1. *Euonymus alatus* (Thunb.) Siebold	SJ2020-4/J.H Kim/2020-08-05	South Korea	Gachon University, Global Campus
2. *Euonymus americanus* L.	980,743/Douglas Ruhren/2020-11-13	North Carolina, United Stated of America	JC Raulston Arboretum
3. *Euonymus bungeanus* Maxim.	SJ2020-5/J.H Kim/2020-06-08	South Korea	Korean National Arboretum
4. *Euonymus chibae* Makino	SJ2020-6/J.H Kim/2022-05-18	South Korea	Suwol Mountain, Geomundo
5. *Euonymus europaeus* L	641/Nikolay Aleksandrovich Trusov and Igor Olegovich Yatsenko/2020-08-05	Kaluga Region, Russia	Tsytsin Main Botanical Garden RAS, Moscow, Russia
6. *Euonymus hamiltonianus* Wall.	SJ2020-8/Akhtar Malik/2020-11-03	Kashmir, Jammu and Kashmir Srinagar India	KASH Herbarium Centre for Biodiversity and Taxonomy, Department of Botany University of Kashmir, Jammu and Kashmir Srinagar India
7. *Euonymus hamiltonianus* Wall.	SJ2021-7/J.H Kim/2021-06-10	South Korea	Mugab Mountain (Mugabsan)
8. *Euonymus pauciflorus* Maxim.	SJ2022-9/J.H Kim/2020-07-27	South Korea	Sangwonsa temple-Jeokmyeol bogung, Odaesan Mountain, Gangwando
9. *Euonymus sieboldianus* Blume	6460/64/Nikolay Aleksandrovich Trusov and Igor Olegovich Yatsenko/2020-08-05	Russia	Tsytsin Main Botanical Garden RAS, Moscow, Russia
10. *Euonymus spraguei* Hayata	CWJ 12,446/Coley, Finlay. WJ, Bleddyn Wynn-Jones/2007-11-29	Taiwan, Tayuling (Dayuling) Old logging Trail	Crug Farm Plants, Caernarfon, GWYNEDD, United Kingdom
11. *Euonymus velutinus* Fisch. & C.A.Mey	262,301/Nikolay Aleksandrovich Trusov and Igor Olegovich Yatsenko/2020-08-05	Azerbaijan	Tsytsin Main Botanical Garden RAS, Moscow, Russia
12. *Euonymus verrucosus* Scop.	3789/Nikolay Aleksandrovich Trusov and Igor Olegovich Yatsenko/2020-08-05	Kaluga region	Tsytsin Main Botanical Garden RAS, Moscow, Russia

Table 1. Plant material of *Euonymus* taxa collected from various areas.

Far East, West Asia, Europe, and the Himalayan region. To gain a better understanding of their biogeographical history. This study will investigate the age of *E. hamiltonianus* complex and allied species across East Asia, West Asia, and Europe. This study will help us better understand the evolutionary history of *E. hamiltonianus* complex within the broad and diversified genus *Euonymus*[1–3,5].

Results

Phylogenetic analysis

To evaluate the phylogenetic position of *E. hamiltonianus* complex, phylogenetic reconstruction was carried out using 80 protein coding genes. The dataset included 70,236 characters (bases) in it, and by utilizing Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI). The phylogenies produced were having same topologies with a 100% BS and 1.0 PP. Seven members of Celastraceae were used as outgroups (Table 3) and 25 taxa of *Euonymus* as ingroups (Table 2).

According to this study's result, the genus *Euonymus* is divided into two main clades (Fig. 1). It is observed that *E. hamiltonianus* is not monophyletic. The placement of different accessions of *E. hamiltonianus* into separate clades make it evident. *E. hamiltonianus* complex can be observed in the Clade I. This complex consists of *E. hamiltonianus*, *E. bungeanus*, *E. maackii* and *E. sieboldianus*. This forms a well-supported clade and suggests a close evolutionary relationship. The presence of multiple *E. hamiltonianus* samples indicates the genetic variation within the species which supports the idea of a species complex. The scattered pattern of *E. hamiltonianus* accessions interspersed with closely related taxa supportd the recognition of a complex. *E. bungeanus*, *E. maackii*, *E. hamiltonianus*, *E. europaeus* and *E. velutinus* share the same ancestor and a resolved relation with a bootstrap value of 100% (100% BB, 1.0 PP). *E. europaeus* showed a close relation to *E. hamiltonianus* but was not the part of the *E. hamiltonianus* complex and can be seen as a distinct species. These results provide compelling evidence for the distinct evolutionary lineages within the *E. hamiltonianus* complex, highlighting the need for integrative taxonomic revision and offering new insights into the diversification of genus *Euonymus*.

The Chinese endemic taxa, *E. yunnanensis* showed a resolved sister relationship (100%, BS, 1.0 PP) with *E. chibae* Makino. The species of *E. pauciflorus*, *E. verrucosus*, *E. phellomanus* Loes. and *E. alatus* (Thunb.) Siebold. are gathered in one subclade (100%, BS, 1.0 PP) and form a sister clade with the rest of the members of sect. *Biloculares*. *E. americanus* L. is forming the basal clade that consists of the members of *E.* sect. *Echinococcus*, *E.* sect. *Ilicifolia* and *E.* sect. *Uniloculares*. *E. spraguei* Hayata. formed a sister relation with members of *E.* sect. *Ilicifolia* (*E. japonicus* Thunb. and *E. fortunei* (Turcz.) Hand. -Mazz.) (100%, BS, 1.0 PP). *ndh*F was observed as a pseudogene in the members of *E.* sect. *Ilicifolia*, *Uniloculares*, and *Echinococcus*. Additionally, *rps*16 is a pseudogene in both the ingroups and outgroups used in the study. Within the species of *E.* sect. *Biloculares*, gene pseudogenization was evident in *E. hamiltonianus* (Mugabsan, South Korea), *E. pauciflorus* and *E. verrucosus* (Fig. 1).

Comparative genome analysis

To calculate the nucleotide diversity (π-value) in *Euonymus* species, we performed an alignment of 114 genes. This dataset comprised both the coding genes, and non-coding tRNAs and rRNAs from the LSC, SSC and IR regions. Out of the 80 protein-coding genes, 13 genes were found to exhibit high nucleotide diversity, ranging from 0.01 to 0.020. The gene *ycf*1 achieved the highest π-value of 0.02, while *rpl*33 had a value of 0.016. In

Scientific Name	NCBI Accession Number	Genes/Pseudogenes	rRNA	tRNA	LSC	SSC	IRa	IRb	Total base pairs	GC %	AT%
1. **Euonymus alatus* (Thunb.) Siebold	OP747447	133/1	8	37	87,132	18,493	26,294	26,294	158,213	37.4	62.6
2. **Euonymus americanus* L.	OP747444	134/2	8	37	86,272	18,684	26,738	26,738	158,432	37.1	62.9
3. **Euonymus bungeanus* Maxim.	OP747445	133/1	8	37	86,559	18,307	26,340	26,340	157,546	37.2	62.8
4. **Euonymus chibae* Makino	OP747446	133/1	8	37	85,771	18,247	26,368	26,368	156,754	37.3	62.7
5. **Euonymus europaeus* L.	OP747448	134/1	8	37	85,860	18,307	26,668	26,668	157,503	37.3	62.7
6. *Euonymus europaeus* L.	MZ567072	133/1	8	37	86,245	18,330	26,344	26,344	157,263	37.3	62.8
7. *Euonymus fortunei* (Turcz.) Hand. -Mazz.	MW288090	134/2	8	37	85,892	18,319	26,700	26,700	157,611	37.3	62.7
8. *Euonymus hamiltonianus* Wall.	KY926695	133/1	8	37	86,399	18,317	26,322	26,322	157,360	37.3	62.8
9. **Euonymus hamiltonianus* Wall. (Mugab Mountain)	OP747450	134/2	8	37	87,222	18,422	26,395	26,395	158,434	37.2	62.8
10. **Euonymus hamiltonianus* Wall. (Kashmir)	OP747449	134/1	8	37	86,291	18,320	26,336	26,336	157,283	37.3	62.7
11. *Euonymus japonicus* Thunb.	KP189362	133/2	8	37	85,941	18,340	26,678	26,678	157,637	37.3	62.7
12. *Euonymus latifolius* (L.) Mill.	OL770076	135/2	8	38	86,161	18,581	26,676	26,676	158,094	37.1	62.9
13. *Euonymus maackii* Rupr.	MW771518	133/1	8	37	86,524	18,337	26,345	26,345	157,551	37.2	62.8
14. *Euonymus macropterus* Rupr.	OL770077	136/2	8	39	85,499	18,460	26,709	26,709	157,377	37.2	62.8
15. *Euonymus oxyphyllus* Miq.	OL770078	134/2	8	37	85,924	18,543	26,697	26,697	157,861	37.1	62.9
16. **Euonymus pauciflorus* Maxim.	OP747439	135/1	8	39	85,968	18,494	26,318	26,318	157,115	37.3	62.1
17. *Euonymus phellomanus* Loes.	MW288092	133/1	8	37	86,299	18,536	26,354	26,354	157,543	37.3	62.7
18. *Euonymus sachalinensis* (F.Schmidth) Maxim.	OL770079	134/2	8	37	85,404	18,476	26,705	26,705	157,290	37.2	62.8
19. *Euonymus schensianus* Maxim	KY511610	133/2	8	37	86,026	18,528	26,574	26,574	157,702	37.2	62.8
20. **Euonymus sieboldianus* Blume	OP747440	134/1	8	37	86,517	18,325	26,353	18,325	157,548	37.3	62.8
21. **Euonymus spraguei* Hayata	OP747441	135/2	8	37	85,731	18,444	26,689	26,689	157,553	37.3	62.7
22. *Euonymus szechuanensis* C.H. Wang	MH853828	134/2	8	37	86,401	18,472	26,296	26,296	157,465	37.2	62.8
23. **Euonymus velutinus* Fisch. & C.A.Mey	OP747442	134/1	8	37	86,227	18,367	26,310	26,310	157,214	37.3	62.2
24. **Euonymus verrucosus* Scop.	OP747443	135/2	8	37	85,090	18,568	27,201	27,201	158,061	37.4	62.7
25. *Euonymus yunnanensis* Franch.	MW770452	134/1	8	37	85,248	18,341	26,646	26,646	156,881	37.3	62.8

Table 2. Chloroplast genome features of *Euonymus* spp (* sign denotes the species whose Chloroplast genomes underwent processing in the present study).

contrast, three of the coding genes, *psb*I, *psb*T and *ycf*15 were found to have no nucleotide diversity (π = 0). Among the 30 tRNAs, 21 genes displayed no nucleotide diversity, resulting in a π value of zero (π = 0). The remaining 9 tRNA genes showed some variation, with *trn*I-GAU having a π value of 0.03 and *trn*I-CAU having a π value of 0.01. The nucleotide diversity of rRNAs, *rrn*23, *rrn*16 and *rrn*4.5, were found to be relatively low, π value ranging from 0.003–0.0002 (Fig. 2) [S-4]. This representation clearly demonstrated that the chloroplast genomes of *Euonymus* species exhibit conserved structure. The graphical representation demonstrates that there is a conservation of protein-coding genes, with less diversification observed in their plots compared to the non-coding region plots in mVISTA (Fig. 3). Comparing the IR region to the LSC and SSC, the genes in the IR regions were more conserved. Among the coding genes variability was evident in *ndh*F and *ycf*1 in the pairwise identification of divergent regions. This showed that despite notable morphological diversifications the genomes are highly conserved.

Plastid genome feature and IR junction

Chloroplast genomes of 25 taxa of *Euonymus* were compared and analyzed in this study. It was indicated clearly that all the taxa had a typical genome that consists of LSC, SSC, and two IR regions (IRa and IRb). The results showed that the plastid genomes ranged from 158,434 bp (*E. hamiltonianus* Mugabsan, South Korea) to 156,754 bp (*E. chibae*). The LSC ranged from 87,222 bp (*E. hamiltonianus* Mugabsan, South Korea) to 85,090 bp (*E. verrucosus*). *E. americanus* had large number of base pairs in the SSC (18,684 bp), whereas *E. chibae* had low number of base pairs (18,247 bp). The base pairs in the IR regions ranged from 27,201 bp (*E. verrucosus*) to 26,310 bp (*E. velutinus*) (Table 2). The IR regions within the chloroplast genomes of flowering plants exhibit minor variations between species[28]. The variability in junctions is evident in Fig. 4. The examination of IR junctions has revealed that the IR expansion and contraction were not constant between the sections and the species. This represents species-specific evolution. The shrinkage of IR junction was observed in *E. hamiltonianus* complex, which was like most of the species of *E.* sect. *Biloculares* analyzed in this study. Comparing the taxa of *E. hamiltonianus* complex less variability can be observed. Within the complex *E. sieboldianus* exhibited an IR junction expansion of 76 base pairs. However, the overall IR length has shown minimal variations among the closely related taxa (*E. bungeanus*, *E. maackii*, *E. hamiltonianus*), ranged from 26,322 bp in *E. hamiltonianus* (KY926695) to 26,395 bp in *E. hamiltonianus* Mugabsan, South Korea. A gap of 19 bp was observed between the IR junction to the end of *ndh*F gene in the taxa of *E. sieboldianus*, *E. hamiltonianus* (KY926695) and *E. hamiltonianus* Kashmir. Within the species of *E.* sect *Biloculares*, IR expansion was noticed in *E. verrucosus*,

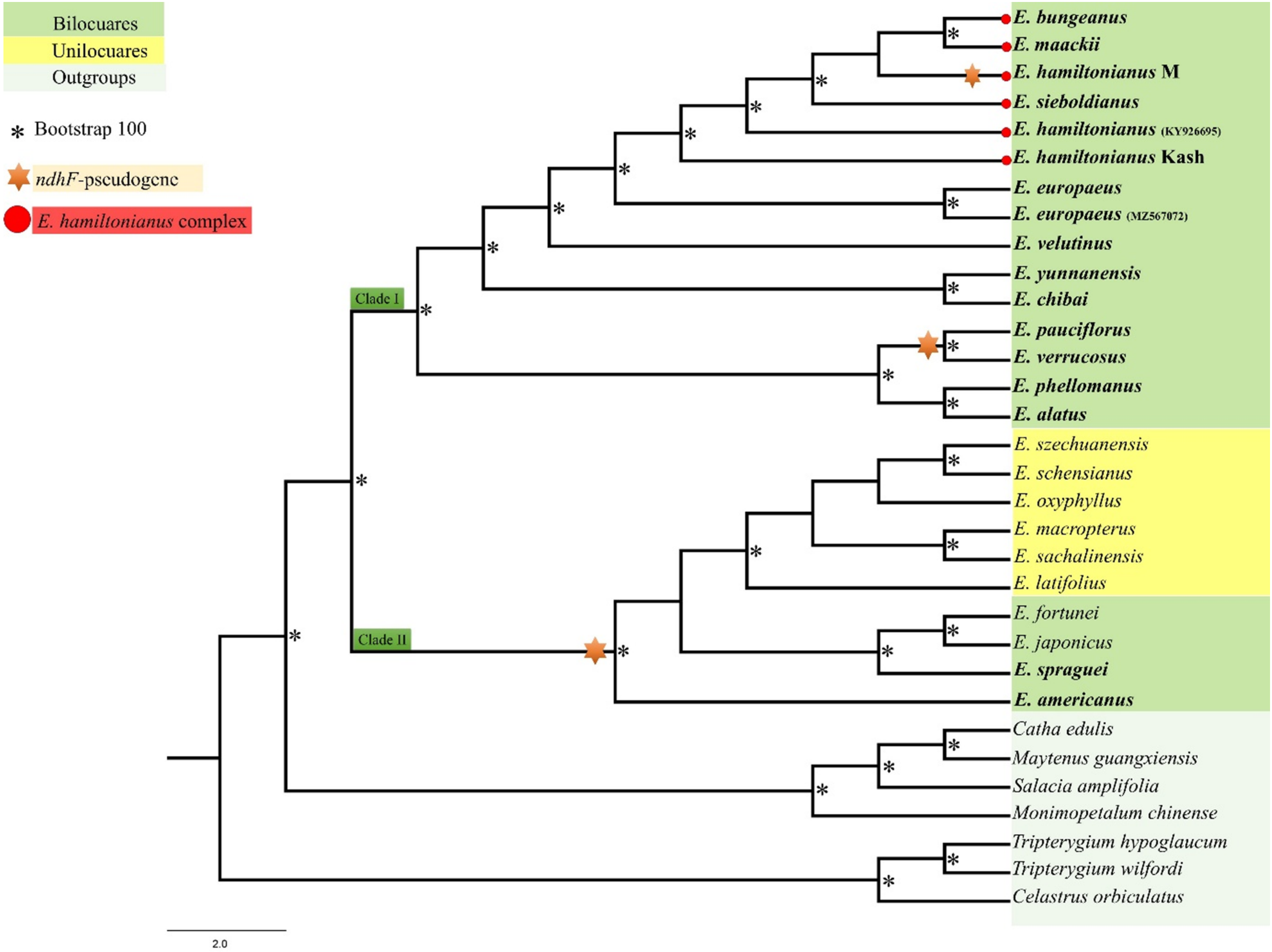

Fig. 1. Phylogenetic tree constructed using Maximum likelihood (ML), and Bayesian inference (BI) based on whole chloroplast genomes of 25 taxa of *Euonymus*. Accession numbers are mentioned with the species which are downloaded from NCBI GenBank. Numbers at each node indicates 100% Bootstrap (PAUP & ML) and 1 posterior probability (BI) resolution (*). Values less than Bootstrap = 60 and PP = 0.5 are ignored. Taxon with accession numbers were downloaded from NCBI GenBank.

E. yunnanensis and *E. europaeus* (MZ567072). *E. verrucosus* displays IR expansion compared to *E. pauciflorus*. Similarly, IR expansion was exhibited in *E. europaeus* (MZ567072) compared to the sister species. IR shrinkage was noticeable in *E. szechuanensis* compared to other nine species in the same clade which represents of *E.* sect. *Echinococcus*, *E.* sect. *Uniloculares* and *E.* sect. *Ilicifolia*.

Divergence time estimation

Based on time divergence, the chloroplast genome study determined the crown age of the genus *Euonymus* to be 66.3 Mya (95% HPD = 35.87–104.5) (Fig. 5). The genus diverged into two distinct clades approximately 34.96 Mya (95% HPD = 18.9–55.3) as observed at Node-15. Node-14, which contains the extant species of *E. pauciflorus* and *E. alatus*, diverged from its ancestors around 19 Mya (95% HPD = 8.99–32.8 Mya). *E. pauciflorus* and *E. verrucosus* (Node-12) were separated 12.07 million years ago (95% HPD = 3.67–21.61) according to the analysis carried in this study. Similarly, *E. velutinus* (Node-8), which is a Western Asian representative of *E.* sect. *Biloculares*, diverged 15.8 Mya (95% HPD = 8–25). Species of *E. hamiltonianus* complex segregated from its western Asian (*E. velutinus*) and European relatives (*E. europaeus*) 12.58 million years ago (95% HPD = 6.57-220.12). The *E. hamiltonianus* complex, which contained *E. hamiltonianus*, *E. sieboldianus*, *E. bungeanus* and *E. maackii* diverged between 9.65 and 1.79 Mya. This divergence occurred between Middle Miocene and Pleistocene Epochs. *E. bungeanus* and *E. maackii* node separated and is most likely the youngest clade (Node-1) in the complex. The divergence was 1.79 Mya (95% HPD = 0.47–3.49). While *E. spraguei* diverged roughly 7.39 Mya (95% HPD = 1.93–14.26)., whereas *E. americanus* diverged at 23.58 Mya (95% HPD = 10.77–37.85) (Fig. 5).

Biogeography

Biogeographical analysis of *E.* sect. *Biloculares* and *E. hamiltonianus* complex suggests that the species in the complex were inferred to have originated in East Asia (Fig. 6). Two dispersal events are recorded. The active region for the dispersal of these species is in East Asia, the Far East and Himalayan region. The ancestral area

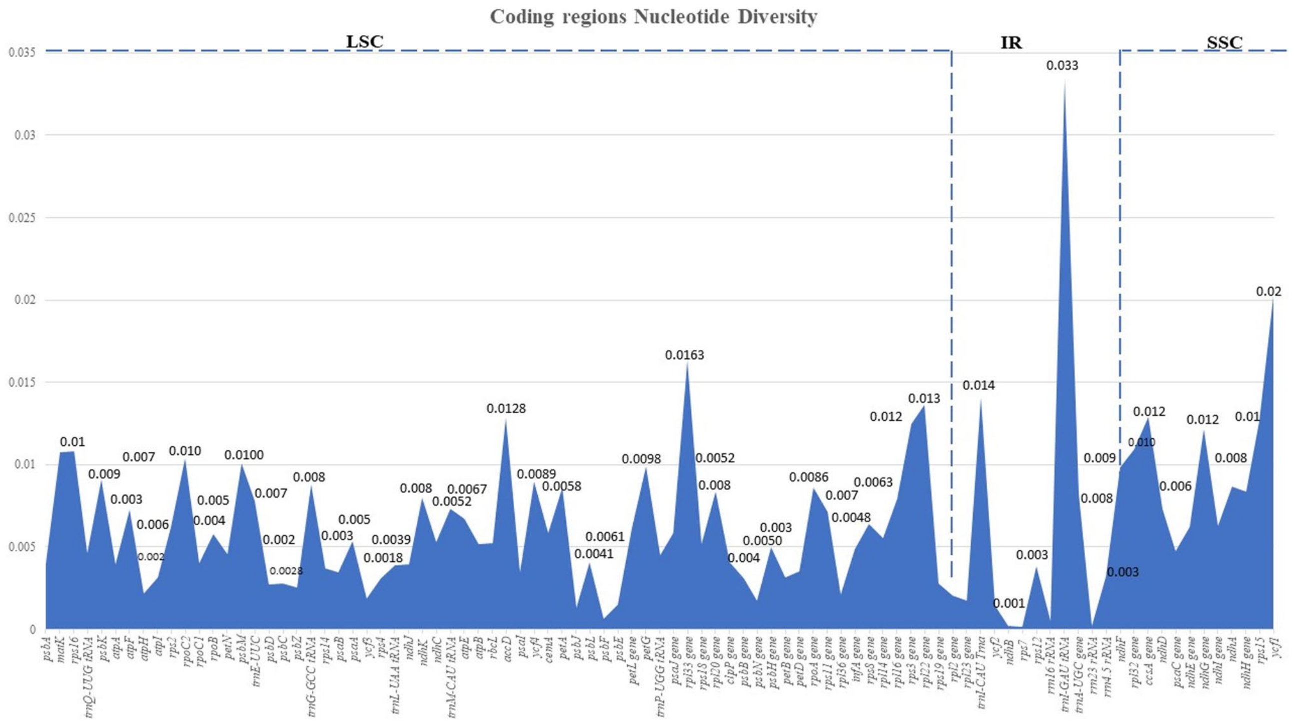

Fig. 2. Nucleotide diversity in the coding genes of 25 species of *Euonymus* is shown in the graph. Genes with zero variability are excluded from the graph.

for *E. bungeanus* and *E. maackii* is recorded as East Asia, Far East, and Southeast Asia with a probability of 0.9 (Node-52) with two dispersal events. *E. europaeus* was inferred to have originated in East Asia, the Far East, West Asia, and the European Region. *E. velutinus*, the western representative of *E.* sect. *Biloculares*, was estimated to have originated in East Asia. The ancestral areas for *E. pauciflorus* and *E. verrucosus* were recorded as East Asia and the Russian Far East (Node-49, relative probability = 0.9). This study found that the region with the highest level of species dispersal was East Asia, as determined with RASP analysis. Additionally, the study found that the region with the highest rate of speciation was also East Asia, with a recorded rate of 25. The Russian Far East had the second-highest rate of speciation, with a recorded rate of 6. Overall, the study found that the global dispersal rate was 57. The rate at which new species develop through time is referred to as speciation[29], and it can be calculated using various techniques. The results of this investigation shed light on the patterns and mechanisms that underlie the distribution and diversification of species across various geographical areas in East Asia, West Asia, Europe etc. Dispersal events from East Asia to different parts of the continent are described in Fig. 6 (S-4).

Discussion

Phylogenetic studies

By using the complete plastid genome, a relationship of twenty-five species of *Euonymus* was discussed in this study with seven species of Celastraceae used as outgroups. An emphasis was on the *E. hamiltonianus* complex and *E.* sect. *Biloculares*. In this study, *E. hamiltonianus* and *E. europaeus* showed a close relationship (Fig. 1) and shared a common ancestral lineage. The same relationship was examined by Park et al., in 2022 and Song et al., in 2014[30,31]. *E. sieboldianus* is embedded within the *E. hamiltonianus* clade, which shows a close evolutionary relation. It suggests that it may represent a lineage within the *E. hamiltonianus* complex. Hara (1996), found few morphological variations between *E. sieboldianus* and *E. hamiltonianus* and suggested that *E. sieboldianus* is a subspecies of *E. hamiltonianus*[32]. Recently, *E. sieboldianus* is described and accepted as a synonym of *E. hamiltonianus*[33]. Our study also investigated the genetic comparison between both the taxa and observed high genetic similarity. LI et al., 2014 gathered *E. hamiltonianus* and *E. europaeus* in one clade due to a similar fruit structure[27]. The Asiatic varieties of *E. hamiltonianus* are closely related to *E. europaeus* based on morphological characters. Blakelock (1951) has distinguished these two species by stating that *E. europaeus* differs in having yellowish anthers and *E. hamiltonianus* (Asian species) had purple to deep red anthers[34]. Species of *E. europaeus*, *E. sieboldianus*, *E. maackii*, and *E. bungeanus* were considered as geographic vicariants by Savinov et al., 2021[35]. They stated that the species share morphological and anatomical similarities, also they grow in the similar conditions. In the past, *E. velutinus* was recognized as a variety of *E. europaeus*[35], but later it was given a species status. *E. velutinus* was not believed as a vicariant species due to morphological delimitations from sister species[35]. In this study *E. velutinus* shows a close relation with *E. europaeus*. *E. alatus* is a member of *E.* sect *Melanocarya* and *E. phellomanus* is representing *E.* sect. *Biloculares*, but it has been claimed that *E. phellomanus* and *E. alatus* belong to the same clade[36]. This conclusion was drawn from *mat*K, *trn*L-F and nuclear genes (ITS and 26 S rDNA), along with morphology. The same relationship was found in the phylogenetic tree of our study

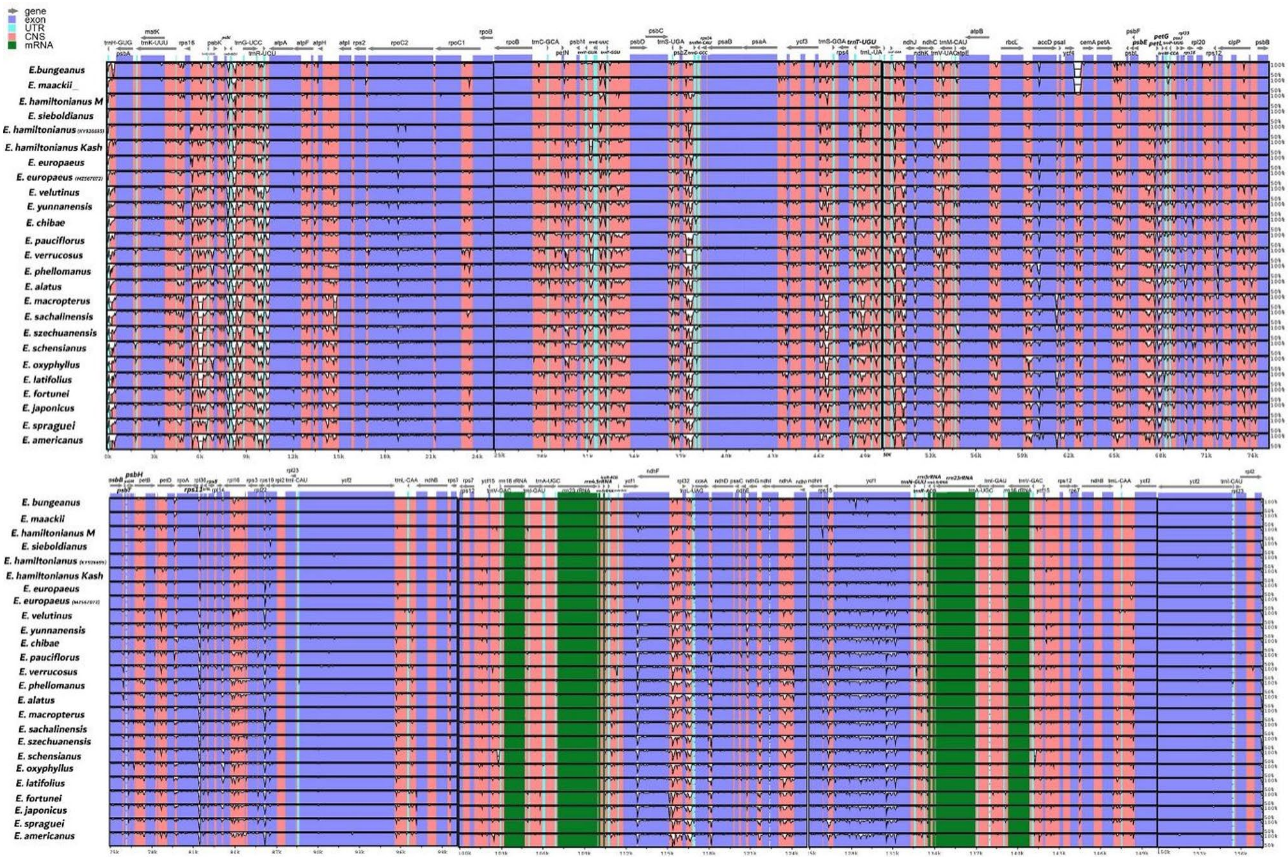

Fig. 3. Using mVISTA, 25 chloroplast genomes of *Euonymus* (15 species of *E.* sect. *Biloculares*) were aligned in parallel with 10 species from other sections of *Euonymus* using *E. hamiltonianus* Jeju (Acc-no, MZ567070) as a reference. The transcriptional directional order of genes is indicated by the arrows. Percent identity on the Y-axis ranged from 50 to 100%. Colour coded regions include exons, introns, and non-coding regions.

where both species were sister and were gathered in one clade (Fig. 1). Morphologically these two species are related due to corky wings on the stem of both species[37]. *E. yunnanensis* which is endemic to China had shown its relatability with sect. *Biloculares* and has demonstrated a sister relationship with *E. chibae* (Bootstrap = 100%, PP = 1.0). *E. americanus* had an unresolved position in the study conducted by Simmons et al., 2012[36]; however, current investigations have demonstrated a resolved connection derived from 80 coding genes in the chloroplast. *E. americanus* is described as a member of sect. *Echinococcus* based on fruit structure, but our analysis based on complete plastid genomes provides a different story. The position of *E. americanus* was slightly different from that of *E. spraguei* (sect. *Echinococcus*), which is endemic to Taiwan. In terms of location of *E.* sect *Uniloculares* and *E.* sect. *Biloculares*, our tree topology resembled the relationship created by LI et al., 2021[13] who used whole plastid genomes of six species of *Euonymus*.

Chloroplast genome features

The total number of protein-coding genes in this study ranged between 133 and 134. The structure of chloroplast genome is a typical structure similar to that of other members of Celastraceae, including LSC, SSC and IR (a typical tetrad)[38,39]. The GC% ranges from 37.4 (*E. verrucosus* and *E. alatus*) to 37.1 (*E. americanus*). *ndh*F was identified to be a pseudogene in three species of *E.* sect. *Biloculares* (Clade I) suggesting a partial loss in this group. In contrast all species in Clade II exhibited *ndh*F pseudogenization. Similarly, *rps*16 was observed as a pseudogene in all Celastraceae members in this study. This indicates a broader range of gene loss in this family. Among the three accessions of *E. hamiltonianus*, in the complex *ndh*F was found pseudogene in *E. hamiltonianus* Mugabsan, South Korea. By comparing it to the previously published work we found that *ndh*F has also reported as a pseudogene in the species of *Saniculiphyllum* C.Y.Wu & T.C.Ku (Saxifragaceae), *Zoaysia* Willd., *Sporobolus* R.Br. (Poaceae), and *Nerine sarniensis* (L.) Herb. (Amaryllidaceae)[40,43]. While pseudogenization of *rps*16 have been observed in species which includes members of Celastraceae, *Gentiana straminea* Maxim[44]., *G. crassicaulis* Duthie ex Burk, *G. robusta* King ex Hook. f. members of Gentianaceae family[45], and *Hepatica* Mill. (Ranunculaceae)[46], *Populus trichocarpa* Torr. & A.Gray ex Hook. (Salicaceae), *Veratrum* L. (Melanthiaceae), non-parasitic species of Fabaceae, and representatives of Orchidaceae[47,49]. Numerous pathways can lead to the emergence of pseudogenes, and spontaneous changes in the coding gene that prevent the gene from being translated or transcribed[50,51]. Chloroplast genomes contain pseudogenes, which are inactive genes that do not produce proteins, but may nonetheless have significant physiological effects because of their ability to maintain

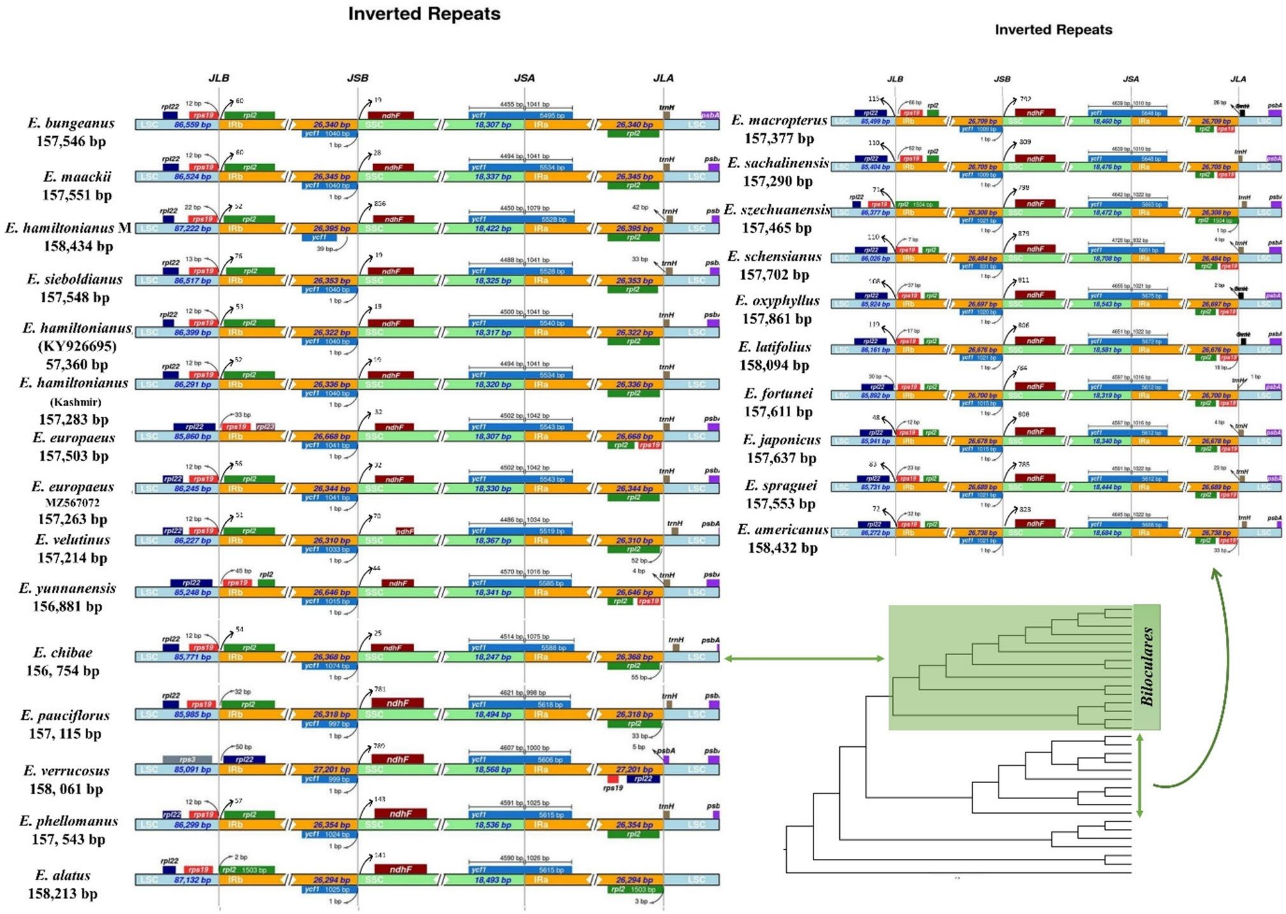

Fig. 4. Comparison of LSC, IR, and SSC junction comparisons between 25 species of *Euonymus* (15 species represented *E.* sect. *Biloculares*).

certain gene regulatory activities[50,52,53]. The relationship between *E. pauciflorus* and *E. verrucosus* was also discussed in this analysis (Fig. 1). *E. pauciflorus* and *E. verrucosus* are regarded as single species[1,2] (*E. pauciflorus* is synonym with *E. verrucosus*). However, in our study, both species displayed certain unique genetic traits. The IR expansion clearly shows this variance. *E. verrucosus* and *E. pauciflorus* were grouped together in a subclade in *E.* sect. *Biloculares*. We observed the variation of IR region in both the species. In *E. verrucosus* the IR junction is expanded and *rpl*22 and *rps*19 were in the IR region compared to *E. pauciflorus*, where both the genes were observed in LSC region (Fig. 4). These two species are described as one species by J. Ma in 2001[1], he ignored the morphological distinctions between the two species with disjunctive distribution and considered them as synonym. The species received the same treatment in Flora of China[2]. Conversely, both the species are treated as independent species by some Japanese and Korean scholars due to some differences in their morphology[11,35,54,55]. According to our analysis, a distinct variation is present according to the IR junction expansion and extraction. The species seems closely related. To determine the exact classification comprehensive research is required.

Age estimation and biogeography

The origin of most of these species used in this study has been recorded in East Asia. The greater climatic diversity of East Asia has been attributed to the exceptionally broad species diversity of vascular plant genera in this area[56]. Numerous variables contribute to the diversity and emergence of new species within a specific region. Such factors include speciation, dispersal, and extinction[57,58]. The climate of Asia is significantly influenced by the size and height of Himalayan mountains and Tibetan Plateau[59,61]. The development of the Gobi Desert, which is in the arid regions of Asia, and changes in the Asian monsoon patterns are also be considered responsible for the evolution and variety of the Asian flora[62]. The last invasion of subtropical plants northward occurred in eastern Asia in the early to middle Miocene. This was followed by the largest maritime transgression in the Neogene, as well as significant species development within numerous woody plant genera[63].

Euonymus is widely distributed, which has sparked interest among many scientists regarding its origin and dispersal patterns. For example, species such as *E. hamiltonianus*, *E. bungeanus*, *E. sieboldianus*, and *E. maackii* are considered to be ecologically and geographically vicariant[35]. In this study, a rough estimation of the time duration was made by determining the period during which each species diverged from its ancestors and sister group, based on the complete plastid genome. Million years ago, *Euonymus* appeared to have evolved during

Node	Mya	95% HPD
1	1.79	0.47-3.49
2	4.2	1.78-7.3
3	4.59	1.88-7.8
4	6.48	2.85-10.6
5	9.65	4.53-15.6
6	12.58	6.57-20.12
7	2.01	0.32-4.38
8	15.88	8.08-25.25
9	26.3	13.45-40.79
10	9.3	2.3-17.9
11	32.22	17.35-50.76
12	12.07	3.67-21.61
13	3.64	0.82-7.8
14	19.92	8.99-32.8
15	34.96	18.9-55.3

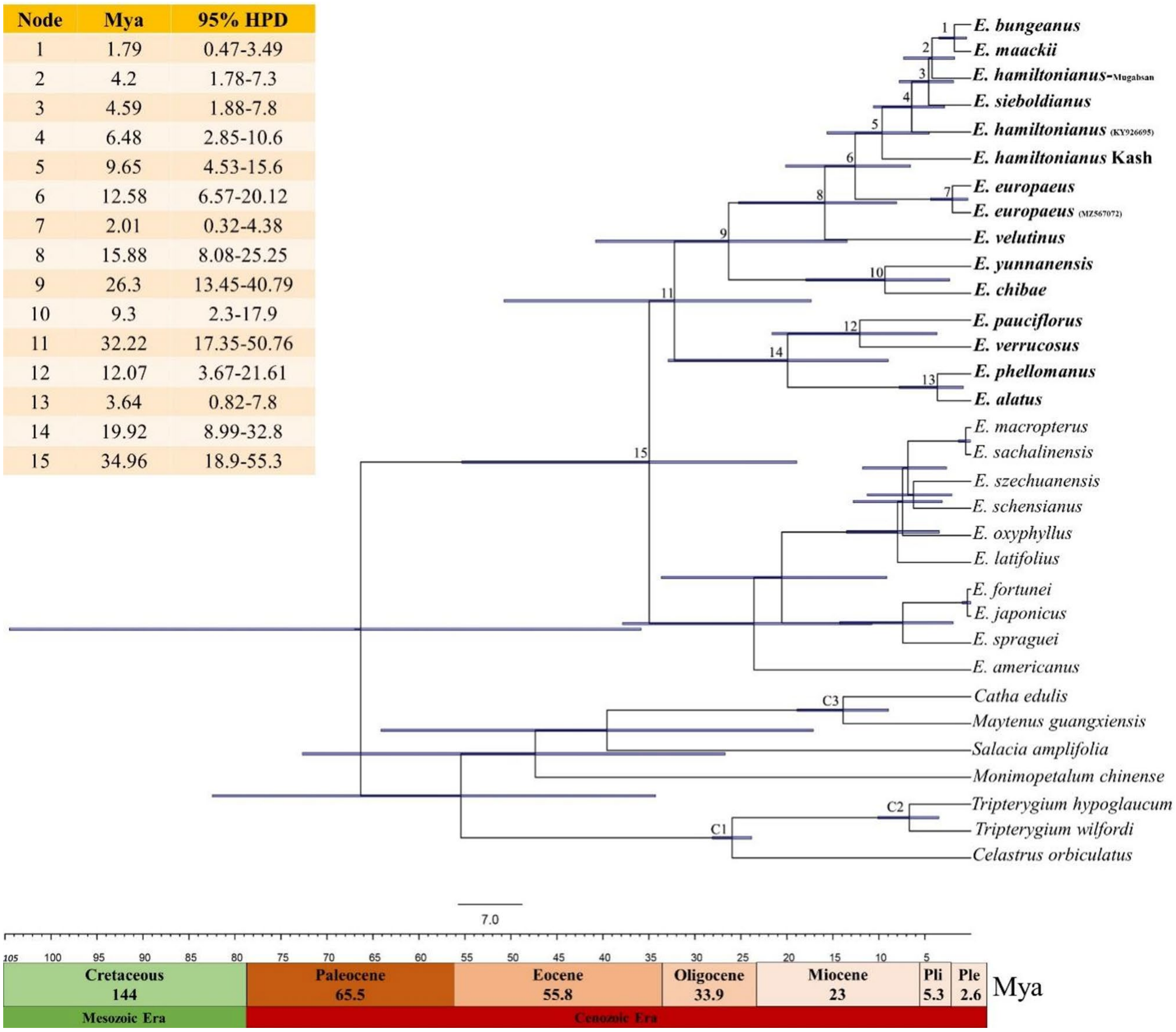

Fig. 5. Chronogram depicting the divergence time estimated in BEAST using chloroplast coding sequences. The 95% posterior probability is represented by a bar for each node. Node 1–15 are nodes of interest, which highlights the diversification in *E.* sect. *Biloculares* and *E. hamiltonianus* complex. The calibration points utilized in the analysis are represented by nodes C1, C2 and C3.

the end of Cretaceous epoch, which spanned from 145 to 66.3 million years ago. Most of the species analysed in this study were reported to originate in East Asia. Specifically, *E.* sect. *Biloculares* appears to have originated around 34.96 Mya, marking the boundary between the Eocene and Oligocene epochs (Figs. 5 and 6). Although this era is not considered an era of massive extinction, the sudden cooling in this era had significant effects on biodiversity. During this time, the Earth's climate changed from being largely devoid of ice to glacial conditions in poles with large ice sheets[64,68].

The origination of *E. europaeus* and *E. hamiltonianus* complex was estimated to be originated in Middle Miocene Epoch, which is considered as an important time period within the Neogene known as the Middle Miocene Transition[69–71]. *E. hamiltonianus* and the species in the complex are widely distributed in East and Southeast Asia, also in some portions of West Asia. Early uplift events in the Himalayan-Tibetan region are thought to be responsible for the beginning of the Indian monsoon in the late Miocene and the strengthening of the East Asian monsoon in the late Miocene and Middle Pliocene[72].

E. americanus is distributed in North America, but its origin is estimated to be in East Asia (Node-47, relative probability-0.89) during the early Miocene epoch. This emphasizes the dispersal and distribution between East Asia and North America. One school of thought asserted that the Bering Land Bridge connected two major globe regions in the Cenozoic era. That bridge is assumed to have contributed to the migration of temperate region flora[73–76]. Also, another school of thought believe that not only the land bridge, but the continental drift and long-distance dispersal might be the reason for such disjunct distribution among the taxa[77].

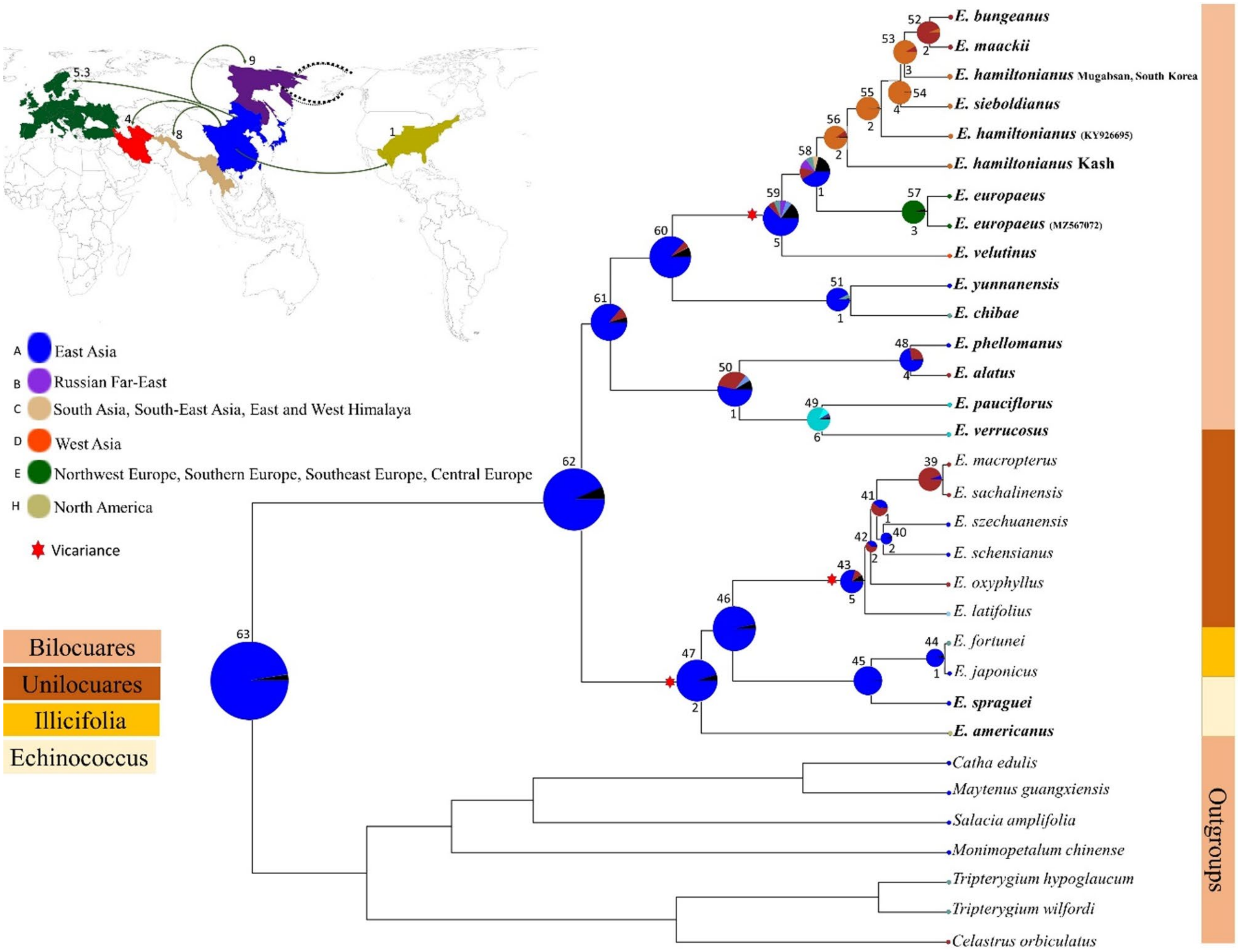

Fig. 6. The Bayesian binary method (BBM) was employed to analyse the biogeography of *E. hamiltonianus* complex and *E.* sect *Biloculares*. The map highlighted six primary areas of interest and the number of dispersal events. The arrows indicate the direction of dispersal between East Asia and other regions. The dotted lines between the continents represents the assumed Bering Land bridge between the big continents. The number above the node indicates the node number. The Number below the node represents the dispersal events recorded at that node. Details of the events at each node are available in S-4 file.

Conclusion

Based on complete chloroplast genome analysis, this study offers a clearer understanding of the evolutionary relationships within the *E. hamiltonianus* complex (*E. hamiltoniaus*, *E. maackii*, *E. bungeanus*, *E. sieboldianus*). This indicates a shared evolutionary history. *E. europaeus* and *E. hamiltonianus* complex shows a strong evolutionary link despite difference in their geographical distributions. *E. velutinus* was not a derived species from the *E. hamiltonianus* group, as it has shown a specific position in the phylogenetic tree in this study. Although the least similarities appeared in the protein coding regions of the species, their position in the phylogenetic tree is clearly distinguishable. *E. verrucosus*, *E. pauciflorus*, *E. alatus* and *E. phellomanus* grouped together in one subclade within *E.* sect. *Biloculares*. All the taxa in *E. hamiltonianus* complex used in this study share many genetic similarities. Far East region is the hotspot distribution region for the species in the complex. The taxa of the complex were inferred to have originated in the Neogene period. East Asia and the Far East are the active region of speciation and diversification of *E. hamiltonianus* complex and related species. Population genetic research on both species from various geographic regions is required for further confirmation of this hypothesis. It was concluded that East Asia is a possible origin of the *Euonymus* and its sections. The species of *Euonymus* spread from East Asia to other continents through a variety of channels. *Euonymus* remains a complex genus that demands further exploration through rigorous research in biogeography and phylogeny, utilizing both morphological and molecular approaches.

Materials and methods

Taxon sampling and DNA extraction

We sampled twelve representative taxa of *Euonymus* in this study (Table 1). Fresh leaves were procured from the International Botanical Gardens and Arboretums and collected from various locations in South Korea

S.no	Species name	Accession numbers
1.	*Celastrus orbiculatus* Thunb.	MW316708
2.	*Tripterygium hypoglaucum* (H. Lev.) Hutch.	MZ064576
3.	*Tripterygium wilfordi* Hook.f.	OK065822
4.	*Monimopetalum chinense* Rehder	MK450440
5.	*Maytenus guangxiensis* C.Y.Cheng & W.L.Sha	MN707924
6.	*Salacia amplifolia* Merr. Ex Chun & F.C.How.	MK799641
7.	*Catha edulis* (Vahl) Endl.	KT861471
8.	*Euonymus europaeus* L.	MZ567072
9.	*Euonymus fortunei* (Turcz.) Hand. -Mazz.	MW288090
10.	*Euonymus hamiltonianus* Wall.	KY926695
11.	*Euonymus japonicus* Thunb.	KP189362
12.	*Euonymus latifolius* (L.) Mill.	OL770076
13.	*Euonymus maackii* Rupr.	MW771518
14.	*Euonymus macropterus* Rupr.	OL770077
15.	*Euonymus oxyphyllus* Miq.	OL770078
16.	*Euonymus phellomanus* Loes.	MW288092
17.	*Euonymus sachalinensis* (F.Schmidth) Maxim.	OL770079
18.	*Euonymus schensianus* Maxim	KY511610
19.	*Euonymus szechuanensis* C.H. Wang	MH853828
20.	*Euonymus yunnanensis* Franch.	MW770452

Table 3. Scientific names and accession numbers of the taxa downloaded from NCBI GenBank.

(Table 1). Leave samples were dried using silica gel. Plant material collected in South Korea were processed as voucher specimens and then submitted to the Gachon University Herbarium. DNA was extracted from the dried materials using the conventional 2x cetyltrimethylammonium bromide method (the common CTAB method)[78]. DNA concentrations were determined using a spectrophotometer (Biospec-nano; Shimadzu). DNA integrity was assessed using gel electrophoresis, and samples with high-quality bands were subjected to quality control testing (QC test). The sites where the 12 species were collected are listed in Table 1, and the GenBank accession numbers are listed in Table 2.

Chloroplast genome assembly annotation

Samples with DNA concentration more than 20 ng/uL were transferred to the next-generation sequencing using the Illumina MiSeq sequencing platform (Illumina, Seoul, South Korea). Sequencing libraries were prepared using Illumina TrueSeq Nano DNA library Preparation Kit. The Raw reads (300-bp paired-end reads) were trimmed to remove error probabilities > 3% per base using Geneious Version 7.1.9[79]. Number of total reads, assembled reads, and coverage is summarized in supplementary file [S1]. Paired end reads were assembled by performing “map to reference” using the already available complete genomes of *Euonymus* spp available in NCBI GenBank. *E. hamiltonianus* (GenBank accession no. KY926695) was used as a reference for assembling nine species of *E.* sect. *Biloculares*. Plastome of *E. maackii* (GenBank Accession no. MW288091) was used to assemble the plastid genome of *E. bungeanus*. To assemble the genome of *E. verrucosus*, *E. pauciflorus* (GenBank Accession no. OP747443 & OP747439) was used as a reference genome. For annotation, Geneious version 7.1.9 was used by applying a 75% similarity index to identify the genes. Once a quadripartite structure was generated, further steps were carried out. GeSeq was used to confirm the annotation patterns of the plastome[80]. The graphical representation of the chloroplast genome was prepared using Orgaellar Genome DRAW (OGDRAW)[81] (S-2).

Comparative genome analysis

To visualize the plastome of *Euonymus* species mentioned in Table 2, it was analyzed in mVISTA in Shuffle-LGAN mode[82]. For this visualization, *E. hamiltonianus* isolate Jeju (accession no. MZ567070) was selected as reference genome. To evaluate the nucleotide diversity (π-value) among the 80 protein coding genes, tRNAs and rRNAs among the plastome of 25 species of *Euonymus* were aligned using MAFFT v7.017 embedded in Geneious 7.1.9[83]. The aligned sequences were extracted individually, and π-value was calculated for each aligned file using DNA sequence polymorphism (DnaSP) v. 6.12.03[84]. The scaled genetic structure of the plastid genomes was visualized using IRscope[85]. The structure and junction between inverted repeats (IR), large singe copy (LSC) and small single copy (SSC) were investigated to observe the shrinkage or expansion of IR regions and SSC regions[85].

Phylogenetic analysis

80 protein coding genes (70,236 bases) from 32 species were concatenated and aligned. These genes were extracted and concatenated from 25 species of *Euonymus* (ingroup) (Table 2) and from 7 species of Celastraceae which were used as outgroups (Table 3). These species include *Celastrus orbiculatus* Thunb. (MW316708), *Tripterygium hypoglaucum* (H. Lev.) Hutch. (MZ064576), and *T. wilfordi* Hook.f. (OK065822), which were also identified as outgroups in Li et al.‘s study[1]. In addition, Simmons et al.[2] noted that *Monimopetalum chinense*

Rehder (MK450440), *Maytenus guangxiensis* C.Y.Cheng & W.L.Sha (MN707924), *Catha edulis* (Vahl) Endl. (KT861471) are relative to *Euonymus*[3]. Furthermore, *Monimopetalum chinense* Rehder (MK450440), *Maytenus guangxiensis* C.Y.Cheng & W.L.Sha (MN707924), and *Salacia amplifolia* Merr. Ex Chun & F.C.How. (MK799641) were included due to the availability of secondary data for age estimation[86]. Twelve species of *Euonymus* (Table 1) were analyzed in the current study, while the rest of the species were obtained from NCBI GenBank (Table 2). The alignment of bases was completed using MAFFT v7.107 embedded in Geneious 7.1.9[83]. PAUP v4.0[87] was used to conduct the maximum parsimony with equal character weighting and gaps were treated as missing data. Searches of 1000 random taxon addition replicates used tree-bisection (TBR) branch swapping, and MulTrees permitted ten trees to be held at each step. Bootstrap analysis was conducted to determine internal support within the same parameters. jModelTest v 2.1.10, was calculated to investigate the applicable model using Akaike's information criterion (AIC)[88] and TVM + I + G was recorded as a best model for Maximum Likelihood analysis. The IQ-TREE web server (http://iqtree.cibiv.univie.ac.at) was used to conduct the ML analysis[89]. Support values were computed using 1000 replicates of ultrafast bootstrap[90]. Bayesian inference analysis was performed using MrBayes v3.2.6[91]. Two runs of at least 1000,000 generations were performed where one tree was sampled for every 1000 generations. 25% of the trees were discarded as burn-in, while the remaining were used to construct a 50% majority-rule consensus tree. The effective sample size (ESS) value was verified for model parameters of at least 200. The phylogenetic tree for ML and Bayesian Inference was observed and edited using FigTree software v1.4.4[92].

Age estimation

The time divergence was estimated by constraining three nodes from the outgroups in this dataset. The C1 calibration point was taken between *Celastrus* and *Tripterygium*, and the estimated age was 26.22 (95% HPD = 24.46–28.17) Mya which was obtained from the studies conducted by Zhu et al.[93],. C2 was the estimated date between the two *Tripterygium* species as 6.85 (95% HPD = 3.92–10.83) Mya[94]. C3 was the calibration point for the age estimation of *Maytenus* sp. The estimated age was 12.2 (95% HPD = 17.4–4.1) Mya[86]. To infer the divergence time, an uncorrelated relaxed clock model was used under the Markov Chain Monte Carlo (MCMC) simulation in BEAST v1.8.3[95] featured by Beauti v1.8.3[95]. The Yule speciation tree prior and normal clock were applied[96]. The length of the MCMC was maintained at a hundred million generations, with parameter sampling every thousand generations. Tracer v1.65[97] was used for convergence, and the ESS were also accessed[98]. After discarding 25% of the trees as burn-in, samples were summarized in an (MCC) maximum clade credibility tree using TreeAnnotator v1.8.3[99]. Ages and 95% HPD were obtained using Tracer v1.6[97] and the results were visualized and arranged using Figtree software v1.4.4[92].

Biogeography

Eight areas were defined for the distribution ranges of the 32 plant species (both ingroup and outgroup). Biogeographic distribution data were compiled from web sources (www.plantsoftheworldonline.org) and flora of China[2,33]. Eight regions were marked based on the geographical distribution of the species with capital letters. (A) East Asia; (B) Russian Far-East; (C) South Asia, South-East Asia, East and West Himalayas; (D) West Asia; (E) Northwest Europe, Southern Europe, Southeast Europe, and Central Europe; (F) North Africa; (G) South Africa and (H) North America. To reconstruct and estimate the spatial patterns of geographic diversification (vicariance, and dispersal) within the species of *Euonymus*, used in this study were inferred by the use of Bayesian binary method (BBM)[100,101] which is implemented in Reconstruct Ancestral State in Phylogeny (RASP) v.3.2[102]. The post burn-in.tree file obtained from BEAST v1.8.3[95] was used for geographical reconstruction.

The fixed state frequencies model (Jukes-Cantor) with equal among-site rate variations was employed to run BBM for 50,000 generations, with 10 chains each and two parallel runs. 1000 trees were discarded during the process. In S-DIVA, the average frequencies of ancestral ranges at a particular node in ancestral reconstructions are determined from all trees. The post burn-in trees from BEAST analysis were utilized for these analyses. To map the ancestral distribution of each node, the consensus tree was obtained through the Compute Condense option in RASP from hundred stored trees in the analysis[103], with the maximum number of ancestral areas set to eight.

Data availability

Twelve chloroplast genomes have been submitted to NCBI (https://www.ncbi.nlm.nih.gov) with accession numbers: OP747439- OP747448 (Table 2).

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

For this study, we collected fresh leaf materials from botanical gardens and arboretums (Table 1). Voucher specimens for the specimens were prepared and submitted to the Gachon University Herbarium (GCU) under the accession numbers (SJ2020-4, SJ2020-5, SJ2020-6, SJ2021-7, SJ2020-8 and SJ2022-9). This study complied with relevant institutional, national, and international guidelines and legislation, and no specific permits were necessary for plant collection. As no endangered or protected plant species were involved, ethical approval or consent was not required.

Consent for publication

Not applicable.

Additional information

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