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NATURAL HYBRIDIZATION BETWEEN ANCIENTLY DIVERGENT *HIPPOPHAE TIBETANA* AND *H. NEUROCARPA* (ELAEAGNACEAE) IN THE EASTERN MARGIN OF THE QINGHAI-TIBET PLATEAU

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Abstract

It is well-regarded that young species are apt to mix their genomes through natural hybridization, whereas the hybridization of old species is rare due to acquired intrinsic reproductive isolation, and alternatively the challenges in detection. In the present study, we identified a hybrid zone of the basal taxon *Hippophae tibetana* mating with *H. neurocarpa* based on morphological and molecular evidence in *Hippophae*. The putative hybrid plants were first discriminated from the sympatric congeneric populations in a field survey, relying on their morphological intermediates of fruit and leaf between the putative parents. Then additivity of the biparental distinctive nucleotide signals of ITS and CHSi, as well as the intermediate features of SSR markers and cpDNA trnS-trnG detected in putative hybrid individuals subsequently, comprehensively support the natural hybridization event that historically occurred. We further estimated that *H. tibetana* and *H. neurocarpa* had diverged 26~27 MYA and hybridization occurred 0.1~0.2 MYA, far from the widely reviewed 5-10 MYA to waiting for hybrid sterility. This is the deepest hybridization event in seed plants studied so far. We speculate that both perennial woody trees and abiotic pollination mechanisms are mainly responsible for the slow evolution of reproductive incompatibility in the genus. Our findings shed light on the incomplete reproductive isolation barriers between members of *Hippophae*, promoting regular hybridization and hybrid speciation in the margins of the Qinghai-Tibet Plateau.

Key words: Hybridization; Nuclear gene morphological characters; *Hippophae*; Chloroplast DNA.

Introduction

Natural hybridization occurs frequently in plants (An, *et al*., 2017), and it frequently results in speciation at the homoploid level or by doubling the genome (Douglas *et al*., 2014; Sarah & Loren, 2014). It is believed to be significant for both plant speciation and diversification (Wang *et al*., 2019, Liao *et al*., 2021). Interbreeding between distinct species is thought roughly about 35% of vascular plant species (Troy *et al*., 2009). Understanding the origins of novel adaptations and the variety of plants can be aided by researching the hybridization process (Li *et al*., 2017). Studies generally show that when more closely associated species are in sympatry, hybridization often takes place and the formation of natural hybrid zones is expected. (Liao *et al*., 2015, Zhang *et al*., 2018, Zheng *et al*., 2021). Heteroploid hybridization is the hybridization of species with various ploidy levels, whereas homoploid hybridization is the hybridization of species with the same ploidy level (Chen *et al*., 2022).

The creation of an entirely novel hybrid species by hybridization without whole-genome duplication and thus without an increase in ploidy is known as homoploid hybrid speciation (Shen *et al*., 2022). Without affecting the number of chromosomes, homoploid hybrid speciation has been shown to be a significant method in the recent past for producing new species and boosting biodiversity (Angélica *et al*., 2022, Wang *et al*., 2022). Furthermore, homoploid hybridization is a fascinating evolutionary process that may result in the introgression of adaptive features into other species or even in the emergence of new species (Adrian *et al*., 2019). Early on in homoploid hybrid speciation, there may be sterility or other fitness barriers that natural selection must get past in order to create new species with distinct genomes and phenotypes (Abbott *et al*., 2010). In addition to having heteropatric and differentiated parental groups, homoploid hybrids also have sympatric parental groups and their hybrid offspring groups. These groups make for great study subjects and natural laboratories for the mechanisms underlying the forces of evolution that drive speciation and differentiation, such as drift, selection, and mutation (Godfrey 1988, Andrea, 2010). Though establishment hybridization and gene flow are anticipated to restrict the evolution of isolation in reproduction and the origin of homoploid hybrid species, the creation of homoploid hybrid species is difficult to explain theoretically, especially when hybrids exhibit homology with their progenitors (Angélica *et al*., 2022, Molly *et al*., 2014).

Since the chromosomal count and genome size of homoploid hybrids are frequently comparable to those of their parental species, the recognition of these hybrids can be challenging (Shen *et al*., 2022). Nonetheless, homoploid hybrids are crucial to the emergence and development of species, particularly in the *Hippophae* species found along the eastern edge of the Qinghai-Tibet Plateau. Eight species make up the tiny genus *Hippophae* within the Elaeagnaceae, according to the most recent systematic treatment (Rousi, 1971). This genus' species are dioecious, wind-pollinated, and capable of seed and clone propagation (Lian *et al*., 1998). Furthermore, diploids (2n = 24) comprise every species and infraspecific variety that is now known (Lian *et al*., 1998). *Hippophae* is known to undergo multiple natural hybridization processes, according to recent research. The *H. goniocarpa* between *H. rhamnoides* ssp. *sinensis* Rousi and *H. neurocarpa* is the earliest diploid hybrid species identified in this genus, in addition to the recently discovered hybridization between *H. rhamnoides* ssp. *yunnanensis* Rousi and *H. neurocarpa*, which may produce *H. gyantsensis* (Liu *et al*., 2016). These investigations have demonstrated the significance of homoploid hybridization in the speciation of *Hippophae* (Sun *et al*., 2003).The categorization places *H. tibetana* and *H. neurocarpa* in the

Sect. *Gyantsenses* of the *Hippophae* genus, which share a geographical distribution that partially overlaps in high altitude regions on the edge of the Qinghai-Tibet Plateau. Both of these species have unique morphologies. The fruit characteristics and reduced height of *H. tibetana* make it easy to differentiate from *H. neurocarpa*. According to a prior phylogenetic analysis, *H. tibetana* is the most basic branch of the genus (Sun *et al*., 2002), and utilizing the *trnT-trnF* region, the divergence time between closely related species is 23.13 Ma before present (Wang *et al*., 2010).

In our field investigations, we observed that some individuals had intermediate morphological characteristics and were distributed sympatrically with *H. tibetana* and *H. neurocarpa*. On the basis of their morphology in the wild, only sixteen natural hybrids have been recognized. Therefore, the purpose of this work was to ascertain if individuals exhibiting morphologically intermediate features are hybrids deriving from *H. tibetana* and *H. neurocarpa* using molecular markers. Plant hybrid assessment has historically been made using a number of parameters derived from crossing experiments, morphology, and distributions (Nora *et al*., 2019). In recent times, different molecular techniques have been employed to identify higher plant hybridization events (Gauri & Park, 2022). The hybrid status of the morphologically intermediate taxon was ascertained in the current work by sequencing biparentally inherited nuclear DNA (nDNA), which included the intron of chalcone synthase-gene (ChSi) and nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS). In order to provide additional genetic evidence to pinpoint the hybridization event, we also use five microsatellite loci. If hybridization is proven to exist then we used maternally inherited chloroplast DNA (cpDNA) (*trnS-trnG* spacer) to determine whether the hybridization is unidirectional or bidirectional, and to establish which the usual paternal or maternal parent is.

Material and Methods

Plant materials: During the field survey in August 2008, only one site where the putative hybrids were living was found on a rocky slope at 4000m above sea level (99°39.185”4′E, 33°46.062′N), near the county of Dari, Qinghai. At the location, several *H. tibetana* were growing adjacent to some individuals of *H. neurocarpa*, while a few wild individuals with apparent intermediate phenotypes of *H. tibetana* and *H. neurocarpa* resided between the two samples with less than 50 meters between them. We collected silica gel dried leaves from almost all individuals (16 individuals) of the putative hybrid. For *H. neurocarpa* and *H. tibetana*, only one tree from every couple or group located fewer 20m apart was sampled to consider clonal reproduction in the genus (Lian *et al*., 2003). Therefore, we obtained 15 individuals of *H. neurocarpa* and 28 of *H. tibetana* within the known range as best as we could find. All possible hybrid voucher specimens as well as putative parental accessions were placed in the Herbarium of Northwest Normal University.

Morphological diagnostic characters: It is anticipated that the morphology of hybrids and hybrid species will lie between that of their paternal progenitors (Zhang *et al*., 2020). In nature, *H. neurocarpa*, *H. tibetana* and the putative hybrid are easily distinguished from each other by their morphological differences. Five to ten ripe fruits as well as mature blades per individual were selected from the three related populations. All materials of the assumed hybrids and both ten individuals of the parental taxa were included. For each individual, four qualitative morphological traits (E.g. Fruit color, ornament etc.) were optically observed, along with seven quantitative ones (E.g. Fruit length, width, etc.) were measured using vernier calipers and so on. In view of the various taxa, statistical parameters such as means and standard deviations were computed using SPSS 11.5 for Windows (SPSS, Chicago, IL, USA) (Table 1, Fig. 1).

DNA extraction, PCR amplification and sequencing: Genomic DNA was isolated from dried leaf tissues using the CTAB method as described by Doyle & Doyle (1987). To uncover nucleotide additivity of nuclear genes in hybrids, the nrDNA ITS region was amplified using the universal primes P1and P4 following the PCR procedures of Sun (Sun *et al*., 2002 & 2023). Additionally, the nuclear CHSi region (intron of Chalcone synthase) was amplified using primers CHSx1F: 5′-AGGAAAAATTCAAGCGCATG-3′ and CHSx2RN: 5 ′-TTCAGTCAAGTGCATGTAACG-3′ (Strand *et al*., 1997) according to the procedures of Bartish (2000). In addition to determining the direction of hybrid mating, the universal primers trn-S and trn-G (Pierre *et al*., 1991) were applied to PCR amplify of cpDNA *trnS-trnG* fragments. PCR products were detected by electrophoresis on a 1% agarose gel. The target band was recovered using a gel extraction kit, and the target fragment was ligated to the pMD19-T vector using a T vector ligation kit. The competent cells (DH5α) were then transformed. The bacteria were coated onto the prepared ampicillin plate, and the positive colonies were screened by an inverted culture overnight. The recombinant plasmid of the target gene was extracted and sent to Beijing Qingke Biotechnology Co., Ltd. for sequencing.

Microsatellites analysis: To assess genetic variation of the suspected hybrid zone and estimate the differentiation among sympatric congeners, two microsatellite loci were newly developed using 5′-anchored PCR in our lab (Fisher *et al*., 1996). One is (TGA)8, which is amplified by NHTP-27 (F: AACCACAGCAAAACAAAAAAC; R: TAA AAATACACCTCCAACTCA), and the other is (A)8…(T)6, amplified by HTI-01 (F: GACG CTTGGC GACAATATAACA; R: CAAACCCAT AGCCTC TACCTCC). In combination with HR-06 from Wang (2008), five pairs of microsatellite primers producing polymorphic bands between the putative hybrids and their parents were selected to amplify the sampled individuals following the procedures of Zhou (2010). Silver-stained bands on urea-polyacrylamide gel (Creste *et al*., 2001) were read and recorded in a Microsoft Excel sheet. Principal coordinate analysis (PCO) was executed in GenAlEx 6.0 (Peakall & Smouse, 2012) to cluster individuals into genetically similar populations, without assuming Hardy-Weinberg equilibrium or linkage equilibrium (Nick *et al*., 2006, Thibaut *et al*., 2009).

Table 1. Variation (mean ± 1 SD) in morphological characters of *H. neurocarpa, H. tibetana*, and putative hybrids.

Morphological character	*H. neurocarpa*	putative hybrids	*H. tibetana*
Plant height (m)	1-3.6	0.5-1	0.1-0.5
Fruit color	Brilliant Black or deep green	Olivedrab	Deep jacinth
Fruit morphology	Cylindrical,curvature	Taper or cylindrical	Ellipse
Longways arris in fruit	Yes	Yes	No
Ornamentation in fruit	No	No	Six top green stellate shaped decorations
Fruit length (mm)	6.40 ± 0.82	10.07 ± 1.09	8.73 ± 0.77
Fruit width (mm)	3.23 ± 0.48	6.19 ± 0.60	8.55 ± 1.01
Stalk length (mm)	0.40 ± 0.06	1.06 ± 0.13	1.48 ± 0.44
Blade length (cm)	2.60 ± 0.46	2.43 ± 0.38	1.68 ± 0.18
Blade width (cm)	0.36 ± 0.06	0.36 ± 0.08	0.33 ± 0.05
Petiole length (cm)	0.11 ± 0.02	0.12 ± 0.03	0.12 ± 0.02

Table 2. Additive nucleotide sites in the aligned chalcone synthase intron (Chsi) sequences and internal transcribed spacer (ITS) sequences that differ between *H.neurocarpa, H. tibetana*, and their putative hybrids.

Taxon	Position in the CHS intron alignment									ITS
	64	66	104	127	141	189	195	264	335	227
H. neurocarpa	A	A	A	C	T	A	-	A	T	C
The putative hybrid	R	W	W	Y	W	W	G/-	W	W	M
H. tibetana	G	T	T	T	A	T	G	T	A	A

DNA sequences analysis and evolutionary time estimation: Using the software CLUSTAL X (Higgins *et al*., 1997), the sequences of three DNA fragments were independently aligned, with manual corrections made where needed. DnaSP 5.10 was used to assign the alignments to different haplotypes (Rozas *et al*., 2003). Further DNA polymorphism parameters, such as Pi and Hd were also calculated. Utilizing the trnS-trnG sequences, the chlorotypes network was built utilizing the NETWORK v. 4.5.1.0 program (http://www.fluxus-engineering.com) in order to ascertain the direction of hybridization. A minimum spanning tree was combined into a single network using the median joining method, and then median vectors according to the parsimony criterion were added (Fig. 2).

To establish a timeline for parental split and historical meeting again, several sequences of *Hippophae* and *Elaeagnus* previously published by Sun (Sun *et al*., 2002) and Jia (2012) were downloaded from GenBank (AF440241-AF440258; HM769690-HM769697; FJ665687-FJ665692; JQ289198, JQ663590, JQ289217, JQ663595-JQ663597). Using J Model Test version 2.1.4, we first selected the DNA substitution model and the associated model parameters. Next, we used MEGA 11 to apply a likelihood ratio test to evaluate the molecular clock hypothesis for ITS sequences. Since there was a substantial difference between constrained and unconstrained analyses for ITS sequences (JC+G+I, 2lnLR=80.7997, d.f. = 18, p<0.01), the idea of a molecular clock could be discarded. Therefore, BEAST version 1.7's relaxed molecular clock technique was used to estimate evolutionary periods based on ITS sequence variation (Alexei *et al*., 2012). A previously published topology of the ITS gene tree (Sun *et al*., 2002) was specified as the starting tree under the guidance of the software. An uncorrelated lognormal distribution across trees with a mutation rate of 0.244×10-9 s/s/y was used to explicitly model the rate change (Wang *et al*., 2010). Every 1000 generations, thirty million Markov chain Monte Carlo (MCMC) searches were conducted and sampled. TRACER 1.7 was used to examine the MCMC chains' convergence (Andrew *et al*., 2018). Using TreeAnnotator 1.8, maximum clade credibility (MCC) trees were annotated; the first 10% of samples were burned in. Fig Tree v1.4.2 showed the posteriors, averages, and 95% highest posterior densities (HPDs) of node ages.

Results

Morphological identification: In Dari field investigation, both *H. neurocarpa* and *H. tibetana* show diagnostic differences in their fruit and leaf features that distinguish them from each other. The putative hybrids display either intermediate traits or a resemblance to one species or the other (Table 1; Fig. 1). The intermediate hybrid is typically exemplified by the plant height: *H. tibetana* is very dwarf about 0.1-0.5 m, *H. neurocarpa* high about 1-3.6 m, while the putative hybrids have a height range of 0.5-1 m. Although the fleshy fruit of the assumed hybrid is analogous to *H. tibetana* in nature, with cylindrical morphology, olivedrab color and longways arris ornamentation, the hybrid fruit contrasts with the opposite taxon, *H. neurocarpa*. And the quantitative distribution of many vegetative traits in the hybrid zone, such as leaves that are 2.60 cm in length and 0.36 cm in width, are also closer to *H. neurocarpa* of 2.43 cm and 0.36 cm than to those of *H. tibetana*. So, the investigated characters of the putative hybrid are more like *H. neurocarpa* than the others to some extent. In addition, only one investigated quantitative morphological character of the suspected hybrids, fruit length, with an average of 10.07 mm, is transgressive to the parents' measurements of 6.40 mm and 8.73 mm in *H. neurocarpa* and *H. tibetana* respectively (Table 1, Fig. 1).

Fig. 1. Fruits and leaves of *H. neurocarpa*, *H. tibetana* and the putative hybrid.

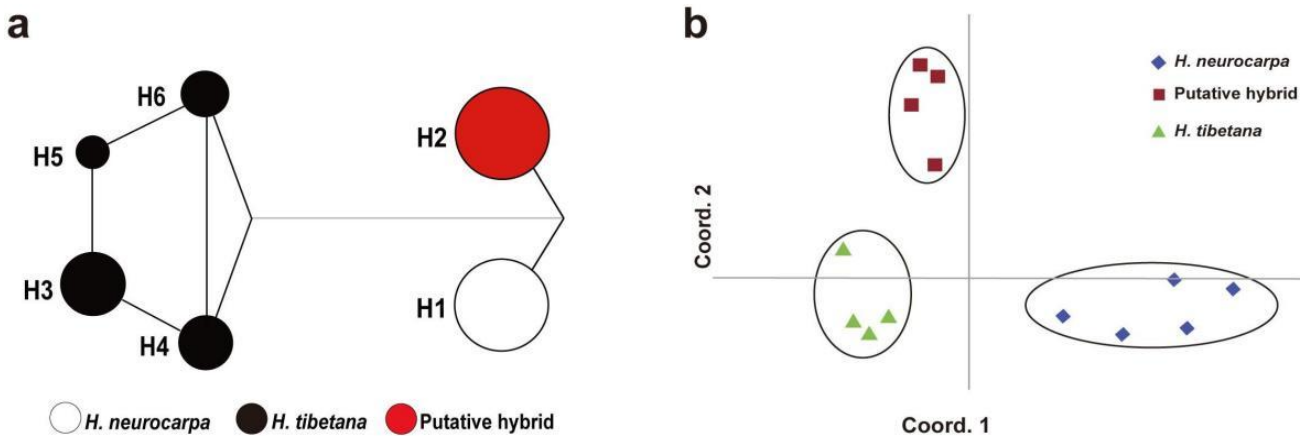

Fig. 2. (a) The most parsimonious network depicts the six chloroplast DNA haplotypes, with haplotypes displayed within or adjacent to each circle. The size of each circle approximately reflects the frequency of the respective haplotype across all three species. The pie charts within each circle indicate the proportional representation of each species (b) Results of the principal coordinate (PCO) analysis of *H. tibetana*, *H. neurocarpa* and putative hybrids used in this study based on genotypic data from five SSR loci.

Table 3. The statistics of amplification sites of the putative hybrid and the putative using SSR primers.

Primer name	HTP-06(bp)	NHTP-27 (bp)	HR-06(bp)	HTI-01(bp)	HTP-08 (bp)
H. neurocarpa	110, 110	240, 242	70, 90	130, 130	127, 127
	110, 116	242, 242	90, 90		
	110, 112		80,92		
Putative hybrid	110, 112	238, 242	70, 87	130, 130	127, 129
		238,238			
H. tibetana	110, 110	242, 242	70, 86	130, 130	127, 129
		238, 238	70, 94	130, 132	
			70, 80	130, 134	

ITS sequences and clones of nuclear DNA CHSi sequences: For both *H. neurocarpa* and *H. tibetana*, the sequences of ITS and CHSi were obtained by cloning and sequencing. The aligned ITS sequences matrix generated a total of 695 bp, comprised of ITS1(270 bp), 5.8S (163 bp), and ITS2 (262 bp) regions. A total of two types of ITS sequences were found in *H. tibetana*. The 15 individuals in the *H. neurocarpa* group all had identical ITS sequence (GenBank accession FJ817083), this sequence was very different from *H. tibetana* (GenBank accession FJ817084, FJ817086). And all the ITS sequences of putative hybrid individuals (GenBank accession FJ817082) were similar to *H. neurocarpa*, besides single nucleotide substitution. Furthermore, only position 229 was polymorphic (C/A) (double peaks on the electropherogram) in all the investigated 16 putative hybrid individuals (Table 2).

We obtained two distinct types of CHSi sequences corresponding to *H. neurocarpa* and *H. tibetana* by cloning and sequencing, respectively (Table 2). The length of the CHSi gene of *H. neurocarpa* was 371 bp, but it was 372 bp for *H. tibetana*. There were 9 different positions, including 8 nucleotide substitutions and a 1-bp indel, between *H. neurocarpa* and *H. tibetana*. These sites explicitly distinguished the sequences of *H. neurocarpa* (GenBank

accession number OP484697) from that of *H. tibetana* (GenBank accession number OP484698). Therefore, the molecular data provided strong evidence for the hybrid status of these 16 accessions.

Nucleotide positions in the whole alignment are represented by numbers. The remaining locations are the same for both species and their hybrids, and polymorphisms are indicated by the IUPAC ambiguous symbols R=A+G, W=A+G, M=A+C, and Y=C+T.

Note: Except for 34 gaps or indeles, there are 93 parsimony informative substitutions in ITS sequences alignment, which may be derived from the ancient divergence between *H. neurocarpa* and *H. tibetana*. Only one additive site between different hybrid individuals is listed in the table.

SSR analysis: Five pairs of effective SSR primers produced a total of eighteen distinctive gel bands representing different alleles in the sampled individuals. These bands showed a co-dominant pattern and resulted in 10, 10 and 6 types of various genotypes in *H. tibetana*, *H. neurocarpa* and the putative hybrid, respectively (Table 2). Unlike the higher heterozygosity observed in Dari populations of the putative parent, four out of the five SSR loci amplified the same bands in all 16 putative hybrids. Only one pair of primers showed identical amplification results in 15 hybrid individuals (NHTP-27: 238, 238), except for one individual that showed a different band (NHTP-27: 238, 242). There are many common bands in the samples, several of which combine into an apparent additive form in the putative hybrid individuals with the two sympatric taxa. For example, the 238-bp band of NHTP-27 was present in *H. tibetana* but absent in *H. neurocarpa*. In contrast, the 112-bp HTP-06 band was present in *H. neurocarpa* but absent in every *H. tibetana*. However, the putative hybrids possessed both of the two bands (238 and 112-bp). The PCO statistical analysis result shows that the two principal coordinates, which account for 65.55% of the variance (37.68% and 27.87% for the X and Y axes, respectively) in the samples, clearly separated all individuals into three clusters corresponding to the three taxa in this investigation. It is clear that the intermediate positions of the putative hybrid individuals were closer to *H. tibetana* than to *H. neurocarpa* along Coordinate 2 (Fig. 2a).

Chloroplast DNA trnS-trnG sequences: Sequences alignment of 662 bp was obtained from the chloroplast trnS-trnG region of samples in *H. tibetana*, *H. neurocarpa* and the putative hybrid, which contains 22 informative substitution sites (3.32%) and 72 indels (10.88%). All the cpDNA sequences are divided into two distinctive groups containing six haplotypes in total, under the condition of taking every gap/indel into account, named as H1-H6. Group I consists of H1 of *H. neurocarpa* and H2 of the putative hybrid, which only differ at three singleton transition sites (- → A, G → A, G → A). The high sequence similarity of the two haplotypes reaches up to 99.55 percent. Group II comprises the remaining four haplotypes: H3, H4, H5, and H6. All of them are found in *H. tibetana* and contribute to generating a relatively high genetic diversity in the Dari population, with Hd = 0.556 (SD=0.075) and Pi = 0.00092 (SD = 0.00012). There are many differences between group I and group II haplotypes of trnS-trnG sequences, which involve ninety-four variable sites. Network analysis reveals the reticulate relationship of the chlorotype lineages in the sympatric populations of the hybrid zone in (Fig. 2a).

Evolutionary history estimation: A relaxed molecular clock approach was used to time the putative parental split and hybridization in the evolutionary process of the genus. The age of the most recent common ancestor (MRCA) of all taxa in *Hippophae* was estimated to be 26.18 Ma (95% HPD, 13.76–46.82 Ma) from the nuclear ITS data (Fig. 3) and 24.14 Ma (12.26–37.56 Ma) from the trnL-trnF data (Fig. 4), which correspond to the divergent time of *H. tibetana* from the other taxa, because it is usually treated as the basal branch in the phylogeny of the genus (Sun *et al*., 2002). The split time of the Dari population from the allopatric congener populations in *H. tibetana* and *H. neurocarp*a is figured out as 6.70 Ma (2.44-14.24) and 1.92 Ma (0.47-4.60), respectively, based on the ITS data. These dates are somewhat consistent with the dates of 3.15 Ma and 0.57-2.76 Ma estimated by Wang (H *et al*., 2010). The only informative site between *H. neurocarpa* N261 and the putative hybrid NT1 was roughly calculated to be 0.18 Ma (0.00-0.88 Ma), appropriately reflecting the re-meeting time or hybridization time of *H. tibetana* and *H. neurocarpa*.

Discussion

Plants frequently experience natural hybridization, which is essential to the evolution and survival of species (Zheng *et al*., 2021). It can promote speciation and innovation by transferring adaptive traits through introgression, creating recombinant forms, or inducing alloploidy (Soltis *et al*., 2009). In comparison to allopolyploidy, homoploid hybridization involves hybridization between parental species without a change in chromosome number, leading to the emergence of new hybrid species or hybrid zones (Angélica *et al*., 2022). In the case of homoploid hybrid speciation, the emergence of a new lineage occurs after the hybrids formed in hybrid zones become reproductively isolated from their parents due to ecological and spatial barriers (Gross & Rieseberg, 2005). Therefore, hybrid zones may represent an important aspect of homoploid hybridization until the hybrids are fully established (Yang *et al*., 2019). Overlapping geographic distributions between species of *Hippophae* provide spatial opportunities for hybridization, and partially overlapping flowering periods may also contribute to natural hybridization. So, there is a possibility of hybridization occurring between *Hippophae* species. Only 16 natural hybrids have been identified on the basis of morphological traits and were distributed sympatrically with *H. neurocarpa* and *H. tibetana* at 4000m above sea level. However, so far there has been no report describing natural hybridization between *H. neurocarpa* and *H. tibetana*. The hybrid characters of the leaf and fruit displayed intermediate or mosaic traits of the parent species (Table 1, Fig. 1). According to previous studies, proofs of the morphology could infer that these individuals are hybrids (Zhang *et al*., 2007), and demonstrate that hybrids, *H. goniocarpa*, correspond to the intermediate traits of their parents (Ma *et al*., 2014). So, we infer that these 16 individuals may be hybrids between *H. neurocarpa* and *H. tibetana*.

We used nuclear genes (ITS and CHSi sequences) in this study to detect whether or not there was a hybridization event between *H. neurocarpa* and *H. tibetana*. The two species varied by complete additivity for the ITS site, which was seen in all hybrid accessions under examination. We first detected hybridization in *Hippophae* using the CHSi gene. And the putative hybrids studied here possess two types of CHSi sequences, each corresponding to that of *H. neurocarpa* and *H. tibetana*, indicating perfect additivity of *H. neurocarpa* and *H. tibetana* CHSi types as shown in Table 2. Genetic admixture between *H. neurocarpa* and *H. tibetana* was detected in the putative hybrids. The sequence evidence has strongly confirmed the occurrence of spontaneous natural hybridization, which was previously presumed based on morphology.

Chloroplast DNA, usually maternally transmitted in angiosperms (Mogensen *et al*., 1996), can be used to determine the maternal parent of hybrids (Andrea & Rieseberg, 2002). In addition, cpDNA has been proven to be maternally inherited in *Hippophae* (Bartish *et al*., 2002). The *trnS-trnG* sequences of the hybrids were very similar to *H. neurocarpa* and extremely different from *H. tibetana* (Table 3), and the chlorotype (H2) of the hybrids in network analysis also showed a closer relationship to *H. neurocarpa* (Fig. 2a). Consequence is that *H. neurocarpa* was the maternal parent and there was unidirectional hybridization. This is not the same situation as found with *H. goniocarpa*. Both *H. rhamnoides* ssp. *Sinensis* and *H. neurocarpa* ssp. *Neurocarpa* appears to have contributed together to the maternal establishment of *H. goniocarpa* (Wang *et al*., 2008). In the present study, the probable cause for asymmetrical hybridization is the difference in flower phenology between *H. neurocarpa* and *H. tibetana*. There may be a high reproductive barrier between the parents because no crossing was found between *H. neurocarpa* as the paternal parent and *H. tibetana* as the maternal parent. *H. neurocarpa* and hybrids do not have identical chloroplast DNA sequences, which does not correspond to other correlational studies (Zha *et al*., 2010). There are several possible causes for the extraparental chloroplast found in hybrids. The reasons for this ambiguity are that some *H. neurocarpa* individuals may still not have been found in the area or could belong to samples that were impossible to measure in the experiment.

In hybrids, the nuclear genome is equally inherited from each of the two parent species. As a result, its PCO analysis ought to reveal intermediate and well-separated sites from the two different parents (Zha *et al*., 2010). PCO analysis using five microsatellite markers revealed distinct *H. tibetana* and *H. neurocarpa* clusters, as well as the presence of separate individuals (Fig. 2b). The hybrid population exhibited a separate group, suggesting a hybridization event occurred between *H. tibetana* and *H. neurocarpa*. From the SSR analysis, the hybrids clearly resemble *H. tibetana* more than *H. neurocarpa* (Fig. 2b). The ITS sequences of hybrids are very similar to those of *H. neurocarpa*, and the hybrids show a closer genetic distance to *H. tibetana* in the SSR analysis. These two results support the conclusion of hybrid origin. The putative hybrids exhibited an additive pattern, with two alleles (238-bp and 112-bp) provided solely by *H. tibetana* and *H. neurocarpa*, respectively. The hybrid individuals' nuclear genomes were shown to be generated from *H. tibetana* and *H. neurocarpa*, according to SSR analysis. Hybrids displayed high amplification consistency of five SSR loci, indicating that all hybrids collected may be reproducing through clonal growth. Stressful situations may benefit from clonal plants' increased fitness (Jacquemyn *et al*., 2005), so the hybrids on rocky slopes can increase resource acquisition through vegetative propagation. According to our germination experiment, hybrids have a lower germination rate than their parents, suggesting that clonal propagation may be more favorable than sexual reproduction of hybrids.

Plant speciation that produces a hybrid species without affecting the number of chromosomes is known as homoploid hybrid speciation. At high altitudes, the geographic distributions of *Hippophae* species frequently overlap in parts on the Qinghai-Tibet Plateau. Furthermore, adaption to a novel or harsh habitat is thought to encourage homoploid hybrid speciation (Gompert *et al*., 2006). The heterogeneous terrain of the Qinghai-Tibet Plateau may provide an opportunity to accelerate hybridization by reducing reproductive isolation barriers in species of *Hippophae*. In the study of reproductive isolation of the *H. goniocarpa* natural hybrid zone, the findings indicated that the parents and hybrid species of *Hippophae* did not have complete prezygotic and postzygotic isolating mechanisms (Gompert *et al*., 2006). *Hippophae* species have a widespread existence, incomplete reproductive isolation and overlapped geographic distribution. These are probably the main reasons for homoploid hybridization. We deduced that the speciation and evolution of *Hippophae* species may have been significantly influenced by homoploid hybrid speciation. Furthermore, understanding the homoploid hybrid created by two sympatric distributions of *Hippophae* is crucial to understanding how the hybrid zone is maintained as well as what happens to the hybrid progeny in terms of evolution.

To date, we have only found natural hybrids between *H. neurocarpa* and *H. tibetana* near the county of Dari. The only plot may be that increasing levels of disturbed habitats promote hybridization (Ma *et al*., 2010). The site of hybridization is located at the foot of a river with a bridge crossing it. So, *H. neurocarpa* and *H. tibetana* were affected by some human activities. Unusual hybridization conditions and favorable habitats for hybrid survival are produced by disturbances. These may encourage species that are typically allopatric to co-colonize (Zha *et al*., 2010, Lamont *et al*., 2003). Furthermore, because human disruption of natural ecosystems breaks the phenological barrier that separates previously isolated species, hybrid establishment has been encouraged more and more (Zha *et al*., 2010). If we conduct inadequate investigations, we may also find hybrid individuals between *H. neurocarpa* and *H. tibetana* in other places.

Overall, our study's combination of molecular and morphological data provides strong evidence for the natural hybridization of *H. neurocarpa* and *H. tibetana*, as well as the identification of *H. neurocarpa* as the maternal parent in this hybridization event. Determining the new hybrid's evolutionary location within *Hippophae* and whether or not new hybrid individuals are more fit than their sympatric parents remain challenging based on current research. To confirm the relationship between *H. tibetana* × *H. neurocarpa* within the genus *Hippophae* and to assess fitness, competition, and resource acquisition with its sympatric parent species in the same regions, more phylogenetic work is thus required. This will offer enough proof to clarify the hybrid zone's maintenance process.

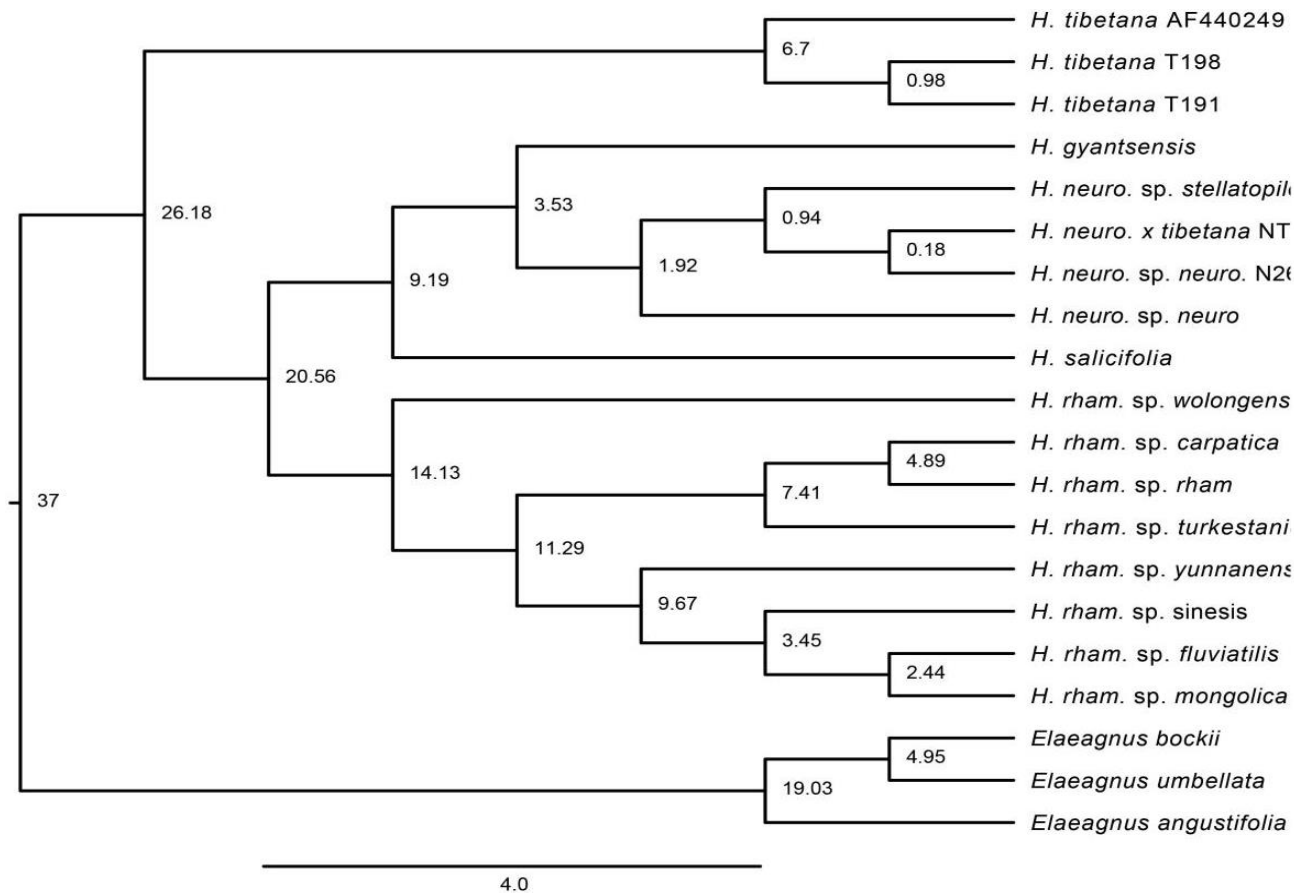

Fig. 3. Estimates of evolutionary divergence between ITS sequences.

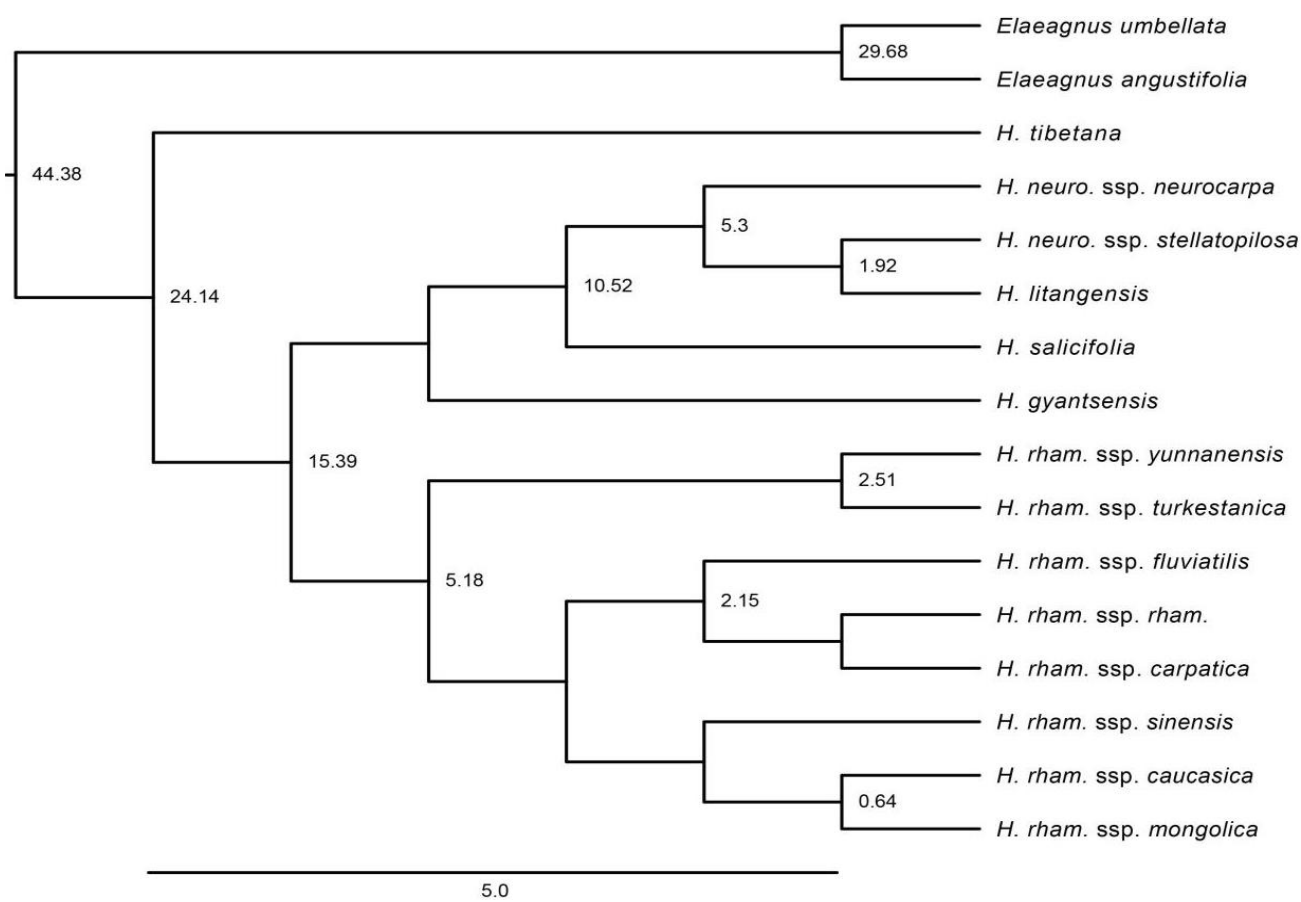

Fig. 4. Estimates of evolutionary divergence between trnL-trnF sequences.

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