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Development and Application of Hypervariable Chloroplast Molecular Markers in Bambusoideae

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Abstract: *Molecular markers of plant hypervariable chloroplast genomes are important genetic markers and have become an important auxiliary means for species identification and phylogenetic analysis. Bambusoideae species are hard to be identified based on external morphology. Therefore, based on the comparative analysis of the whole genome sequences of chloroplasts of five published Bambusoideae species, the hypervariable chloroplast molecular markers of bamboos were selected and developed, and the genetic diversity and phylogeny analyses of nine Bambusoideae species based on five chloroplast molecular markers were performed. The five markers are rbcL, matK, trnH-psbA, trnL-trnF, and trnG-trnT, with mutations 2 - 40 including indels; the π values of nucleotide diversity are 0.00058-0.03256, and θw values are 0.00097 - 0.02743. The π and θw values of trnG-trnT are the highest, indicating the genetic diversity of trnG-trnT is the highest. Phylogenetic analysis shows that nine Bambusoideae species are clustered into two branches, with Pleioblastus amarus,Pseudosasa japonica and Yushania nana clustered into one branch, and Bambusa blumeana,Dendrocalamus latiflorus,D. minor, Sarocalamus yongdeensis, D. barbatus and D. giganteus clustered into another branch. This study is of great significance for phylogenetic research, species identification and new cultivars development of Bambusoideae.*

Keywords: *Bambusoideae; Chloroplast; Hypervariable region; Molecular markers*

1. Introduction

The Bambusoideae are a natural group of Poaceae [1]. The distribution of Bambusoideae are primarily concentrated in tropical and subtropical regions, with a limited number of species also found in temperate and subfrigid regions [2]. The Bambusoideae are a highly significant non-wood renewable forestry resource and an essential component of garden greening, which has high economic and ecological value [3].

The classification of Bambusoideae poses a challenging conundrum of taxonomy [4]. In the systematic studies of Bambusoideae, due to different research purposes and limited sampling, different experimental materials and fragment markers were used in various studies, which made the results obtained by analysis not completely uniform, resulting in unclear division of some branches of Bambusoideae. For example, the relationship between the three tribes of Bambusoideae were contradictory [5]; which about the four old World woody bamboo subfamilies, Hickelinae and Racemobambosinae have no systematic position resolution or only low support [6,7]. In addition, the boundary between the Hickelinae and the Bambusoideae was unclear. And some species of Greslania, which originally belonged to the Bambusoideae, were congenered with the Hickelinae [8].Previous studies supported a sister-group relationship between Chusqueinae and Guaduinae + Arthrostylidiinae [7]. The division of species within individual genera is also unclear; for example, the *S. yongdeensis* belonged to *Bashania*, but now belongs to *Sarocalamus* [9,10]. Up to now, Bambusoideae has been divided into three tribes: Bambuseae, Arundinarieae and Olyreae [7]. The Hickelinae, Racemobambosinae, Chusqueinae,Guaduinae and Arthrostylidiinae are now part of Bambuseae. There are nearly 1,700 species of bamboo in 127 genera worldwide [11,12]. However, due to the diversity of Bambusoideae, the classification system of Bambusoideae needs to be constantly updated and revised [11].

With the development of molecular systematics, the new methods for bamboo classification at home and abroad are mainly based on chloroplast molecular markers [13,14]. The molecular information provided by chloroplast genome sequence is a good resource for plant systematics, phylogenetic studies and population genomics [15,16], for example, Liu [17]and Zhou [18] are based on *B. basihirsuta*, *De.*

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bambusoides, *De. barbatus*, *De. birmanicus* and *Pl. amarus* provided new molecular data for elucidating the evolution of Bambusoideae; Wang et al [19]and Ma et al[20]compared the whole chloroplast genome sequence by using mVISTA, and found that mutation-rich regions, such as hypervariable regions, in some plant lineages were valuable sources of genetic markers, which could provide a theoretical basis for plant species identification [21,22]. Feng et al [23] proposed a new strategy of high-mutation fragment mining based on the materials of the genus *Primulina*, and developed high-mutation chloroplast molecular markers from the true hypervariable regions (Con_Seas) of the entire chloroplast genome. Therefore, mutations in chloroplast genomes are widely used for species identification, molecular marker development, evolutionary analysis, and high-resolution phylogenetic analysis [24,25].

Currently, there has been a lack of systematic research on the development and application of hypervariable chloroplast molecular markers in Bambusoideae. In this study, the chloroplast hypervariable fragments were selected by comparing the whole chloroplast genome of 5 species of Bambusoideae, then the primers are designed for the hypervariable fragments, and PCR and Sanger sequencing were performed to verify the generalizability and validity of the primers. Furthermore, the mutation and genetic diversity of Bambusoideae were analyzed and phylogenetic trees were constructed, the ultimate goal is to provide essential data and theoretical foundation for classification and identification of Bambusoideae, as well as genetic breeding, germplasm resource identification, classification protection.

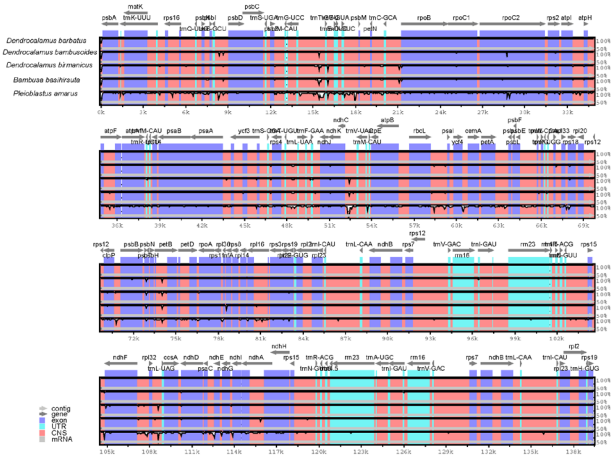
2. Results and analysis

2.1 Screening of hypervariable fragments of chloroplast genome in Bambusoideae

There were 7 chloroplast hypervariable fragments selected by the method of Feng et al [23], as shown in Table 1, and their length are range from 115-593 bp. The hypervariable fragments *trnH-psbA*, *ndhE*, *trnV-UAC* and *trnL-UAG* in chloroplast genome were analyzed based on mVISTA (Figure 1). The hypervariable fragments, including *matk*, *trnL-trnF*, *rbcL*, *trnG-trnT*, and *trnH-psbA*, were subjected to development primer, PCR amplification, and sequencing validation.

Table 1: Seven hypervariable fragments in chloroplast genome of five species in the Bambusoideae

Number	Start site	End site	Length (bp)	Annotation
1	1561	1769	209	*matK*
2	7805	7991	187	*trnS-GCU*
3	15542	15949	408	*trnG-trnT*
4	16015	16607	593	*trnE-UUC*
5	20413	20970	378	*trnC-GCA*
6	49457	49571	115	*trnL-trnF*
7	56999	57216	218	*rbcL*



Note: The vertical and horizontal axes in the figure represent the consistency degree of the sequences from 50% to 100% and the position in the chloroplast genome, respectively; Annotated genes are displayed along the top.

Figure 1: Structural alignment of chloroplast genomes of five species in Bambusoideae with De. barbatus genome as reference genome

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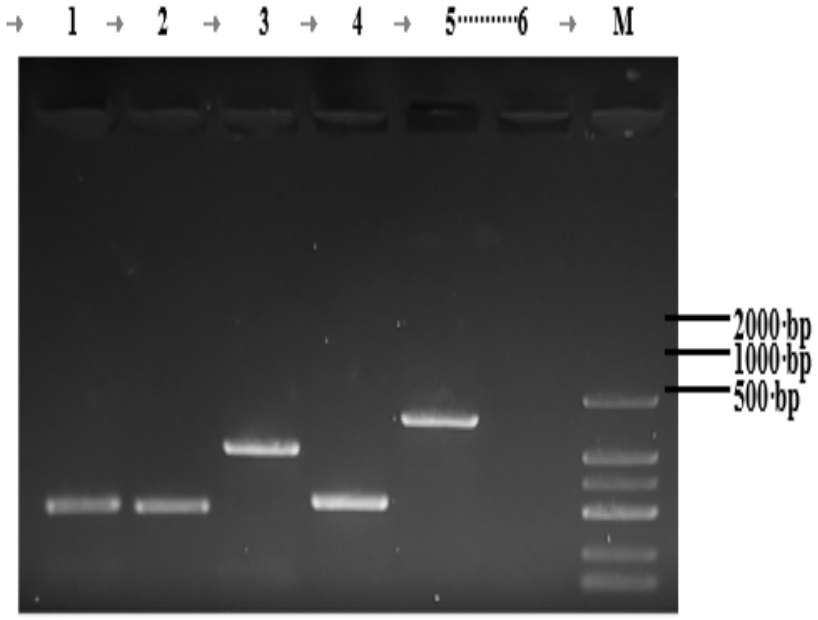
2.2 Primers design, PCR amplification and sequencing of hypervariable fragments of chloroplast genome in Bambusoideae

Primers were designed according to the selected region of highly variable fragments. The primers of 5 hypervariable chloroplast DNA fragments were shown in Table 2, and the length of PCR products ranged from 459-1500 bp. PCR amplification was performed on 9 species of Bambusoideae with 5 pairs of primers, then agarose gel electrophoresis and Sanger sequencing were performed, the results showed that all five hypervariable DNA fragments could be successfully amplified and sequenced, and the PCR bands were single and bright, which indicated that the primers had good universality and specificity. The primer sequence, region length, annealing temperature, and GenBank number of the five hypervariable chloroplast DNA fragments are shown in Figure 2.

Table 2: Primer sequence, region length, and annealing temperature of hypervariable fragments

Molecular marker	Primer(5'-3')	PCR products length /bp	Annealing temperature /°C	GenBank number
trnG-trnT	F:GAGGAGTTAGGTATGTAGGG R:TTTTATTGGCTGATCTTATG	459	47	OQ338140-OQ338146 OR268943-OR268944
**matK*	F: AAACAGAAATCTCGTCAA R: AGGGTTCACCAGGTCATT	1500	45	OQ348039-OQ348045 OR286207- OR286208
rbcL	F: CCGGAGTATGAAACCAAGGA R: AAATCAAGTCCACCGCGTAG	518	50	OQ348046-OQ348052 OR286205-OR286206
trnH-psbA	F: GAATTTGCAATAATGCGATGG R: ATCCGACTAGTTCCGGGTTC	530	53	OQ348053-OQ348059 OR295458-OR295459
trnL-trnF	F: CCTGAGCCAAATCCGTGT R: GGATGATGCACAAGAAAAGG	711	50	OQ348060-OQ348066 OR268945-OR268946

**matK* adopted the primers in Li et al [26], and primers for other fragments were designed in this study.



Note: 1: trnH-psbA primer pair; 2: trnG-trnT primer pair; 3: trnL-trnF primer pair; 4: rbcL primer pair; 5: matK primer pair; 6: blank; M: DNA Marker DL 2000

Figure 2: Agarose gel electrophoresis of five hypervariable fragments PCR products from De.giganteus

2.3 Analysis of mutation in hypervariable fragments of chloroplast genome in Bambusoideae

Table 3: Mutation sites of five hypervariable DNA fragments in Bambusoideae species

Mutation site location of *trnG-trnT*/bp																	
Species	7	17	27	66	94	96	102	104	105	114	125	130-133	147-152	198	226	251	256
1	C	A	T	T	T	G	T	G	G	C	T	----	------	G	C	G	C

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2	A	A	T	T	T	G	T	G	G	C	T	----	------	G	C	G	C
3	C	A	T	T	T	G	T	G	G	C	T	----	------	G	C	G	C
4	A	A	T	T	T	G	T	G	G	C	T	----	------	G	C	G	C
5	C	A	T	T	T	G	T	G	G	C	T	----	------	G	C	T	C
6	C	A	T	T	T	G	T	G	G	C	T	----	------	G	C	T	C
7	A	G	G	C	C	G	T	G	G	C	C	AAGG	------	T	T	T	T
8	A	G	G	C	C	A	C	T	T	A	C	TTGG	------	T	T	T	T
9	A	G	G	C	C	G	T	G	G	C	C	TTGG	TAAAAA	T	T	T	T
	259	272	282	296	299	303	320	343	353	359	361	385	389	392			
1	T	T	C	C	A	G	A	C	C	A	G	A	T	T			
2	G	T	C	C	C	G	G	C	G	T	G	A	T	C			
3	T	T	C	C	A	G	A	C	C	A	G	A	T	T			
4	G	T	C	C	C	G	G	C	G	T	G	A	T	C			
5	T	T	C	C	A	G	A	C	C	A	G	A	T	T			
6	T	T	C	C	A	G	A	C	C	A	G	A	C	T			
7	T	C	A	A	C	A	A	T	G	G	G	G	T	T			
8	T	C	A	A	C	A	A	T	G	G	A	G	T	T			
9	T	C	A	A	C	A	A	T	G	G	G	G	T	T			
Mutation site location of *ttrnL-trnF*/bp																	
	13	34	38	67	142	167	179	334	337-379	381	384-385	387-391					
1	G	A	G	A	A	T	G	C	CCT	A	CT	ACTAT					
2	G	A	G	A	A	T	G	C	CCT	A	CT	ACTAT					
3	G	A	G	A	A	T	G	C	CCT	A	CT	ACTAT					
4	G	A	G	A	A	T	G	C	CCT	A	CT	ACTAT					
5	G	A	G	A	A	T	G	C	CCT	A	CT	ACTAT					
6	G	A	G	A	A	T	G	C	CCT	A	CT	ACTAT					
7	C	G	A	G	T	C	A	T	---	-	--	-----					
8	C	G	A	G	T	C	A	T	---	-	--	-----					
9	C	G	A	G	T	C	A	T	---	-	--	-----					
	429	446	505-507	542	552	553	611										
1	C	A	---	A	C	T	C										
2	C	A	---	A	C	T	C										
3	C	A	---	A	C	T	C										
4	C	A	---	A	C	T	C										
5	C	A	---	A	C	T	C										
6	C	A	---	A	C	T	C										
7	G	G	GCT	C	T	C	T										
8	G	G	GCT	C	T	C	T										
9	G	G	GCT	C	T	C	T										
Mutation site location of *trnH-psbA*/bp																	
	52	140															
1	-	G															
2	-	G															
3	-	G															
4	-	G															
5	-	G															
6	-	A															
7	A	G															
8	A	G															
9	A	G															
Mutation site location of *rbcL*/bp																	
	11	119	134	163	297	319	428										
1	G	A	C	A	A	T	T										
2	G	A	C	A	C	T	T										
3	G	A	C	A	A	T	T										
4	G	A	C	A	C	T	T										
5	G	A	C	A	A	T	T										
6	G	A	C	A	A	T	T										
7	C	G	T	A	C	A	C										
8	C	G	C	T	C	A	C										
9	C	G	T	A	A	A	C										
Mutation site location of *matK*/bp																	
	154	160	188	213	272	281	286	293	317	319	332	341					
1	G	T	C	-	G	-	-	-	-	T	-	-					
2	G	T	C	-	G	-	-	-	-	T	-	-					
3	G	T	C	-	G	-	-	-	-	T	-	-					
4	G	T	C	-	G	-	-	-	-	T	-	-					
5	G	T	C	-	G	-	-	-	-	T	-	-					
6	G	T	C	-	G	-	-	-	-	T	-	-					
7	T	C	A	-	C	-	-	-	-	C	-	-					
8	T	C	A	-	G	-	-	-	-	C	-	-					
9	T	C	A	-	G	-	-	-	-	C	-	-					

Note:1: De. latiflorus; 2:B. blumeana; 3: De. minor; 4: S. yongdeensis; 5: De. giganteus; 6: De. barbatus; 7: Ps. japonica; 8: Y. nana; 9: Pl. amarus

The mutation site location of hypervariable DNA fragments *rbcL*, *matK*, *trnH-psbA*, *trnL-trnF*, and

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t*rnG-trnT* in the chloroplast genome of Bambusinae are shown in Table 3, including indels. *trnG-trnT* has the largest number of mutation, with 42 mutations, including 16 transitions and 16 transversions, and 10 indels, the occurrence of simultaneous transversion and indel at sites 130-132, as simultaneous transition and transversion at site 359, was also observed; the second largest number of mutation is *trnL-trnF*, with 29 mutations, including 10 transitions and 4 transversions, and 15 indels; next is *matK*, with 12 mutations, including 2 transitions and 3 transversions, and 7 indels; next is *rbcL*, with 7 mutations, including 3 transitions and 4 transversions; the least is *trnH-psbA*, with 2 mutations, including 1 transversion and 1 indel. The highest frequency of indel is 35.9% in these mutations, the second is conversion, the frequency of transition is 34.8%; the lowest frequency of transversion was 29.3%.

2.4 Genetic diversity and neutrality of chloroplast DNA fragments in Bambusoideae

In this study, the genetic diversity of 5 chloroplast DNA fragments of Bambusoideae was analyzed and neutral test was performed (Table 4). The length of five chloroplast DNA fragments are 381-599 bp. The results of single nucleotide polymorphism showed that the number of segregating sites of 5 chloroplast DNA fragments range from 1-29, Haplotype diversity (*H*d) are 0.222 - 0.917, nucleotide diversity θw and π values were 0.00097-0.02743 and 0.00058-0.03256, respectively. Among the five chloroplast DNA fragments, *trnG-trnT* has the highest θw value, π value and *H*d, *trnH-psbA* is the lowes*t*, it indicated that *trnG-trnT* has the highest level of genetic diversity. The neutral test results of the whole analysis of these 5 DNA fragments showed that *Tajima's D* values, *Fu and Li's D** values, *Fu and Li's F** values were positive in *rbcL*, *matK*, *trnL-trnF*, *trnG-trnT* and *trnH-psbA*. But there was no significant difference. So these five chloroplast DNA fragments fit the neutral evolutionary model, and it can be used for phylogenetic analysis of Bambusoideae (Table 4).

Table 4: Genetic diversity and neutrality tests of five DNA fragments by DnaSP

DNA fragments	Number of sequences	Length	θ_w Nucleotide diversity	π Nucleotide diversity	Number of polymorphic segregating sites	H*d*	Neutrality tests		
							D	D*	F*
trnG-trnT	9	389	0.02743	0.03256	29	0.917	0.73999(ns)	0.69910(ns)	0.79537(ns)
matK	9	421	0.00437	0.00528	5	0.556	0.88079(ns)	0.68670(ns)	0.81551(ns)
rbcL	9	458	0.00562	0.00691	7	0.806	1.02379(ns)	0.92083(ns)	1.05421(ns)
trnH-psbA	9	381	0.00097	0.00058	1	0.222	-1.08823(ns)	-1.18990(ns)	-1.28293(ns)
trnL-trnF	9	599	0.00921	0.01206	15	0.556	1.48909(ns)	1.28707(ns)	1.49522(ns)
Overall	9	2248	0.04760	0.08995	57	3.057	3.045	2.404	2.877

Note: Length without indels; D: Tajima's D value; D*: Fu and Li's D* value; F*: Fu and Li's F* value; ns: non-significant, P > 0.10;θ_w:Theta (per site) from S

2.5 Construction of chloroplast genome phylogenetic tree in Bambusoideae

In order to determine the position of nine Bambusoideae species in Bambusoideae, the complete genome sequences of 48 species of Bambusoideae were obtained from NCBI after blastn with the *trnG-trnT* fragments of nine species of Bambusoideae, represented by *trnG-trnT* with the most mutation, phylogenetic tree was then constructed using highly similar sequences to get more information(Figure 3). *Pl. amarus* is associated with the genus of *Oligostachyum*,*Shibataea*,*Indosasa*,*Gelidocalamus* and *Fargesia*; *Ps. japonica* goes with *Thamnocalamus spathiflorus*; *Y. nana* goes with *Chimonobambusa hirtinoda*; *B. blumeana* and *S. yongdeensis* are associated with the genus of *Thyrsostachys*; and *D. barbatus*, *D. giganteus*, *D. latiflorus*, *D. minor* associated with the genus of *Melocalamus.* Phylogenetic trees constructed based on *matK*, *rbcL*, *trnL-trnF*, *trnG-trnT* and their combined chloroplast DNA fragments showed similar topological structures (Figure 4). Nine species of Bambusoideae were successfully divided into two branches. Among them, the *De. latiflorus*,*B. blumeana*,*De. Minor*,*S. yongdeensis*,*De. Giganteus*,*De. barbatus* are clustered into one, belonging to the Bambuseae, its approval rating is above 80%; *Ps. Japonica*,*Y. nana* and *Pl. amarus* are clustered into another, belonging to the Arundinarieae, its approval rating is above 80%. Phylogenetic trees of *trnH-psbA* alone could not distinguish the nine species of Bambusoideae due to their low mutation. Phylogenetic tree based on *rbcL*, *trnG-trnT* and combined chloroplast DNA fragments

Phylogenetic trees based on *rbcL*, *trnG-trnT* and combined chloroplast DNA fragments were compared to phylogenetic trees based on other fragments, these fragments have sites that distinguis, Which *B. blumeana* and *S. yongdeensis* are together, *De. latiflorus*,*De. Minor*,*De. Giganteus* and *De.*

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Barbatus are together.

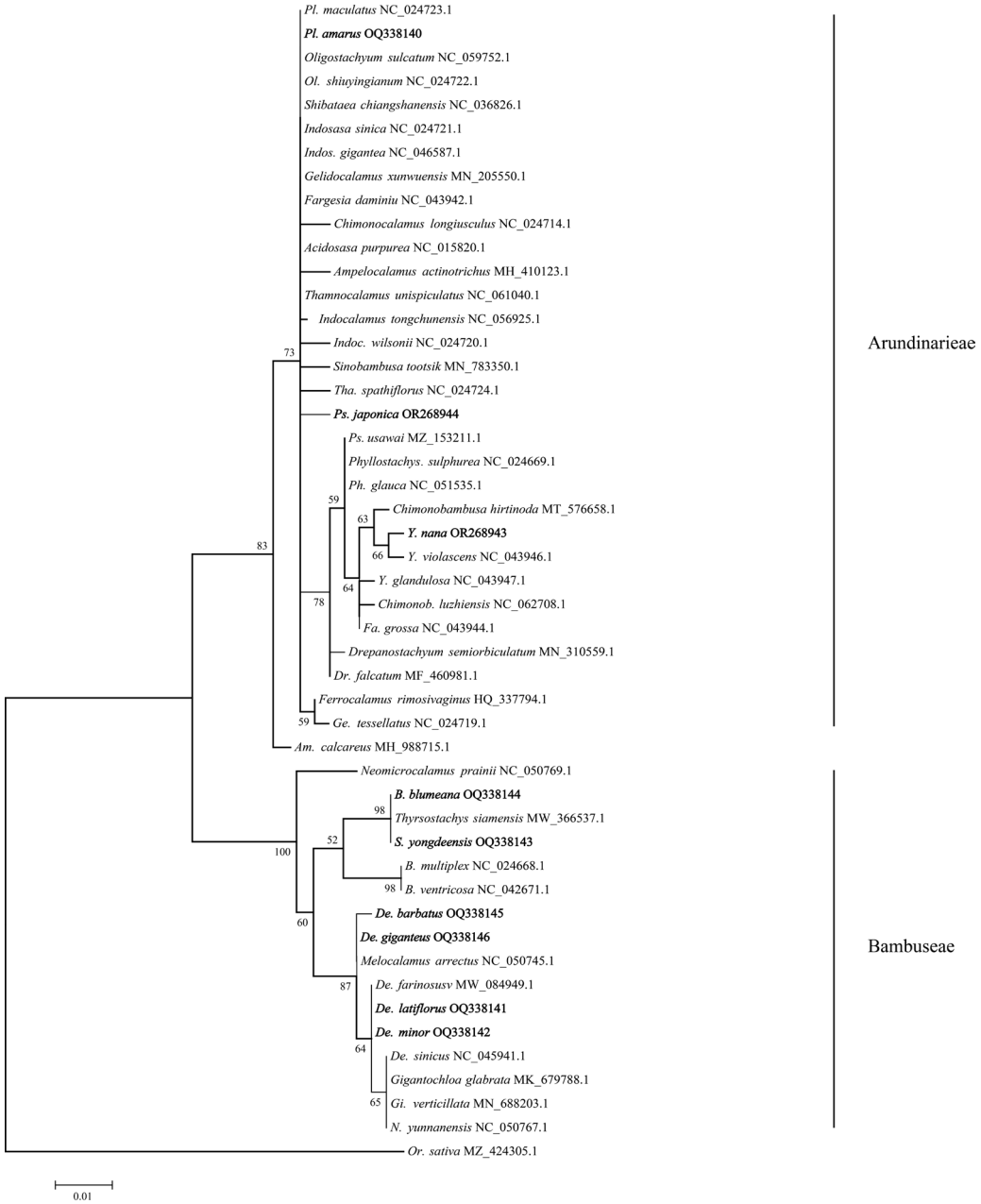

Figure 3: ML phylogenetic tree was constructed based on the chloroplast genome sequences of 48 Bambusoideae species and the trnG-trnT fragment of 9 excellent economic bamboo species; Hide branches with a support rate of less than 50%; Or. sativa was used as the outgroup.

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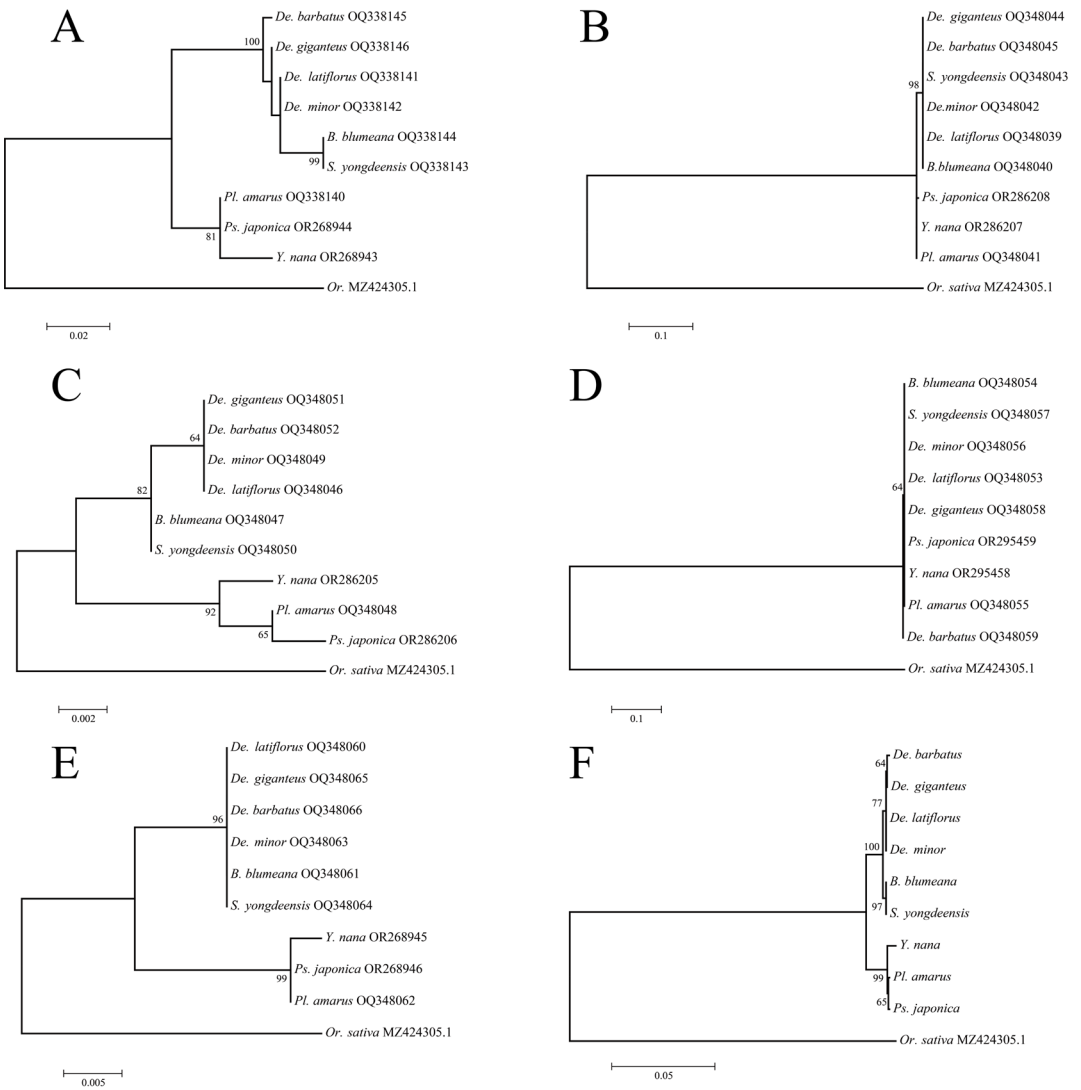

Note: A: ML Phylogenetic Tree Based on trnG-trnT; B: ML Phylogenetic Tree Based on matK; C: ML Phylogenetic Tree Based on rbcL; D: ML Phylogenetic Tree Based on trnH-psbA; E: ML Phylogenetic Tree Based on trnL-trnF;F: ML Phylogenetic Tree by Combining Five Gene fragments

Figure 4: ML Phylogenetic Tree Based on DNA fragment of 9 Excellent Economic Bamboo Species; Hide branches with a support rate of less than 50%; Or. sativa was used as the outgroup.

3. Summary

Bambusoideae is an important forest resource, which can replace wood products and provide food, and has great social and economic value [27]. Therefore, the development of molecular markers of Bambusoideae is helpful to distinguish and utilize Bambusoideae better.

At present, chloroplast sequencing and comparative genomes have been used to screen hypervariable regions as specific DNA molecular markers for species identification [21,22]. However, there are few studies combining the method of Feng et al[23] and mVISTA to screen hypervariable regions.

After constructing a single phylogenetic tree and a combined phylogenetic tree of 5 hypervariable region DNA fragments in this study, the results showed that the two branches of the phylogenetic tree were in agreement with the classification of Bambusoideae by Kelchner et al [7], these two tribes are Bambuseae and Arundinarieae. The *S. yongdeensis* belonged to *Bashania*, but now belongs to *Sarocalamus*, and is a separate genus. The reason is because that and strong similarities between the Himalayan type species and a species from N Yunnan were evident, which species from the Himalayas and China appear congeneric on morphological and phylogenetic grounds, so *Bashania* is transferred into *Sarocalamus* [10].

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In this study, DNA markers based on *matK*, *rbcL*, *trnL-trnF* and *trnG-trnT* fragments can effectively identify nine species of Bambusoideae, and the combination of four hypervariable region DNA fragments can also effectively identify nine species of Bambusoideae, providing scientific molecular basis for identification of Bambusoideae. It is hoped that this study will provide a new tool and choice for the identification of bamboo species and the study of the relationship between bamboo species.

4. Materials and methods

4.1 Data collection

In this study, 5 species of Bambusoideae were selected as the research objects for screening the chloroplast genome hypervariable region of Bambusoideae, and then screened the bamboo from the *B. basihirsuta* (NC_050773.1), *De. bambusoides* (NC_050762.1), *De. barbatus* (NC_050747.1), *De. birmanicus* (NC_0507501) and *Pl. amaru*s (MH988736.1) from GenBank.

4.2 Plant material

In this study, nine species of Bambusoideae were used to verify whether the selected chloroplast genome regions are hypervariable. They are *B. blumeana*,*D. latiflorus*,*D. minor*,*S. yongdeensis*,*D. barbatus*,*D. giganteus*,*Pl. amarus*,*Ps. japonica* and *Y. nana*. The plant material were mainly sampled in Yibin, Sichuan Province. *Pl. amarus* was taken from Cuiping District, Yibin (104°37′E ,28°46′N), and other samples were all taken from Jinglei Zhuhai, Sichuan (104°36′E,29°08′N). The leaves were put into sampling bags and brought back to the laboratory to be dried with silica for further DNA extraction.

4.3 Identification of hypervariable region of chloroplast genome in Bambusoideae

In this study, In this study, Feng et al's method and LAGAN model mVISTA program were adopted [28,29], the chloroplast genome was used as reference to compare the chloroplast genome with that of *B. basihirsuta*, *D. bambusoides*, *D. barbatus*, *D. birmanicus* and *Pl. amaru*s to determine the hypervariable region.

Feng et al's method needs to determine the determinism and polymorphism index of the Con_Sea region before determining the hypervariable region. The region labeled "Con_Islands" in conserved sites in the chloroplast genome was identified for the first time, and the region between two adjacent Con_Islands was named "Con_Seas." In this study, the Perl script of Feng et al's was used to obtain the locations of chloroplast mutation regions of *B. basihirsuta*, *D. bambusoides*, *D. barbatus*, *D. birmanicus* and *Pl. amarus*, and the parameter "Con_Island minimum length" (50 in this study) could be changed. Potential information traits (PICs) calculated by the sum of SNP and Indel between the two chloroplast genomes can be used to evaluate the polymorphism of each region. Then, according to the starting and ending positions of chloroplast mutation fragments obtained by Perl script, the fragments with length greater than 100bp and PICs value greater than 1 are selected as the hypervariable region.

4.4 Design of primers for the hypervariable region of chloroplast genome in Bambusoideae

In this study, the whole chloroplast genome sequence of *D. barbatus* was selected for primers design. Primers were designed using software Primer Premier v 5.0 [30] according to the hypervariable region. Finally, the 5 pairs of primers with the highest score were selected from the 5 fragments. The primers were synthesized by Beijing Liuhe Huada Gene Technology Co., Ltd.

4.5 DNA extraction, PCR amplification and sequencing of plants

By improving the method described by Fulton et al. [31], total DNA was extracted from the dried silica gel leaves, then it was detected by agarose gel electrophoresis and ultraviolet spectrophotometry, and then the extracted DNA was diluted 4 times and stored at -20℃ until PCR amplification [32]. Then, pairs of hypervariable primer selected from comparative chloroplast genome analysis of five Bambusoideae species were selected for amplification of target bands. The PCR experiment used the Thermal Cycler for PCR. PCR amplification was performed at a final volume of 50 μL, including 5 μL diluted DNA by a factor of four, 25 μL of 2×Taqmix enzyme, 10 mM forward primers and reverse primers are 3 microliters each (Beijing Liuhe Huada Gene Technology Co., Ltd, China), and 14 μL ultrapure water. The PCR was performed at 95℃ and predenaturated for 5 min. 95℃, denaturation 45 s; annealing for

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30 seconds at different temperatures (the annealing temperature of each primer pair is provided in Table 2), extended for 1min at 72℃, and 30 cycles were performed; The product was extended at 72℃ for 5 min and cooled to 4℃ for 10 min. The PCR products were detected by 1% agarose gel electrophoresis and then sent to BGI for sequencing on ABI 3730 sequencer.

4.6 Data analysis

The sequences were performed using SeqMan (DNA STAR package; DNA Star Inc., Madison, WI, USA) edited and assembled.

The software DnaSP 5.0 [33] was used to calculate the number of mutations in five DNA fragments, including the number of polymorphic segregating sites (S), the Haplotype diversity (H*d*), the average difference value for each base of two random sequences (π) [34], and Watterson's parameter (θ) based on the number of segregating sites [35].

In order to test whether interspecies evolved neutrally, DnaSP was also used to calculate the values of *Tajima's D* [36], *Fu* and *Li's D** and *Fu* and *Li's F** [37].

4.7 Phylogenetic analysis of hypervariable fragments of chloroplast genome in Bambusoideae

To verify whether the hypervariable fragments distinguish the nine Bambusoideae species, and to determine the interspecies relationship of these nine Bambusoideae specie, with *trnG-trnT* as the representative, the whole genome sequences of thirty-nine species of NCBI were compared with nine assembled gene fragments to obtain highly similar sequences, and then the phylogenetic tree was constructed. The phylogenetic tree of the five hypervariable DNA fragments and the phylogenetic tree combined with the five hypervariable DNA fragments were constructed. Among these phylogenetic trees, *Or. sativa* as an outgroup.

The software MEGA7.0 [38] was used to analyze and build the phylogenetic trees, and the PhyloSuite 1.2.3 [39] was used to concatenate five DNA fragments; then MEGA7.0 software was used to find the best models. Tamura [40] is the best nucleotide replacement model for *trnG-trnT* and combined fragments, and is an equal-input model, JC [41] is the best nucleotide replacement model of other four DNA fragments: *matK*, *rbcL*, *trnH-psbA*, *trnL-trnF*, and it is the simplest single-parameter replacement model; then, phylogenetic analysis were conducted, including Maximum-likelihood (ML), Neighbor-Joining (NJ), Minimum evolution (ME). The phylogenetic tree constructed by the *rbcL*, *matK*, *trnH-psbA*, *trnL-trnF*, *trnG-trnT* and the phylogenetic tree constructed by combining five DNA fragments were very similar, therefore, only ML phylogenetic trees are listed in this paper. At the same time, the Bootstrap test was used to repeat 1000 times in the process of establishment, and the support rate of each branch was tested. Finally, the number of branches in the phylogenetic trees with a support rate of less than 50% were hidden.

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References

[1] Zhu Z X, Zhang F Y, Song S, et al. Research Advances in Bambuseae Taxonomy. World Forestry Research. Vol. 30 (2017) No. 3, p. 35-40.
[2] Zhou F C. Bamboo resources in the world. Bamboo research. Vol. 1 (1998) , p. 4-10.
[3] Dai X D, Xu C B, Dai Q M. Bamboo resources and research progress. Journal of Shandong Forestry Sicence and Technology. Vol. 1 (2009), p. 107-111.
[4] Bai Q, Yu L X, Yan B. Classification and research progress of bamboo. Journal of Henan Sicence and Technology. (2014) No. 5, p. 188-189.
[5] Triplett J K, Clark L G, Fisher A E, et al. Independent allopolyploidization events preceded speciation in the temperate and tropical woody bamboos. New Phytologist. Vol. 204 (2014) No. 1, p. 66-73.
[6] Goh W, Chandran S, Franklin D, et al. Multi-gene region phylogenetic analyses suggest reticulate evolution and a clade of Australian origin among paleotropical woody bamboos (Poaceae:

ISSN 2616-5910 Vol. 4, Issue 1: 43-53, DOI: 10.25236/AJALS.2023.040107

Bambusoideae: Bambuseae). Plant systematics and evolution. Vol. 299 (2013) No. 1, p.239-257.
[7] Kelchner S A, Group B P. Higher level phylogenetic relationships within the bamboos (Poaceae: Bambusoideae) based on five plastid markers. Molecular phylogenetics and evolution. Vol. 67 (2013) No. 2, p. 404-413.
[8] Zhang X Z, Zeng C X, Ma P F, et al. Multi-locus plastid phylogenetic biogeography supports the Asian hypothesis of the temperate woody bamboos (Poaceae: Bambusoideae). Molecular Phylogenetics and Evolution. Vol. 96 (2016), p. 118-129.
[9] Shi J Y,Yi T P, Yao J, et al. A New Species of Bashania Keng f et Yi on Western Yunnan,China. Forest Research. Vol. 20 (2007) No. 6, p. 864-866.
[10] Stapleton C M A .New combinations in Sarocalamus for Chinese alpine bamboos (Poaceae: Bambusoideae)[J].Nordic Journal of Botany, 2019, 37(7).DOI:10.1111/njb.02361.
[11] Clark L, Londoño X, Ruiz-Sanchez E. Bamboo taxonomy and habitat. Bamboo: The plant and its uses. (2015), p.1-30.
[12] Clark L G, Oliveira R. Diversity and evolution of the new world bamboos. Proceedings World Bamboo Congress, Mexico. (2018), p. 14-18.
[13] Gong Y, Chen H J, Xu Z E. Polymorphisms of Dwarf 14(D14) Gene in Bambusoideae. Journal of Nuclear Agricultural Sciences. Vol. 32 (2018) No. 1, p. 48-57.
[14] Zhu S, Liu T, Tang Q, et al. Evaluation of bamboo genetic diversity using morphological and SRAP analyses. Russian Journal of Genetics. Vol. 50 (2014), p. 267-273.
[15] Wang Y, Zhan D F, Jia X, et al. Complete chloroplast genome sequence of Aquilaria sinensis (Lour.) Gilg and evolution analysis within the Malvales order. Frontiers in plant science. Vol. 7 (2016), p. 280-293.
[16] Zhou J, Cui Y, Chen X, et al. Complete chloroplast genomes of Papaver rhoeas and Papaver orientale: molecular structures, comparative analysis, and phylogenetic analysis. Molecules. Vol. 23 (2018) No.2, p. 437-452.
[17] Liu J X, Zhou M Y, Yang G Q, et al. ddRAD analyses reveal a credible phylogenetic relationship of the four main genera of Bambusa-Dendrocalamus-Gigantochloa complex (Poaceae: Bambusoideae). Molecular phylogenetics and evolution. Vol. 146 (2020), p. 106758-106810.
[18] Zhou Y, Zhang Y Q, Xing X C, et al. Straight from the plastome: molecular phylogeny and morphological evolution of Fargesia (Bambusoideae: Poaceae). Frontiers in Plant Science. Vol. 10 (2019), p. 981-998.
[19] Wang M Y, Zhang X M, Ding Y. Comparison and Evolutionary Analysis of Chloroplast Genomes in Hemiparasitic Plants of the Santalaceae. Vol. 21 (2023) No.9, p. 2908-2924.
[20] Ma L H, Ning J Q, Wang Y J. Comparative genomics on chloroplasts of Sinopodophyllum hexandrum. Chinese Journal of Biotechnology. Vol. 38 (2022) No.10, p. 3695-3712.
[21] Chong X, Li Y, Yan M, et al. Comparative chloroplast genome analysis of 10 Ilex species and the development of species-specific identification markers. Industrial Crops and Products. Vol. 187 (2022), p. 115408-115421.
[22] Xia C, Wang M, Guan Y, et al. Comparative analysis of the chloroplast genome for Aconitum species: genome structure and phylogenetic relationships. Frontiers in Genetics. Vol. 13 (2022), p. 878182-878201.
[23] Feng C, Xu M, Feng C, et al. The complete chloroplast genome of Primulina and two novel strategies for development of high polymorphic loci for population genetic and phylogenetic studies. BMC Evolutionary Biology. Vol. 17 (2017), p. 1-16.
[24] Song W, Chen Z, He L, et al. Comparative chloroplast genome analysis of wax gourd (Benincasa hispida) with three Benincaseae species, revealing evolutionary dynamic patterns and phylogenetic implications. Genes. Vol. 13 (2022) No. 3, p. 461-479.
[25] Park I, Song J H, Yang S, et al. Comparative analysis of Actaea chloroplast genomes and molecular marker development for the identification of authentic Cimicifugae Rhizoma. Plants. Vol. 9 (2020) No.2, p. 157-171.
[26] Li L B, Liu L, Yuan J L, et al. Feasibility of the Chloroplast 5S rDNA ITS and matK Gene Sequence to the Phylogenetic Relationships in the Genus of Phyllostachys. Molecular Plant Breeding. Vol. 7 (2009) No.1, p. 89-94.
[27] Xu Q, Liang C, Chen J, et al. Rapid bamboo invasion (expansion) and its effects on biodiversity and soil processes+. Glob. Ecol. Conserv. Vol. 21 (2020), p. e00787-e00797.
[28] Mayor C, Brudno M, Schwartz J R, et al. VISTA: visualizing global DNA sequence alignments of arbitrary length. Bioinformatics. Vol. 16 (2000) No.11, p. 1046-1047.
[29] Dubchak I, Ryaboy D V. VISTA family of computational tools for comparative analysis of DNA sequences and whole genomes. Gene Mapping, Discovery, and Expression: Methods and Protocols. Vol. 338 (2006), p. 69-89.

ISSN 2616-5910 Vol. 4, Issue 1: 43-53, DOI: 10.25236/AJALS.2023.040107

[30] Lalitha S. Primer premier 5. Biotech Software & Internet Report: The Computer Software Journal for Scient. Vol. 1 (2000) No. 6, p. 270-272.
[31] Fulton T M, Chunwongse J, Tanksley S D. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Molecular Biology Reporter. Vol. 13 (1995) ,p. 207-209.
[32] Duan H, Wang W, Zeng Y, et al. The screening and identification of DNA barcode sequences for. Rehmannia. Vol. 9 (2019), p. 17295-17307.
[33] Rozas J. DNA sequence polymorphism analysis using DnaSP. Bioinformatics for DNA sequence analysis. (2009), p. 337-347.
[34] Nei M. Molecular evolutionary genetics[M]. Columbia university press, 1987.
[35] Watterson G. On the number of segregating sites in genetical models without recombination. Theoretical population biology. Vol. 7 (1975) No. 2, p. 256-276.
[36] Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics. Vol. 123 (1989) No. 3, p. 585-595.
[37] Fu Y X, Li W H. Statistical tests of neutrality of mutations. Genetics. Vol. 133 (1993) No. 3, p. 693-709.
[38] Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution. Vol. 33 (2016) No. 7, p. 1870-1874.
[39] Zhang D, Gao F, Jakovlić I, et al. PhyloSuite: An integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. Molecular ecology resources. Vol. 20 (2020) No. 1, p. 348-355.
[40] Tamura K. The rate and pattern of nucleotide substitution in Drosophila mitochondrial DNA. Molecular biology and evolution. Vol. 9 (1992) No. 5, p. 814-825.
[41] Jukes T H, Cantor C R. Evolution of protein molecules. Mammalian protein metabolism. Vol. 3 (1969), p. 21-132.