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Phylogenetic Relationships among Tibet *Rubus* (Rosaceae) Species Inferred from Multiple Chloroplast and Nuclear DNA Sequences

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Abstract. In this study, we collected 20 Tibet *Rubus* taxa from 4 sections, *Idaeobatus*, *Malachobatus*, *Chamaebatus*, and *Cylactis* of genus *Rubus*, as well as two outgroups, *Fragaria vesca* and *Rosa banksiae*. To investigate the phylogenetic relationships among Tibet *Rubus* species, the phylogeny was reconstructed by using chloroplast *rbc*L, *rpl*20-*rps*12, and *trn*G-*trn*S, nuclear ITS, *GBSSI*-2 and *PEPC* sequences. The final combined chloroplast DNA matrix consisted of 22 taxa and 2124 bp, of which 204 (9.60%) were variable and 94 (4.43%) parsimony informative. The nuclear DNA dataset included 22 accessions and 1897 aligned nucleotides that contained 374 (19.72%) variable and 146 (7.70%) parsimony informative sites. TIM1+I+G and TrN+G were selected as the best-fit models for the combined chloroplast and nuclear datasets, respectively. The Tibet *Rubus* is resolved as a monophyletic group and well supported through both chloroplast and nuclear datasets. Sect. *Idaeobatus* species have at least two independent evolutionary routes. Two sect. *Malachobatus* species clustered together with high support values. Then they were sister to three sect. *Cylactis* species in chloroplast tree, while to the subclade of major sect. *Idaeobatus* species in nuclear tree. This could be interpreted by frequent hybridization and genetic introgression. *Rubus calycinus* of sect. *Chamaebatus* forms a separate clade, suggesting its monophyly. To be concluded, the combined chloroplast and nuclear DNA sequences provided abundant information for the phylogenetic relationships among Tibet *Rubus* species.

INTRODUCTION

The genus *Rubus* L. comprises 750-1000 species and has a worldwide distribution [1-3]. Chinese *Rubus* is composed of eight sections, *Idaeobatus*, *Lampobatus*, *Rubus*, *Malachobatus*, *Dalibardastrum*, *Chamaebatus*, *Cylactis* and *Chamaebatus* [4]. As one of important distribution centers in China, there are abundant *Rubus* germplasms in Tibet Province. It seems that abundant genetic diversity is predicted among *Rubus* species in Tibet due to its special geographical and climatic condition, which can provide potentiality for raspberry breeding program. To investigate the phylogenetic relationships among Tibet *Rubus* species, we tried to reconstruct the phylogeny of Tibet *Rubus* by using multiple chloroplast and nuclear DNA sequences.

MATERIALS AND METHODS

Plant Materials

We sampled 20 *Rubus* taxa belonging to four sections, *Idaeobatus*, *Malachobatus*, *Chamaebatus*, and *Cylactis* of genus *Rubus*, from Tibet, China (**TABLE 1**). *Fragaria vesca* and *Rosa banksiae* were selected as the outgroups.

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TABLE 1 Voucher information and locality of *Rubus* species from Tibet province, China used in this study

Taxa	Locality	Altitude / m	Latitude (N) / Longtitude (E)	Voucher	GenBank No.					
					*rbc*L	*rpl*20-*rps*12	*trn*G-*trn*S	ITS	*GBSS*I-2	*PEPC*
Section *Idaeobatus* Focke										
Subsect. *Idaeanthi* (Focke) Yü et Lu										
R. niveus	Chayu	2315	28°38.555′ / 97°27.077′	L. Liu *R2502*	KU881270	KU881414	KU881558	KU881126	KU926791	KU891141
R. idaeus	Motuo	820	29°19.389′ / 95°19.456′	L. Liu *R2516*	KU881242	KU881386	KU881530	KU881098	KU926764	KU891116
R. aurantiacus	Lang County	3133	29°09.322′ / 93°12.010′	L. Liu *R2512*	KU881204	KU881348	KU881492	KU881060	KU926730	KU891085
R. austro-tibetanus	Milin	2971	29°09.564′ / 94°11.361′	L. Liu *R2524*	KU881205	KU881349	KU881493	KU881061	KU926731	KU891086
R. irritans	Linzhi	3743	29°39.594′ / 94°43.098′	L. Liu *R2527*	KU881254	KU881398	KU881542	KU881110	KU926776	KU891128
Subsect. *Pileati* Yü et Lu										
R. pubifolius	Yanjing	4222	29°15.575′ / 98°40.486′	L. Liu *R2536*	KU881296	KU881440	KU881584	KU881152	KU926816	KU891166
Subsect. *Stimulantes* Yü et Lu										
R. ellipticus	Motuo	1630	29°30.089′ / 95°34.371′	L. Liu *R2517*	KU881223	KU881367	KU881511	KU881079	KU926746	KU891100
R. ellipticus var. *obcordatus*	Chayu	1835	28°36.030′ / 97°11.311′	L. Liu *R2503*	KU881224	KU881368	KU881512	KU881080	KU926747	KU891101
R. mesogaeus	Linzhi	2695	29°53.152′ / 94°47.088′	L. Liu *R2522*	KU881266	KU881410	KU881554	KU881122	KU926787	KU891137
R. biflorus	Chayu	1705	28°36.568′ / 96°54.251′	L. Liu *R2504*	KU881207	KU881351	KU881495	KU881063	KU926733	KU891088
R. alexeterius var. *acaenocalyx*	Lang County	2984	29°11.226′ / 94°00.486′	L. Liu *R2513*	KU881199	KU881343	KU881486	KU881055	KU926725	KU891080
Subsect. *Pungentes* (Focke) Yü et Lu										
R. stans	Linzhi	3743	29°39.594′ / 94°43.098′	L. Liu *R2528*	KU881314	KU881458	KU881602	KU881170	KU926833	KU891181
R. macilentus	Xiachayu	2315	28°38.555′ / 97°27.077′	L. Liu *R2501*	KU881262	KU881406	KU881550	KU881118	KU926783	—
Subsect. *Rosaefolii* (Focke) Yü et Lu										
R. eustephanus var. *glanduliger*	Motuo	1688	29°27.107′ / 95°34.371′	L. Liu *R2518*	KU881227	KU881371	KU881515	KU881083	KU926750	—
Sect. *Malachobatus* Focke										
Subsect. *Moluccani* (Focke) Yü et Lu										
R. reticulatus	Motuo	973	29°27.335′ / 95°25.244′	L. Liu *R2521*	KU881302	KU881446	KU881590	KU881158	KU926822	KU891172
R. hypopitys var. *hanmiensis*	Motuo	3543	29°48.177′ / 95°41.535′	L. Liu *R2533*	KU881238	KU881382	KU881526	KU881094	KU926760	KU891112
Sect. *Chamaebatus* Focke										
R. calycinus	Damu	1688	29°27.107′ / 95°34.371′	L. Liu *R2519*	KU881209	KU881353	KU881497	KU881065	KU926735	KU891090
Sect. *Cylactis* Focke										
R. fockeanus	Galongla	3156	29°09.140′ / 94°12.547′	L. Liu *R2523*	KU881232	KU881376	KU881520	KU881088	KU926755	KU891108
R. nyalamensis	Motuo	2978	29°43.266′ / 95°37.491′	L. Liu *R2534*	KU881271	KU881415	KU881559	KU881127	KU926792	KU891142
R. fragarioides var. *pubescens*	Motuo	3498	29°09.132′ / 94°12.581′	L. Liu *R2530*	KU881233	KU881377	KU881521	KU881089	KU926756	KU891109
Outgroups										
Fragaria vesca	—	—	—	Y. Wang *R0155*	KU881195	KU881339	KU881483	KU881051	KU926722	XM011462481
Rosa banksiae	—	—	—	Y. Wang *R0156*	KU881196	KU881340	KU881484	KU881052	KU926723	—

DNA Extraction and Sequence Amplification

Total genomic DNA was isolated from silica-gel dried leaf tissues using a modified CTAB method [5]. Three chloroplast regions (*rbc*L, *rpl*20-*rps*12, and *trn*G-*trn*S), nuclear ribosomal internal transcribed spacers (ITS), and two single copy nuclear genes coding for granule-bound starch synthase I (*GBSS*I-2), and phosphoenolpyruvate carboxylase (*PEPC*) were used in this study (**TABLE 2**).

PCR amplification was performed in a 25 μL volume, which contained 20 ng of genomic DNA, 1.2 μL of $MgCl_2$ (25 mmol·L^{-1}), 1.4 μL of dNTP mix (10 mmol·L^{-1}), 1 μL of each primer (5 μmol·L^{-1}), 1.5 U of PfuDNA polymerase (Tiangen, Beijing), and 2.0 μL of 10× PCR buffer (10 mmol·L^{-1} pH 8.0 Tris-HCl, 50 mmol·L^{-1} KCl, 1.5 mmol·L^{-1} EDTA). Conditions for amplification consisted of an initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 45 s, then at 55-58°C for 1 min and at 72°C for 1 min, with a final extension at 72°C for 10 min. Amplifications were carried out using a PTC-200 thermocycler (Bio-rad, Hercules, CA).

Sequence Alignment and Phylogenetic Analyses

After PCR amplification and agarose gel electrophosis, the products were sequenced directly in both directions using Big Dye Terminator Cycle Sequencing kit. All the sequences were deposited in the GenBank database to obtain the accession numbers (**TABLE 1**). Sequences were edited and assembled using CLC Genomics Workbench, aligned with Muscle [6], and adjusted in the Molecular Evolutionary Genetics Analysis software (MEGA7) [7]. DNA substitution models were selected out from JModelTest v2.1.1 [8] according to Akaike Information Criterion (AIC) [9] for each gene. We carried out the Maximum Likelihood (ML) and Bayesian Inference (BI) methods to reconstruct the phylogeny of Tibet *Rubus* by using IQ-TREE v1.4.2 [10, 11] and MrBayes v3.2.1 [12].

TABLE 2. Primers for chloroplast and nuclear DNA sequences were used in this study

Region	Primer sequence (5'→3')	Tm (°C)	Amplified length (bp)
*rbc*L [13]	1F: ATGTCACCACAAACAGAAAC 724R: TCGCATGTACCTGCAGTAGC	55	700
*rpl*20-*rps*12 [14]	F: TTTGTTCTACGTCTCCGAGC R: GTCGAGGAACATGTACTAGG	55	800
*trn*G-*trn*S [14]	F: GAACGAATCACACTTTTACCAC R: GCCGCTTTAGTCCACTCAGC	58	700
ITS [15]	ITS5: GGAAGTAAAAGTCGTAACAAGG ITS4: TCCTCCGCTTATTGATATGC	55	700
*GBSS*I-2 [16]	F2: TGGTCTTGGTGATGTTCTTGG R2: GTGTAGTTGGTTGTCCTTGTAATCC	58	530~600
PEPC [17]	F: CCGKCTTGCWACACCWGAGCTGGAG R: CCRGGWGCRTACTCGC	58	750

RESULTS

Sequence Variation Among Tibet *Rubus* Species

After treating the gaps as missing characters, the final combined chloroplast DNA matrix consisted of 22 taxa and 2124 bp, of which 204 (9.60%) were variable and 94 (4.43%) parsimony informative. For *PEPC*, we failed to obtain sequences for *R. eustephanus* var. *glanduliger* and *R. macilentus*. Based on the theoretic research by Wiens and Moen [18], we treated the failure sequences as missing data for subsequent analyses. The nuclear DNA dataset included 22 accessions and 1897 aligned nucleotides that contained 374 (19.72%) variable and 146 (7.70%) parsimony informative sites. The TIM1+I+G and TrN+G were selected as the best fit models for combined chloroplast and nuclear datasets by JModelTest software, respectively (**TABLE 3**).

TABLE 3. The sequences variation of combined chloroplast and nuclear datasets and the best-fitting model utilized in Tibet *Rubus*

Datasets	cpDNAs	nDNAs	Datasets		cpDNAs	nDNAs
Number of accessions			AIC selected model		TIM1+I+G	TrN+G
Rubus plus outgroups	22	22	Base frequencies	A	0.3147	0.2478
Rubus	20	20		C	0.1784	0.2214
Aligned nucleotide length (bp)				G	0.1755	0.2331
Rubus plus outgroups	2124	1897		T	0.3314	0.2978
Rubus	2087	1858	Substitution model	A-C	1.0000	1.0000
Variable sites (%)			(rate matrix)	A-G	0.9175	2.1562
Rubus plus outgroups	204 (9.60%)	374 (19.72%)		A-T	0.5301	1.0000
Rubus	131 (6.28%)	210 (11.30)		C-G	0.5301	1.0000
Parsimony informative sites (%)				C-T	1.4799	3.7956
Rubus plus outgroups	94 (4.43%)	146 (7.70%)		G-T	1.0000	1.0000
Rubus	55 (2.64%)	78 (4.20%)	pinvar		0.6580	0
			G		0.9670	0.3210

Note: AIC, Akaike Information Criterion; pinvar, proportion of invariable sites; G, gamma shape.

Phylogenetic Analyses for Tibet *Rubus* Species

Phylogeny of Tibet *Rubus* using both ML and BI methods did not show obvious incongruence except for support values. The Tibet *Rubus* is resolved as a monophyletic group and well supported through both chloroplast and nuclear datasets (**FIGURE 1**). Phylogenetic tree through combined chloroplast datasets suggested three clades A, B and C (**FIGURE 1-1**). *Rubus ellipticus*, *R. ellipticus* var. *obcordatus* from subsect. *Stimulantes*, *R. macilentus* from subsect. *Pungentes* and *R. eustephanus* var. *glanduliger* from subsect. *Rosaefolii* of sect. *Idaeobatus* were assigned into clade A, while the remaining *Idaeobatus* species into clade C. Clade B was divided into two clusters, corresponding to *R. calycinus* of sect. *Chamaebatus*, and three sect. *Cylactis* and two sect. *Malachobatus* species, respectively. Phylogeney through combined nuclear datasets indicated four clades D-G (**FIGURE 1-2**). Clades D, E, and F corresponded to clade A by cpDNA tree. The clade G contained four subclades. Subclade G1, G2 and G3 corresponded to *R. calycinus* (B1), three *Cylactis* species and two *Idaeobatus* species (B2), respectively. The subclade G4 was almost consistent with clade C by cpDNA tree.

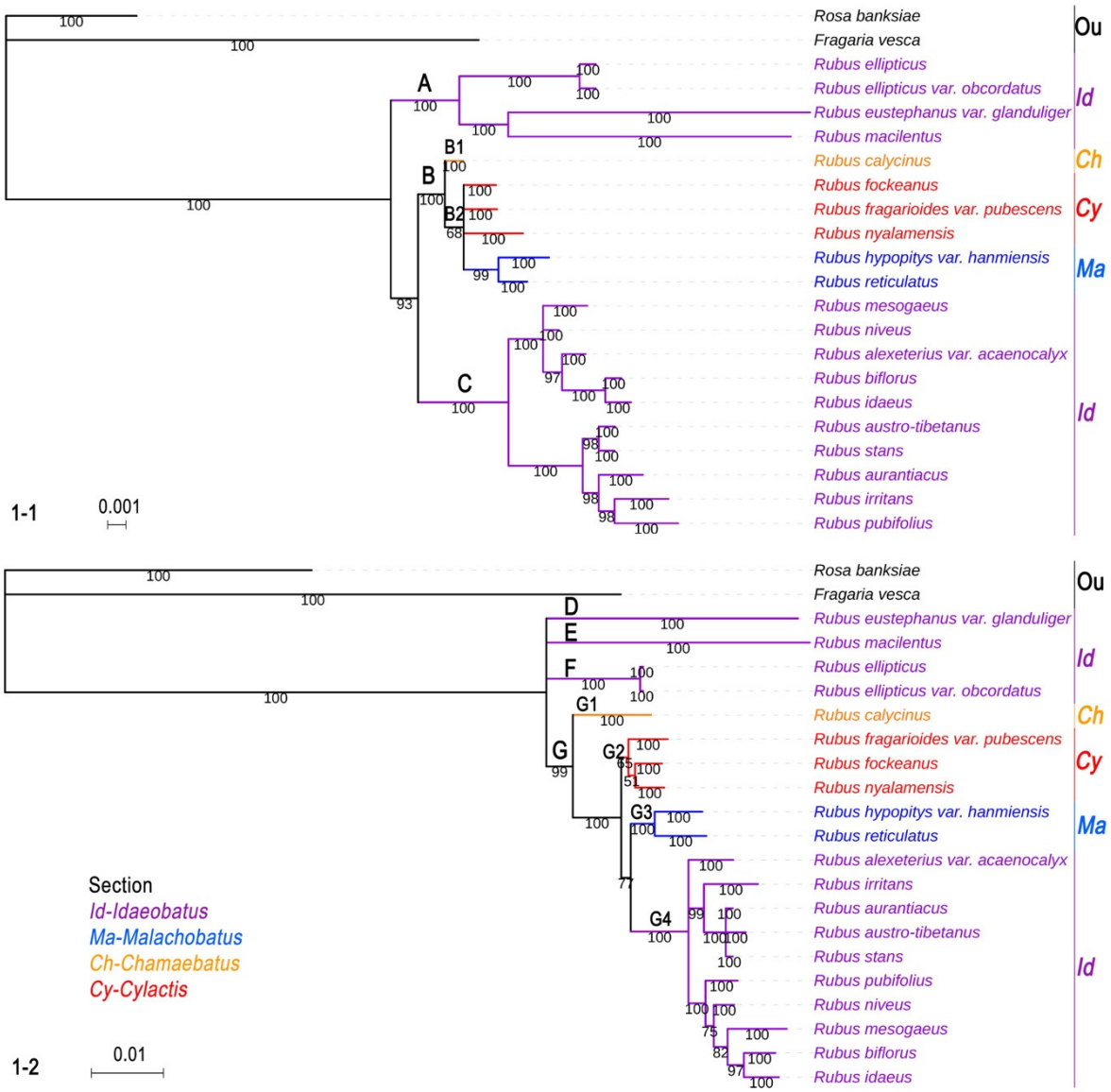

FIGURE 1. Phylogeny of Tibet *Rubus* through combined chloroplast (1-1) and nuclear (1-2) datasets. Poster probabilities from BI analysis > 50 are provided below the branches.

DISCUSSION

This study based on both chloroplast and nuclear sequences supported that sect. *Idaeobatus* is a polyphyletic group among Tibet *Rubus* species. The result indicated that sect. *Idaeobatus* species have at least two independent evolutionary routes, which is consistent with previous study by Morden et al [19]. There were some incongruences between chloroplast and nuclear trees. Two sect. *Malachobatus* species clustered together with high support values. Then they were sister to three sect. *Cylactis* species in chloroplast tree, while to the subclade of major sect. *Idaeobatus* species in nuclear tree. This could be interpreted by frequent hybridization and genetic introgression [20, 21]. *Rubus calycinus* of sect. *Chamaebatus* forms a separate clade in the chloroplast and nuclear trees. Its monophyly has been demonstrated by Alice and Campbell [22]. Three examined species of sect. *Cylactis* form a clearly polyphyletic group. This is also in agreement with its morphological heterogeneity, confirming their different origin patterns by cytological

study [4, 23]. To be concluded, this study provided abundant information for phylogenetic relationships among Tibet *Rubus* species by combined chloroplast and nuclear DNA sequences.

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