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Molecular Phylogenetic Distinction between Taiwan Endemic *Rhynchotechum brevipedunculatum* and *R. discolor* (Gesneriaceae) Widespread in Subtropical and Tropical Asia

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Abstract Molecular analyses using ITS of nrDNA and *trnSG* intron of cpDNA were conducted to test the phylogenetic distinction between Taiwanese endemic *Rhynchotechum brevipedunculatum* and *R. discolor* widespread in subtropical and tropical Asia. The analyses revealed the reciprocal monophyly of the two species. This result indicates that *R. brevipedunculatum* is an independent species from *R. discolor*, adding molecular support for the taxonomic concept based on morphology.

Key words: endemic, Gesneriaceae, ITS, *Rhynchotechum*, Ryukyus, Taiwan, *trnSG*.

Introduction

The genus *Rhynchotechum* Blume (Gesneriaceae) consists of 16 species and primarily occurs in tropical and subtropical Asia (Wang and Wang, 2000). In Taiwan, two *Rhynchotechum* species has long been recognized: *R. discolor* (Maxim.) B.L.Burtt widely distributing from Japan to New Guinea (Burtt, 1962) (Fig. 1A) and *R. formosanum* Hatusima occurring in China and Taiwan (Wang *et al.*, 1998). In 2000, a new species *R. brevipedunculatum* J.C.Wang was described as a Taiwanese endemic species (Wang and Wang, 2000) (Fig. 1B).

Rhynchotechum brevipedunculatum and *R. discolor* are apparently distinguishable from *R. formosanum* by their morphological characters. On the other hand, *R. brevipedunculatum* is morphologically similar to *R. discolor*, but the two species are distinguished based on some floral traits. The former has peduncles less than 2 mm long, compact simple cyme inflorescences, and calices of 10 to 12 mm long × 1.5 to 2.0 mm wide; while the latter has peduncles more than 18 mm long, loose compound cyme inflorescences, and calices of 5 to 8 mm long × 0.5 mm wide (Wang and Wang, 2000). Distribution ranges of the two species mostly overlapped in Taiwan, from New Taipei City to Pingtung. Habitats of the two *Rhynchotechum* species are moist and slightly shaded environments under broadleaf forest canopy, and the two species rarely sympat-

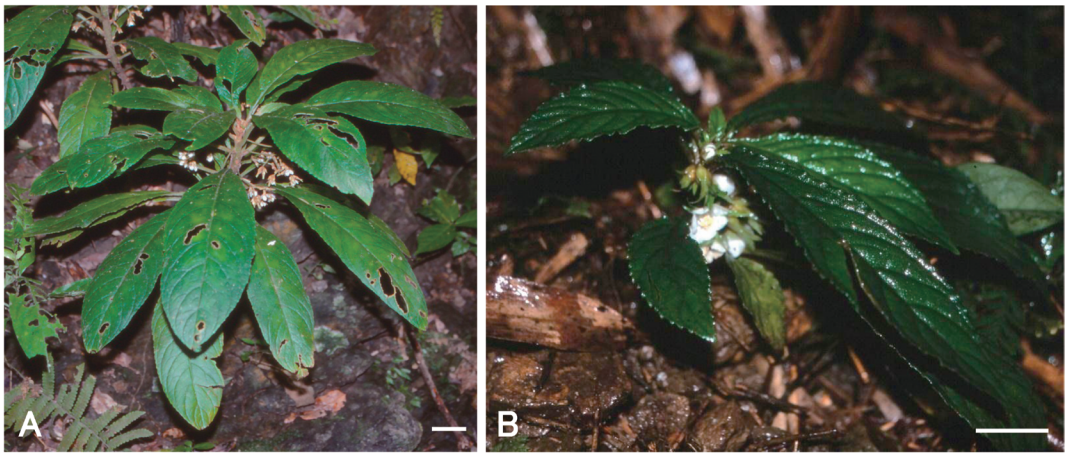

Fig. 1. Habits of *Rhynchotechum brevipedunculatum* and *R. discolor*. A. *R. discolor* (GK212). B. *R. brevipedunculatum* (GK4155). Bars indicate 2 cm.

rically occur (Wang and Wang, 2000). In this study, we conducted molecular analyses to test the phylogenetic distinction between *R. brevipedunculatum* and *R. discolor* based on the internal transcribed spacer (ITS) region of nuclear ribosomal DNA and a spacer region between *3'trnS* and *trnG* (*trnSG*) of chloroplast DNA.

Materials and Methods

Plant materials

We morphologically identified *Rhynchotechum brevipedunculatum* and *R. discolor* following the key of Wang and Wang (2000). Fourteen samples of *R. brevipedunculatum* from Taiwan Island, and 39 samples (including 15 Japanese, 20 Taiwanese and 4 Philippine samples) of *R. discolor* were collected for the molecular analyses (Table 1 and Fig. 2). In the present study, we found two localities where the two *Rhynchotechum* species occurred within 50 m from each other in Shihting (T5) and Sanhsia (T6) of Taiwan (Table 1 and Fig. 2). *Rhynchotechum formosanum* distributed in China and Taiwan (Wang and Wang, 2000) was selected as an outgroup taxon (Table 1). Total number of operational taxonomic units was 54 including the 53 ingroup members and an outgroup member (Table 1). Voucher specimens for the molecular analyses were deposited in the herbarium of the National Museum of Nature and Science (TNS).

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from silica gel dried leaves using the DNeasy Plant Mini Kit (Qiagen, Qiagen, Valencia, CA) following the manufacturer's protocol. The genomic DNA samples were deposited in the Molecular Biodiversity Research Center of the National Museum of Nature and Science. The ITS and *trnSG* regions were amplified by the polymerase chain reaction (PCR) using an iCycler (BIO RAD, California, USA). Forward primer 'AB101' (5′-ACG AAT TCA AGG TCC GGT GAA GTG TTC G-3′) and reverse primer 'AB102' (5′-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3′) were used for ITS (Douzery *et al.*, 1999); and forward primer '*trnS* (GCU)' (5′-GCC GCT TTA GTC CAC TCA GC-3′) and reverse primer '*trnG* (UCC)' (5′-GAA CGA ATC ACA CTT TTA CCA C-3′) were used for *trnSG* (Hamilton, 1999). Amplifications were performed using Takara Sapphire-Amp Fast Master Mix (Takara, Otsu, Japan) with Sapphire Amp (Takara, Tokyo, Japan). The PCR profile was 35 cycles of 5 sec at 98°C, 5 sec at 51°C, and 5 sec at 72°C after an initial denaturing for 3 min at 94°C for both of ITS and *trnSG*. The PCR products were checked by electrophoresis before purification using illustra ExoProStar (GE

Table 1. Plant materials of *Rhynchotechum* species investigated in the present study

Species	Collection locality (altitude)	Abbreviation*	Voucher no.	ITS		*trnSG*	
				Accession no. **	Type	Accession no. **	Type
R. brevipedunculatum	Taiwan: Keelung (45 m)	T5	GK4173	AB871639	A	AB871721	a
	Taiwan: Taipei, Shihting (122 m)	T6	GK4155	AB871629	A	AB871711	b
	Taiwan: Taipei, Shihting (100 m)	T6	GK14982	AB871633	A	AB871715	a
	Taiwan: Taipei, Shihting (100 m)	T6	GK14984	AB871634	A	AB871716	a
	Taiwan: Taipei, Shihting (100 m)	T6	GK14985	AB871635	A	AB871717	a
	Taiwan: Taipei, Sanhsia (535 m)	T12	GK14226	AB871636	A	AB871718	a
	Taiwan: Taipei, Sanhsia (535 m)	T12	GK14227	AB871637	A	AB871719	a
	Taiwan: Taipei, Sanhsia (494 m)	T12	GK14233	AB871638	A	AB871720	a
	Taiwan: Miaoli, Shitan (487 m)	T16	GK5177	AB871640	A	AB871722	a
	Taiwan: Pingtung, Shihtzu (388 m)	T20	GK15039	AB871641	A	AB871723	c
	Taiwan: Pingtung, Shihtzu (388 m)	T20	GK15040	AB871642	A	AB871724	c
	Taiwan: Pingtung, Shihtzu (388 m)	T20	GK15047	AB871630	A	AB871712	b
	Taiwan: Pingtung, Shihtzu (388 m)	T20	GK15048	AB871631	A	AB871713	b
	Taiwan: Pingtung, Shihtzu (388 m)	T20	GK15059	AB871632	A	AB871714	b
R. discolor	Japan: Ryukyus, Nakanoshima Island (100 m)	R1	GK11337	AB871590	B	AB871672	d
	Japan: Ryukyus, Nakanoshima Island (150 m)	R2	GK11341	AB871591	B	AB871673	d
	Japan: Ryukyus, Tokunoshima Island, Amagi (140 m)	R3	GK12120	AB871592	B	AB871674	d
	Japan: Ryukyus, Tokunoshima Island, Isen (140 m)	R4	GK12478	AB871593	B	AB871675	d
	Japan: Ryukyus, Tokunoshima Island, Isen (132 m)	R5	GK12594	AB871594	B	AB871676	d
	Japan: Ryukyus, Tokunoshima Island, Isen (180 m)	R6	GK12493	AB871595	B	AB871677	d
	Japan: Ryukyus, Okinawa Island, Kunigami (400 m)	R7	GK342	AB871596	B	AB871678	d
	Japan: Ryukyus, Okinawa Island, Kunigami (150 m)	R8	GK8983	AB871597	B	AB871679	d
	Japan: Ryukyus, Okinawa Island, Higashi (50 m)	R9	GK10073	AB871598	B	AB871680	d
	Japan: Ryukyus, Okinawa Island, Higashi (150 m)	R10	GK9026	AB871599	B	AB871681	d
	Japan: Ryukyus, Okinawa Island, Motobu (300 m)	R11	GK196	AB871600	B	AB871682	d
	Japan: Ryukyus, Ishigaki Island (305 m)	R12	GK11814	AB871601	B	AB871683	d
	Japan: Ryukyus, Ishigaki Island (300 m)	R13	GK212	AB871602	B	AB871684	d
	Japan: Ryukyus, Iriomote Island (70 m)	R14	GK67	AB871603	B	AB871685	d
	Japan: Ryukyus, Iriomote Island (230 m)	R15	GK207	AB871604	B	AB871686	d
	Taiwan: Taipei, Sanchih (837 m)	T1	GK8493	AB871620	B	AB871702	e
	Taiwan: Taipei, Sanzhi (820 m)	T2	GK14938	AB871609	B	AB871691	d
	Taiwan: Taipei, Sanzhi (820 m)	T2	GK14939	AB871610	B	AB871692	d
	Taiwan: Taipei, Sanzhi (820 m)	T2	GK14940	AB871611	B	AB871693	d
	Taiwan: Keelung (45 m)	T3	GK4172	AB871619	B	AB871701	e
	Taiwan: Taipei, Shitan (275 m)	T4	GK4150	AB871607	B	AB871689	d
	Taiwan: Taipei, Shihting (100 m)	T5	GK14990	AB871616	B	AB871698	e
	Taiwan: Taipei, Sanhsia (427 m)	T6	GK14235	AB871617	B	AB871699	e
	Taiwan: Taipei, Pingxi (183 m)	T7	GK14974	AB871614	B	AB871696	d
	Taiwan: Taipei, Pingxi (183 m)	T7	GK14973	AB871615	B	AB871697	e
	Taiwan: Taipei, Taipei, Shiting (250 m)	T9	GK3099	AB871605	B	AB871687	d
	Taiwan: Taipei, Shihting (504 m)	T10	GK15119	AB871613	B	AB871695	d
	Taiwan: Taipei, Shihting (414 m)	T10	GK15112	AB871612	B	AB871694	d
	Taiwan: Taipei, Wulai (231 m)	T11	GK10045	AB871618	B	AB871700	e
	Taiwan: Hsinchu, Chienshih (1339 m)	T13	GK5215	AB871621	B	AB871703	e
	Taiwan: Miaoli, Chienshih (1110 m)	T14	GK5185	AB871623	B	AB871705	e
	Taiwan: Hsinchu, Wufen (940 m)	T15	GK5120	AB871606	B	AB871688	d
	Taiwan: Ilan, Nannao (262 m)	T17	GK6231	AB871622	B	AB871704	e
	Taiwan: Miaoli, Sanyi (371 m)	T18	GK5153	AB871608	B	AB871690	d
	Taiwan: Pingtung, Wutai (1075 m)	T19	GK8902	AB871624	B	AB871706	e
	Philippines: Banaue (1712 m)	P1	GK9655	AB871625	C	AB871707	f
	Philippines: Isabela, Dinapigue (130 m)	P2	GK13141	AB871628	D	AB871710	f
	Philippines: Aurora, Dipaculao (363 m)	P3	GK13132	AB871626	B	AB871708	f
	Philippines: Lucena (unknown)	P4	GK911	AB871627	B	AB871709	f
R. formosanum	Taiwan: Taipei, Pinglin (350 m)	T8	CIP18195	AB871643	F	AB871725	g

* Referring Fig. 2.

** Deposited Sequences in the DDBJ database.

Helthcare, Tokyo, Japan). Cycle sequencing reaction was carried out with the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA) using the same PCR primers. The Sanger sequencing products were then purified by ethanol precipitation. Automated sequencing was carried out on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, California, USA). The electropherograms were analysed using ATGC ver. 4.01

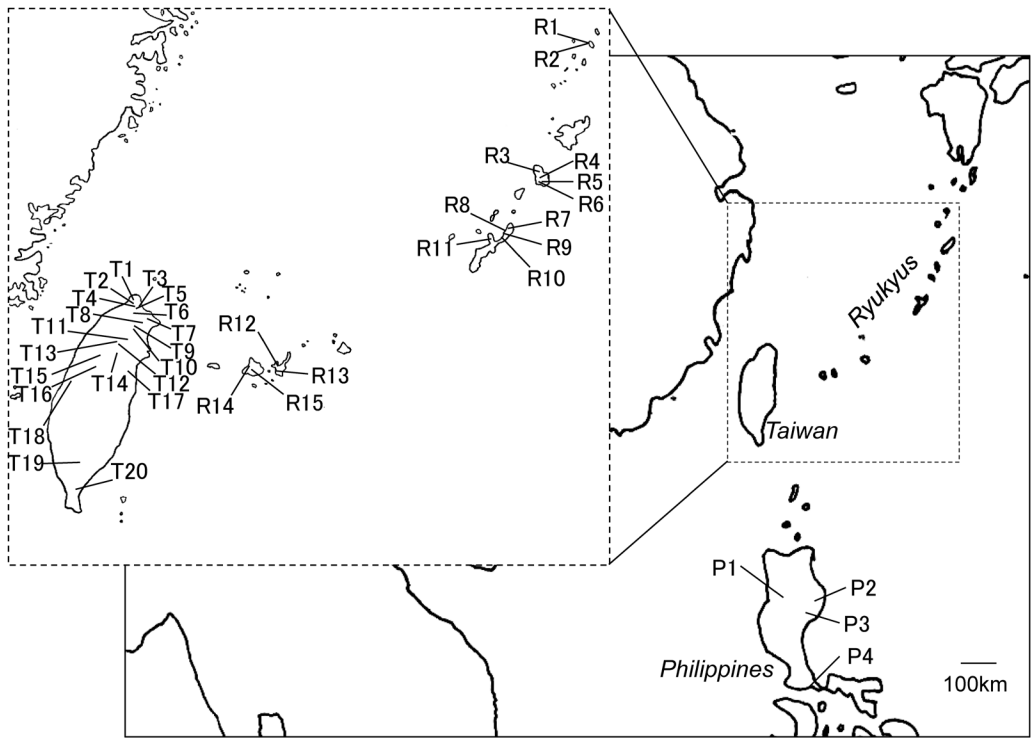

Fig. 2. Map showing the 39 collection localities of the 54 individuals of three *Rhynchotechum* species from the Ryukyus of Japan, Taiwan, and the Philippines. Abbreviations refer to localities listed in Table 1.

(GENETYX Co., Tokyo, Japan). Sequence data from this study were deposited to the DDBJ database (http://www.ddbj.nig.ac.jp/) (Table 1).

Phylogenetic analysis

DNA sequences were aligned using the program ClustalW 1.8 (Thompson *et al.*, 1994) and then manually adjusted. The combinability between the ITS and *trnSG* regions was assessed using the incongruence length difference (ILD) test (Farris *et al.*, 1994).

Phylogenetic analyses were conducted based on a Bayesian approach using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and a maximum parsimony (MP) criterion using PAUP* version 4.0b10 (Swofford, 2002). In the Bayesian phylogenetic analysis, the Hierarchical Likelihood Ratio Tests (hLRT) implemented in MrModeltest 2.2 (Nylander, 2004) was used to estimate the appropriate evolutionary model of nucleotide substitutions. Based on the model selected, two separate runs of Metropolis coupled Markov chain Monte Carlo (MCMCMC) analyses were performed, each with a random starting tree and four chains (one cold and three heated). The MCMCMC length was two million generations, and the chain was sampled every one hundredth generation from the cold chain. The first 5000 sample trees (25% of the total 20000 sample trees) were discarded as burn-in after checking that the average standard deviation of split frequencies (ASDSF) reached a stationary state at <0.01 thereafter. As a guide to convergence, the potential scale reduction factors (PSRFs) were ascertained to be reasonably close to 1.0 for all parameters in an output table. A 50% majority consensus tree of the output tree file from MrBayes was generated by TREEVIEW (Page, 1996).

In the MP phylogenetic analysis, characters

were treated as unordered, and character transformations were equally weighted. Indels were scored as binary states indicating presence or absence, following the simple indel-coding strategy of Simmons and Ochoterena (2000). The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony search was performed with 200 replicates of random additions of sequences with ACCTRAN character optimization, tree bisection–reconnection (TBR) branch swapping, and MULTREES and STEEPEST DESCENT options on. Statistical support for each clade was assessed by bootstrap analysis (Felsenstein, 1985). A thousand replicates of heuristic searches, with the TBR branch swapping switched on and MULTREES options off, were performed to calculate bootstrap values (BS).

Results and Discussion

Phylogenetic distinction based on ITS and trnSG

After alignment of the sequences from the 54 samples, a matrix of 636 bp was obtained for ITS. Four ITS types (A–D) were recognized in the ingroup; only type A was found in *R. brevipedunculatum* and the other three types B–D were found in *R. discolor* (Table 1). A matrix of 704 bp was obtained for *trnSG* after alignment. Six haplotypes (a–f) were recognized in the ingroup; three haplotypes (a–c) were found in *R. brevipedunculatum* and the other three haplotypes (d–f) were found in *R. discolor* (Table 1). The ILD test did not show significant incongruence between the two regions ($p = 1.00$), and thus they were combined for the present Bayesian and MP analyses. The length of the combined matrix was 1340 bp.

The model of F81 was selected in the Bayesian analysis. The 50% majority rule consensus tree of all the post-burn-in trees is depicted with mean branch lengths and posterior probabilities (*PP*) (Fig. 2). In the MP analysis, 14 of 26 variable characters were parsimony-informative, and two equally parsimonious trees of 26 steps were obtained with CI of 1.000 and RI of 1.000. Because the topology of the MP strict consensus tree (not shown) was compatible with that of the Bayesian 50% majority rule consensus tree, the bootstrap percentages (*BP*) were plotted on the Bayesian tree (Fig. 3).

The Bayesian and MP analyses (Fig. 3) showed two clades in the ingroup: one composed of all the 14 plants of *R. brevipedunculatum* (clade I; *PP*/*BS* = 1.00/86%) and the other clade composed of all the 39 plants of *R. discolor* (clade II; *PP*/*BS* = 1.00/87%). In clade I, twelve plants of *R. brevipedunculatum* formed a subclade (*PP*/*BS* = 0.55/—%). In clade II, 10 Taiwanese plants of *R. discolor* formed a subclade (*PP*/*BS* = 1.00/85%).

As mentioned in the introduction, *R. brevipedunculatum* is morphologically similar to *R. discolor* (Wang and Wang, 2000). In addition, during sample collection, we found that the two species occurred almost sympatrically within 50 m from each other in the two localities (T5 and T6) in Taiwan. However, the present molecular analyses revealed that the two species were clearly separated. This result indicates that *R. brevipedunculatum* is an independent species from *R. discolor*, adding molecular support for Wang and Wang (2000).

In the present analyses, no hybrid of the two species was found, although it was expected from their sympatric occurrence. Therefore, there likely is some barrier to the genetic exchange between the two species. The corolla tube of *R. brevipedunculatum* (ca. 2 mm) is longer than that of *R. discolor* (ca. 1 mm) (Wang and Wang, 2000). It is possible that the two species have different pollinators, because of which they are reproductively isolated.

Molecular polymorphism of R. brevipedunculatum *and* R. discolor

In the present molecular survey, both *R. brevipedunculatum* and *R. discolor* had multiple ITS/*trnSG* types. However, the geographical distribution of the different ITS/*trnSG* types did not correlate with geographical coordinate or elevation of the sampling localities (Table 1). Additionally, in two localities T6 and T20 in Taiwan, *R. brevi-*

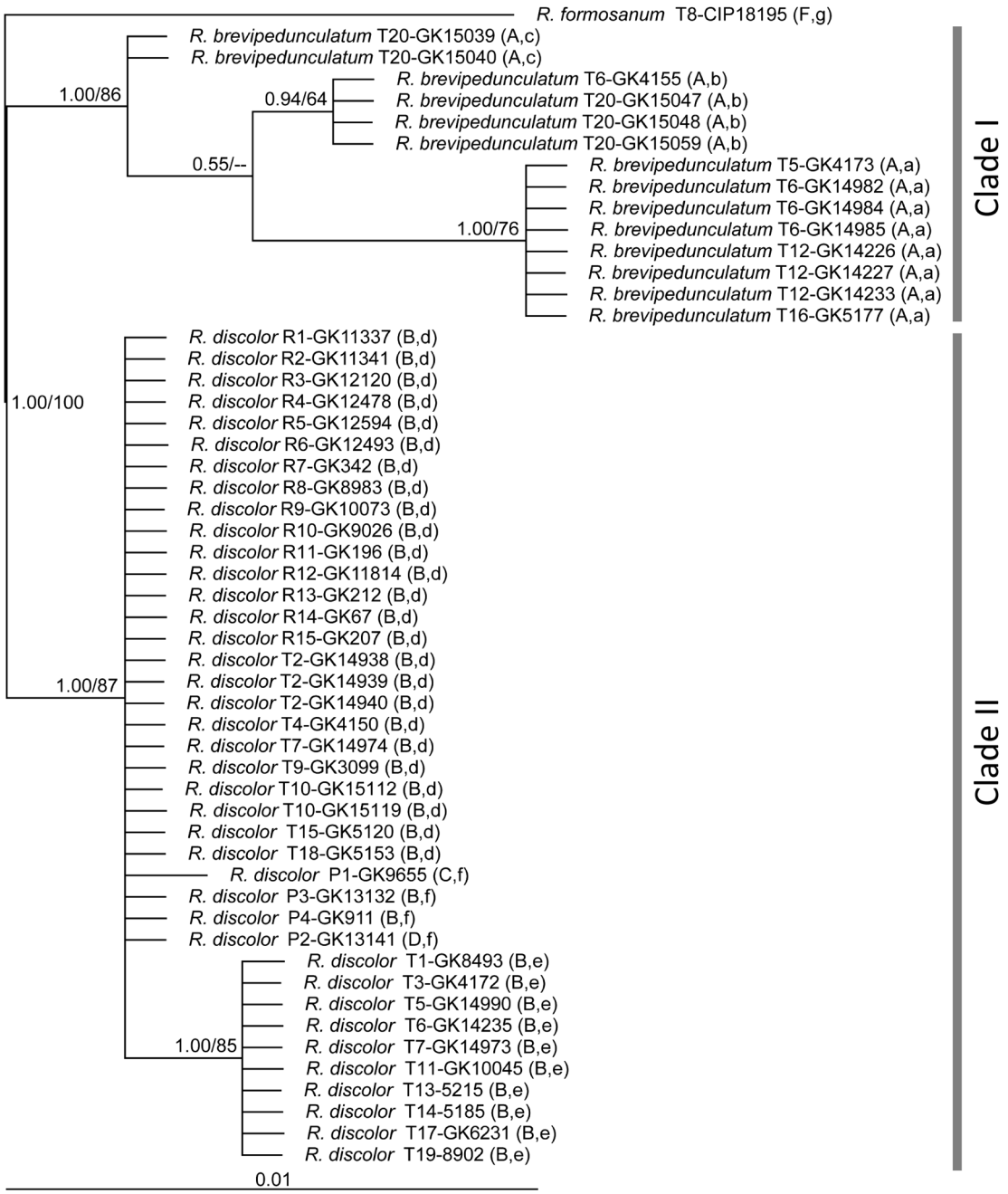

Fig. 3. The Bayesian 50% majority rule consensus tree of *Rhynchotechum* based on nrITS and cpDNA *trnSG* sequences. The topology of the maximum parsimony strict consensus tree was compatible with the Bayesian tree. Alphabets in parentheses indicate nrITS and *trnSG* types. Numerals above branches indicate Bayesian posterior probabilities (*left*) and bootstrap percentages in the maximum parsimony analysis (*right*; –<50%).

pedunculatum had intrapopulation polymorphism in *trnSG*. *Rhynchotechum* species have fresh fruits (Wang and Wang, 2000), which are expected to be dispersed by birds. The absence of geographical pattern in the ITS/*trnSG* type distribution and the intrapopulation polymorphism may be caused by

high mobility of fruits/seeds via bird dispersal. To further discuss the phylogeography of the two species, more detailed analyses with dense sampling of populations are needed.

Acknowledgments

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