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Phylogenetic analyses and species delimitation of *Aconurella* Ribaut (Hemiptera: Cicadellidae: Deltocephalinae: Chiasmini) in China based on molecular data

MINHUI YAN[1,2], JIANGWEI BAO[1,3], MIAOQING LUO[1,4], YAO GAO[1,5], CHRISTOPHER H. DIETRICH[6] & YANI DUAN[1*]

[1]*Anhui Province Key Laboratory of Integrated Pest Management on Crops, Key Laboratory of Biology and Sustainable Management of Plant Diseases and Pests of Anhui Higher Education Institutes, School of Plant Protection, Anhui Agricultural University, Hefei, Anhui 230036, China.*

[2] *1319710269@qq.com; https://orcid.org/0000-0003-0748-5445*

[3] *1966703017@qq.com; https://orcid.org/0000-0003-3676-6385*

[4] *1260270732@qq.com; https://orcid.org/0000-0003-2346-5700*

[5] *1161934262@qq.com; https://orcid.org/0000-0001-5700-5660*

[6]*Illinois Natural History Survey, Prairie Research Institute, University of Illinois, Champaign, IL 61820, USA.*
http://orcid.org/0000-0003-4005-4305

**Corresponding author. duanyani@hotmail.com; http://orcid.org/0000-0002-5952-0778*

Minhui Yan, Jiangwei Bao and Miaoqing Luo contributed equally to this article.

Abstract

The grassland leafhopper genus *Aconurella* is widespread in the Old World. Species of this genus are difficult to identify by traditional morphological characters but the morphology-based species classification in this genus has not previously been tested using molecular data. This study analysed DNA sequence data from two mitochondrial genes (COI, 16S) and one nuclear gene (ITS2) to infer the phylogenetic relationships and status of five previously recognized *Aconurella* species and compare the performance of different molecular species-delimitation methods using single and multiple loci. The analysis divided the included haplotypes into five well-supported subclades, most corresponding to existing morphology-based species concepts. However, different molecular species delimitation methods (jMOTU, ABGD, bPTP, GMYC and BPP) yielded somewhat different results, suggesting the presence of between 4 and 8 species, sometimes lumping the haplotypes of *Aconurella diplachnis* and *Aconurella sibirica* into a single species or recognizing multiple putative species within *Aconurella prolixa*. Considering the different results yielded by various methods employing single loci, the BPP method, which combines data from multiple loci, may be more reliable for delimiting species of *Aconurella.* Our results suggest that the morphological characters previously used to identify these species are reliable and adequately reflect boundaries between genetically distinct taxa.

Key words: DNA barcoding, jMOTU, ABGD, bPTP, GMYC, BPP

Introduction

Species of the Old World leafhopper genus *Aconurella* Ribaut 1948 (Hemiptera: Cicadellidae: Deltocephalinae: Chiasmini) are distributed throughout the Palearctic, Oriental and Afrotropical regions and feed on grasses. Currently, 25 species of *Aconurella* are recognized (Vilbaste 1965; Zahniser 2008) but most appear to have relatively narrow distributions (Duan & Zhang 2012). Several common and widespread Palearctic species in this genus are difficult to identify. Unlike in most other leafhoppers, the aedeagus is rather conservative in shape and species recognition is based more on differences in other structures of the male genitalia. Species-level taxonomy and identification of *Aconurella* are based on the darkly sclerotized posteroventral margin of the pygofer, usually bearing spines, and by the concave lateral margin and attenuated apex of the subgenital plate with a few macrosetae in a marginal row (Duan & Zhang 2012). However, such differences may be subtle, not easily recognized by non-experts, and the reliability of such characters remains uncertain. For rapid and accurate identification of *Aconurella*, molecular species delimitation methods may complement existing morphology-based methods.

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In this study, we performed phylogenetic analyses and tested molecular species delimitation methods on five species of *Aconurella* that occur in China: (*Aconurella prolixa* (Lethierry 1885), *Aconurella sibirica* (Lethierry 1888), *Aconurella montana* (Distant 1908), *Aconurella diplachnis* Emeljanov 1964 and *Aconurella furcata* Duan & Zhang 2012). We inferred their phylogenetic relationships based on two mitochondrial genes (COI, 16S) and one nuclear gene (ITS2). We compare the performance of five species-delimitation methods (jMOTU, ABGD, GMYC, bPTP, BPP). The goals of this study are: (i) analyse the phylogenetic relationships for some taxa of this genus; (ii) evaluate the validity of the current classification; and (iii) explore suitable species-delimitation methods.

Material and methods

Taxa sampling

Leafhopper samples included in this study were mainly collected between 2010 and 2019 from various locations in China. To document possible geographic variation within species, individuals of the same species were collected from multiple localities throughout their known ranges: 23 *A. montana* samples were collected from 23 localities in 4 provinces, 15 *A. prolixa* samples were collected from 15 localities in 8 provinces, 12 *A. sibirica* samples were collected from 12 localities in 10 provinces, 5 *A. diplachnis* samples were collected from 4 localities in 2 provinces, and 4 *A. furcata* specimens were collected from 2 localities. We chose three species of a related genus of Chiasmini, *Doratura* (*Doratura gravis* Emeljanov 1966, *Doratura homophyla* (Flor 1861) and *Doratura stylata* (Boheman 1847)), as the outgroup. Specimens were collected directly into 95% or 100% ethanol and stored in -80 °C prior to study. Identification of each individual was based on examination of external morphology and male genitalia of the adult using an Olympus SZX10 stereoscopic microscope (Olympus Corporation, Tokyo, Japan) using keys provided in Duan & Zhang (2012). *Aconurella* specimens used in this study are deposited at Northwest A&F University, Yangling, China. All specimens used for molecular identification and phylogenetic analysis are listed in Table 1 and geographical distributions are illustrated in Fig. 1. Whenever possible, specimens of different intraspecific morphological variants were selected and only male specimens were examined.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from abdominal muscle of individual, non-parasitized adult male specimens using an EasyPure Genomic DNA Kit (EE101; Transgen, Beijing, China) following the manufacturer's standard protocol, except that 20 µl proteinase K was mixed with 100 µl buffer for overnight lysis at 56 °C and the final elution volume was 60 µl due to small specimen size. After DNA extraction, the DNA solution was stored at -20 °C for subsequent molecular experiments. The abdominal exoskeleton of each extracted individual was stored in glycerin in a micro vial as a morphological voucher specimen.

Standard PCR methods were used to amplify partial sequences of the two mitochondrial genes (COI, 16S) and one nuclear gene (ITS2). Primer sequences are shown in Table 2 and the amount of template DNA was adjusted according to the DNA concentration and varied between 2 and 3 µl (Folmer et al. 1994; Simon et al. 1994; Colgan et al. 1998; Ji et al. 2003), combined with 12.5 µl of 2 Taq MasterMix, 1 µl each of forward and reverse primer, and ddH2O added to make a total volume of 25 µl for each reaction.

The PCR conditions differed according to the gene and the specific primers, especially the annealing temperature, which was the most critical factor influencing product quality. Thermal cycling conditions for each gene were as follows: an initial denaturing step at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min; annealing at 52, 54 and 56 °C for 1 min for COI, 16S and ITS2, respectively; an extension at 72 °C for 1 min; and a final extension step of 72 °C for 10 min, and ending with incubation at 12 °C . The PCR products were examined using 1% agarose gel electrophoresis with ethidium bromide stain to ensure the products were the target size. DNA products were subsequently sequenced in both directions by Qingke Biotech (Xi'an) Co., Ltd, using the original PCR primers.

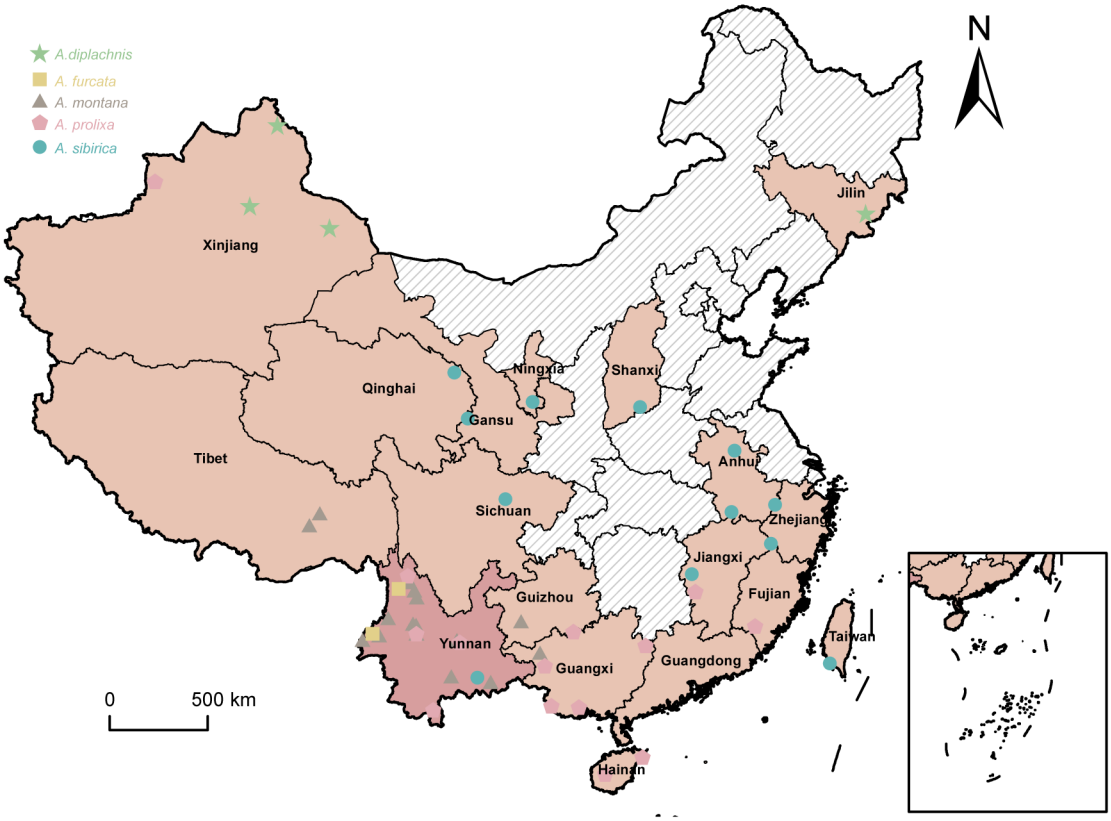

FIGURE 1. Distribution of sampled specimens of *Aconurella* in China.

TABLE 1. Specimens used in this study (all from China) with morphological identifications, haplotype classifications, collecting localities and GenBank accession numbers.

Species	Size	Code	Locality	COI		16S		ITS2	
				Haplotype	Accession Number	Haplotype	Accession Number	Haplotype	Accession Number
A. diplachnis	5	Hm084057	Erdaobai River, Jilin Province	Hap 6	MZ508723	Hap 3	MZ509015	Hap 2	MZ509050
		Hm086373	Balikun, Xinjiang	Hap 6	MZ508709	Hap 3	MZ509004	Hap 2	MZ509049
		Hm086302	Balikun, Xinjiang	Hap 6	MZ508725	Hap 3	MZ509017		-
		Hm087535	Bole City, Xinjiang	Hap 6	MZ508706		-		-
		| Hm086470	Xiaodonggou, Xinjiang	Hap 6	MZ508724	Hap 3	MZ509016	Hap 2	MZ509051
A. furcata	4	Hm084045	Weixi County, Yunnan Province	Hap 7	MZ508726	Hap 7	MZ509018	Hap 1	MZ509047
		Hm084044	Weixi County, Yunnan Province	Hap 10	MZ508707	Hap 1	MZ509002		-
		Hm080180	Zhanxi Town, Yunnan Province	Hap 7	MZ508710	Hap 4	MZ509005		-
		Hm080181	Zhanxi Town, Yunnan Province		-		-	Hap 1	MZ509048

.....*Continued on the next page*

TABLE 1. (Continued)

Species	Size	Code	Locality	COI		16S		ITS2	
				Haplotype	Accession Number	Haplotype	Accession Number	Haplotype	Accession Number
		Hm087348	Cen Wang Laoshan, Guangxi Province	Hap 3	MZ508727	Hap 8	MZ509019	Hap 3	MZ509066
		Hm086200	Yongning Town, Guizhou Province	Hap3	MZ508728	Hap 2	MZ509020	Hap 3	MZ509067
		Hm086867	Motuo Xiachayu, Tibet	Hap 27	MZ508711	Hap 2	MZ509006	Hap 3	MZ509054
		Hm087498	Nyingchi Pailong, Tibet		-		-	Hap 3	MZ509053
		Hm087702	Tongmai Town, Tibet	Hap 3	MZ508712	Hap 5	MZ509007	Hap 3	MZ509055
		Hm087500	Yigong Town, Tibet	Hap 1	MZ508729		-	Hap 3	MZ509068
		Hm086953	Cangshan, Yunnan Province	Hap1	MZ508708	Hap2	MZ509003	Hap3	MZ509052
		Hm080262	Dali, Yunnan Province	Hap 1	MZ508713	Hap 2	MZ509008	Hap 3	MZ509056
		Hm083461	Heping Village, Yunnan Province	Hap 2	MZ508715	Hap 6	MZ509010	Hap 3	MZ509058
		Hm087711	Gongshan County, Yunnan Province	Hap 3	MZ508719	Hap 2	MZ509013	Hap 3	MZ509062
		Hm087354	Laomdeng Village, Yunnan Province	Hap 1	MZ508718		-	Hap 3	MZ509061
A. montana	23	Hm087552	Nanuo County, Yunnan Province	Hap 1	MZ508735		-		-
		Hm087560	Pianma Town, Yunnan Province	Hap 5	MZ508722		-	Hap 3	MZ509065
		Hm081781	Qinglong Town, Yunnan Province	Hap 8	MZ508730	Hap 2	MZ509021	Hap 3	MZ509069
		Hm082273	Tengchong City, Yunnan Province	Hap 9	MZ508731	Hap 2	MZ509022	Hap 3	MZ509070
		Hm081831	Tiger Leaping Gorge, Yunnan Province	Hap 1	MZ508716	Hap 2	MZ509011	Hap 3	MZ509059
		Hm083839	Wangshan, Yunnan Province	Hap 4	MZ508720		-	Hap 3	MZ509063
		Hm083968	Weishan County, Yunnan Province	Hap 1	MZ508714	Hap 2	MZ509009	Hap 3	MZ509057
		Hm084048	Weixi County, Yunnan Province	Hap 1	MZ508717	Hap 2	MZ509012	Hap 3	MZ509060
		Hm083137	Xima Town, Yunnan Province	Hap 11	MZ508733	Hap 2	MZ509024	Hap 3	MZ509072
		Hm087365	Yaodian Village, Yunnan Province	Hap 3	MZ508732	Hap 2	MZ509023	Hap 3	MZ509071
		Hm087426	Yulong Snow Mountain, Yunnan Province	Hap 1	MZ508721	Hap 2	MZ509014	Hap 3	MZ509064
		Hm080171	Zhanxi Town, Yunnan Province	Hap 1	MZ508734	Hap 2	MZ509025		-

.....Continued on the next page

TABLE 1. (Continued)

Species	Size	Code	Locality	COI		16S		ITS2	
				Haplotype	Accession Number	Haplotype	Accession Number	Haplotype	Accession Number
		Hm080617	Longtan, Fujian Province	Hap 12	MZ508736	Hap 12	MZ509026	Hap 4	MZ509073
		Hm083842	Dinghu Mountain, Guangdong Province	Hap 13	MZ508737	Hap 12	MZ509027	Hap 4	MZ509074
		Hm087347	Guzhang Town, Guangxi Province	Hap 12	MZ508747		-	Hap 4	MZ509085
		Hm087156	Lingyun County, Guangxi Province	Hap 12	MZ508746		-	Hap 4	MZ509084
		Hm087496	Shangsi County, Guangxi Province	Hap 12	MZ508748		-	Hap 4	MZ509086
		Hm087016	Xiashi Town, Guangxi Province	Hap 12	MZ508745		-		-
		Hm086253	Maolan Town, Guizhou Province	Hap 17	MZ508743	Hap 12	MZ509034	Hap 4	MZ509082
A. prolixa	15	Hm084054	Jianfengling, Hainan Province	Hap 15	MZ508740	Hap 12	MZ509031	Hap 4	MZ509079
		Hm084156	Tongguling, Hainan Province	Hap 15	MZ508741	Hap 12	MZ509032	Hap 4	MZ509080
		Hm086192	Xincheng Area, Jiangxi Province	Hap 16	MZ508742	Hap 12	MZ509033	Hap 4	MZ509081
		Hm084053	Shangrila City, Yunnan Province		-		-	Hap 4	MZ509075
		Hm084002	Weishan County, Yunnan Province	Hap 14	MZ508738	Hap 12	MZ509028	Hap 4	MZ509076
		Hm084158	Mengla County, Yunnan Province	Hap 14	MZ508739	Hap 12	MZ509029	Hap 4	MZ509077
		Hm081572	Qinglong Town, Yunnan Province		-	Hap 12	MZ509030	Hap 4	MZ509078
		Hm086345	Yili River Wetland Park, Xinjiang	Hap 18	MZ508744		-	Hap 4	MZ509083

.....Continued on the next page

TABLE 1. (Continued)

Species	Size	Code	Locality	COI		16S		ITS2	
				Haplotype	Accession Number	Haplotype	Accession Number	Haplotype	Accession Number
A. sibirica	12	Hm083152	Bengbu City, Anhui Province	Hap 19	MZ508749	Hap 9	MZ509035	Hap 5	MZ509087
		Hm083231	Wangjiang County, Anhui Province	Hap 19	MZ508750	Hap 9	MZ5090363	Hap 5	MZ509088
		Hm083891	Xiahe County, Gansu Province	Hap 21	MZ508752	Hap 10	MZ509038		-
		Hm084198	Wanlongshan, Jiangxi Province	Hap 26	MZ508759	Hap 11	MZ509046	Hap 5	MZ509094
		Hm084056	Dalongtan, Ningxia	Hap 24	MZ508757	Hap 11	MZ509043	Hap 5	MZ509092
		Hm083457	Zhaobi Mountain, Qinghai Province	Hap 20	MZ508751	Hap 9	MZ509037		-
		Hm083956	Yangcheng County, Shanxi Province	Hap 22	MZ508753	Hap 11	MZ509039	Hap 5	MZ509089
		Hm084051	Mianyang City, Sichuan Province	Hap 22	MZ508756	Hap 11	MZ509042		-
		Hm084038	Sun Yat-Sen University, Taiwan	Hap 19	MZ508755	Hap 9	MZ509041	Hap 5	MZ509091
		Hm084036	Gelaihe Village, Yunnan Province	Hap 23	MZ508754	Hap 10	MZ509040	Hap 5	MZ509090
		Hm084196	Qingliangfeng, Zhejiang Province		-	Hap 11	MZ509045		-
		Hm084155	Xianxialing, Zhejiang Province	Hap 25	MZ508758	Hap 11	MZ509044	Hap 5	MZ509093
D. gravis	1	Hm086974	Jiangxi ditch, Qinghai Province	Hap 28	MZ544645	Hap 13	MZ557385	Hap 6	MZ557382
D. homophyla	1	Hm086296	Kanas, Xinjiang	Hap 29	MZ544646	Hap 14	MZ557386	Hap 7	MZ557383
D. stylata	1	Hm087339	Kanbutangla, Tibet		-	Hap 15	MZ5573857	Hap 8	MZ557384

GenBank accession numbers for COI, 16S and ITS2 sequences are provided; "-" indicates no number.

TABLE 2. Primer sequences for PCR amplification and sequencing.

Gene segment	Primer Name	Primer sequence (5'-3')	Reference
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
16S	LR-J-12887	CCGGTCTGAACTCAGATCACGT	Simon et al. (1994)
	LR-N-13398	CGCCTGTTTAACAAAAACAT	Simon et al. (1994)
ITS2	ITS2-F	TGAACATCGACATTTYGAACGCACAT	Ji et al. (2003)
	ITS2-R	TTCTTTTCCTCCSCTTAYTRATATGCTTAA	Ji et al. (2003)

Data analysis

To obtain single consensus sequences, chromatograms, including sense and antisense strands, were analyzed and assembled using Seqman software (Swindell & Plasterer 1997). In order to ensure that the correct target gene fragment was obtained, the Basic Local Alignment Search Tool (BLAST) was used to check all sequences against the NCBI database (Altschul et al. 1990). Concurrently, for the encoding gene fragments, to ensure that stop codons and pseudogenes did not exist, we translated the assembled contigs into amino acids using MEGA 6 (Tamura et al. 2013). Multiple alignments were obtained using MAFFT, and the sequences were then adjusted and trimmed manually using MEGA 6 (Tamura et al. 2013; Katoh & Standley 2013). Conserved sites (C), variable sites (V), parsimony-informative sites (PI), and average nucleotide composition for each region were calculated by MEGA 6 (Tamura et al. 2013). Genetic diversity parameters including the haplotype number, haplotype diversity (Hd), and nucleotide diversity (Pi) were calculated by DNASP 5.0, with gaps/missing data not included in computation of Pi (Jukes and Cantor) (Librado & Rozas 2009). Finally, before combining multiple genes to build trees, scatter plots of transitions/transversions of each gene were made by using DAMBE5.0 software (Xia 2013) to test for substitution saturation. Samples with identical sequences for a particular gene belong to the same haplotype for that gene, and a single representative of each haplotype was included in the analyses of individual genes.

Phylogeny

Sequences for each gene were aligned separately in MAFFT. Regions of ambiguous alignment were deleted, and the alignments of all three genes (COI, 16S, ITS2) were concatenated into a single dataset for analysis. The three gene fragments and individual codon positions within the protein-coding gene (COI) were treated as separate data partitions in phylogenetic analyses. Phylogenetic reconstruction was conducted using Bayesian inference (BI) and Maximum likelihood (ML). The most suitable substitution models and partition scheme were determined for the combined data sets using PartitionFinder 2.1.1 (Lanfear et al. 2012). The selected models are as follows: the three codon positions of COI were (GTR, GTR+G, GTR+G); 16S was (GTR+G); ITS2 was (GTR).

BI analyses were conducted using MrBayes 3.2 with partitioned models (Ronquist et al. 2012). Two simultaneous runs of 10,000,000 generations were conducted for each matrix. The trees were sampled every 1000 generations. Convergence and stability were evaluated in Tracer v.1.7 ensuring effective sample size > 200 for all parameters (Rambaut et al. 2018). With the first 25% of trees discarded as burn-in, Bayesian posterior probabilities were calculated for a 50% majority rule consensus tree of the remaining trees. For comparison, we also reconstructed the phylogenetic relationships of *Aconurella* using ML. We conducted ML phylogenetic analysis on each locus separately and on the concatenated gene data sets with RAxML v8 (Felsenstein 1985; Stamatakis 2014). We used 1,000 bootstrap replicates in a rapid bootstrap analysis and under the GTRCAT model to search for the best scoring ML tree. Trees were visualized using FigTree v1.4.2.

Species delimitation

We used four independent species delimitation methods that do not require a priori taxonomic information and use data from single loci: jMOTU (Jones et al. 2011), ABGD (Puillandre et al. 2012), GMYC and bPTP (Fujisawa & Barraclough 2013; Zhang et al. 2013). In addition, we used one method that requires a priori taxonomic information and used multiple loci to verify the results from analyses of single loci: Bayesian coalescent method using BPP software (Yang 2015).

jMOTU

The jMOTU software divides the sequences into different molecular operational taxonomic units (MOTUs) based on the magnitude of the genetic distances, and combines sequences into the same MOTU whenever the genetic distance difference of the sequence is smaller than the specified threshold. Thresholds specified include a range of

sequence cut off values (1–30), the shortest sequence matching percentage (95%), and the lowest BLAST value (97%). The number of MOTUs was plotted against the threshold values to determine the number of species inferred from each value of the different distance thresholds (Jones et al. 2011).

ABGD

This method assumes that intraspecific genetic distances are significantly lower than interspecific differences, giving rise to a barcode gap that can be used for species delimitation (Puillandre et al. 2012). Using the web server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) we selected the following parameters for analysis: prior intraspecific divergence values (Pmin=0.001 and Pmax=0.1), relative gap width (X=1.0) and the Kimura 2-P (K80) distance model. Other parameters were set to default values.

GMYC

The GMYC model-based likelihood method identifies the transition point between coalescent and speciation events using an ultrametric tree as input. Different haplotypes were identified using DNASP 5.0 (Librado & Rozas 2009) as defined above and PartitionFinder was used to determine the most suitable substitution model for each gene (Lanfear et al. 2012). The models for the three genes (COI, 16S, ITS2) are respectively GTR, GTR+G and HKY. We used the uncorrelated relaxed clock and the Yule speciation model, with 10 million generations with sampling every and 1,000 generations with the first 25% of samples removed as burn-in (Drummond et al. 2012). After the effective sample size (ESS) of each parameter was greater than 200, TreeAnnotator v.1.7.0 (BEAST package) was used to assemble the maximum clade credibility tree (Rambaut et al. 2018). We used the ultrametric tree generated for GMYC analysis. The GMYC model was implemented in the R splits package (available at http://r-forge.rproject.org/projects/splits), performing single and multiple-threshold analyses separately.

bPTP

The bPTP analysis, which assumes independent exponential distributions to model the branch lengths for speciation and for coalescence, was conducted on the online server (http://species.h-its.org) using the maximum-likelihood tree from IQ-TREE (Zhang et al. 2013), specifying an outgroup, with 100,000 MCMC generations, a thinning value of 100, burn-in of 10%, and default values for other parameters.

BPP

For BPP analysis, we used the concatenated three-gene data set (COI, 16S, ITS2) as input with the guide tree generated by BEAST v1.7.0 (Drummond et al. 2012). We included data for *D. gravis*, *D. homophyla* and *D. stylata* for the BPP analyses because the statistical power of BPP can be increased when closely related outgroups are included (Rannala & Yang 2013). The analysis was run for 10 million generations, with a sample frequency of 1000. Convergence and stability were evaluated in Tracer v.1.7. ensuring an effective sample size > 200 for all parameters (Rambaut *et al.* 2018). A maximum clade credibility tree was assembled using TreeAnnotator v.1.7.0 (BEAST package) with the first 25% of samples removed as burn-in.

Species delimitation in BPP requires a priori estimation of two evolutionarily significant parameters: ancestral population size (θ) and degree of divergence among species (τ). We followed the procedure suggested by Yang (2015) in conducting runs with the following combinations: 1. Θ: G (2: 1000), τ: G (2: 2000); 2. Θ: G (2: 100), τ: G (2: 200); 3. Θ: G (2: 100), τ: G (2: 2000); 4. Θ: G (2: 1000), and τ: G (2: 200). All BPP analyses were run for 500,000 generations with sampling every five generations, after discarding an initial burn-in of 20,000 generations. For verification, every analysis was run twice to check for convergence between runs and agreement on the posterior probability of the species delimitation models.

Results

Data analyses

PCR and sequencing yielded 54 COI sequences (5 *A. diplachnis*, 3 *A. furcata*, 11 *A. sibirica*, 22 *A. montana*, 13 *A. prolixa*), 45 16S sequences (4 *A. diplachnis,* 3 *A. furcata*, 12 *A. sibirica*, 17 *A. montana*, 9 *A. prolixa*), and 48 ITS2 sequences (3 *A. diplachnis,* 2 *A. furcata*, 8 *A. sibirica*, 21 *A. montana*, 14 *A. prolixa*) from the available samples. Trimmed alignments for the three genes are 597bp, 516bp and 582bp, respectively. C, V, PI, average nucleotide composition, haplotype number, Hd, and Pi are listed in Table 3. All included species were represented by multiple haplotypes for at least one locus. The sequences of two mitochondrial genes showed high haplotype number and Hd, with multiple species having more than one haplotype, while the nuclear gene showed low haplotype number and Hd (no more than one haplotype per species). Tests of substitutional saturation using DAMBE indicated no significant saturation in any of the included loci.

TABLE 3. C, V, PI, Average Nucleotide Composition, Haplotype Number, Hd, and Pi

Gene	C	V	PI	T(%)	C(%)	A(%)	G(%)	A+T(%)	Haplotype Number	Hd	Pi
COI	364	233	212	33.5	14.4	32.9	19.1	66.4	27	0.943	0.09554
16S	249	267	265	37.8	12.7	31.4	18.1	69.2	12	0.819	0.17000
ITS2	481	151	143	20.7	28.4	23.4	27.5	44.1	5	0.716	0.01338

Phylogeny

The tree topologies reconstructed by both BI and ML analyses based on the one concatenated data set were very similar. The inferred phylogenetic relationships were robust for the major clades (Fig. 2). The BI and ML analyses grouped the ingroup taxa into two identical monophyletic clades with high support values (PP=1, BS=61). All analyses consistently supported the monophyly of the five species *A. montana*, *A. diplachnis*, *A. sibirica*, *A. furcata* and *A. prolixa*. Trees from both analyses consistently yielded the following topology (*A. montana* + ((*A. diplachnis* + *A. sibirica*) + (*A. furcata* + *A. prolixa*))). Monophyly of *A. montana* is highly supported in ML. In clade II, *A. diplachnis* and *A. sibirica* (PP=1, BS=1) were strongly supported as monophyletic sister clades. The other subclade included sister groups *A. furcata and A. prolixa* (PP = 0.85, BS = 83).

Species delimitation

jMOTU

For the COI dataset, when the sequence cut off values were 23–30, although the number of MOTU remained stable at 5 and the species recognized are different from the morphological species: *A. diplachnis* and *A. sibirica* are combined into one MOTU and *A. prolixa* is divided into two MOTUs. The success rate of identification was 80%. For the 16S dataset, jMOTU yielded the same number of MOTUs for the range of cut off values of 7–8. However, for this gene, the five morphological species each corresponded to a separate MOTU. For the ITS2 dataset, as the sequence cut off value increases gradually, jMOTU places all the sequences into a single MOTU, indicating that this gene is unable to distinguish species of *Aconurella* that have been recognized based on traditional morphological criteria (Figs. 3–5).

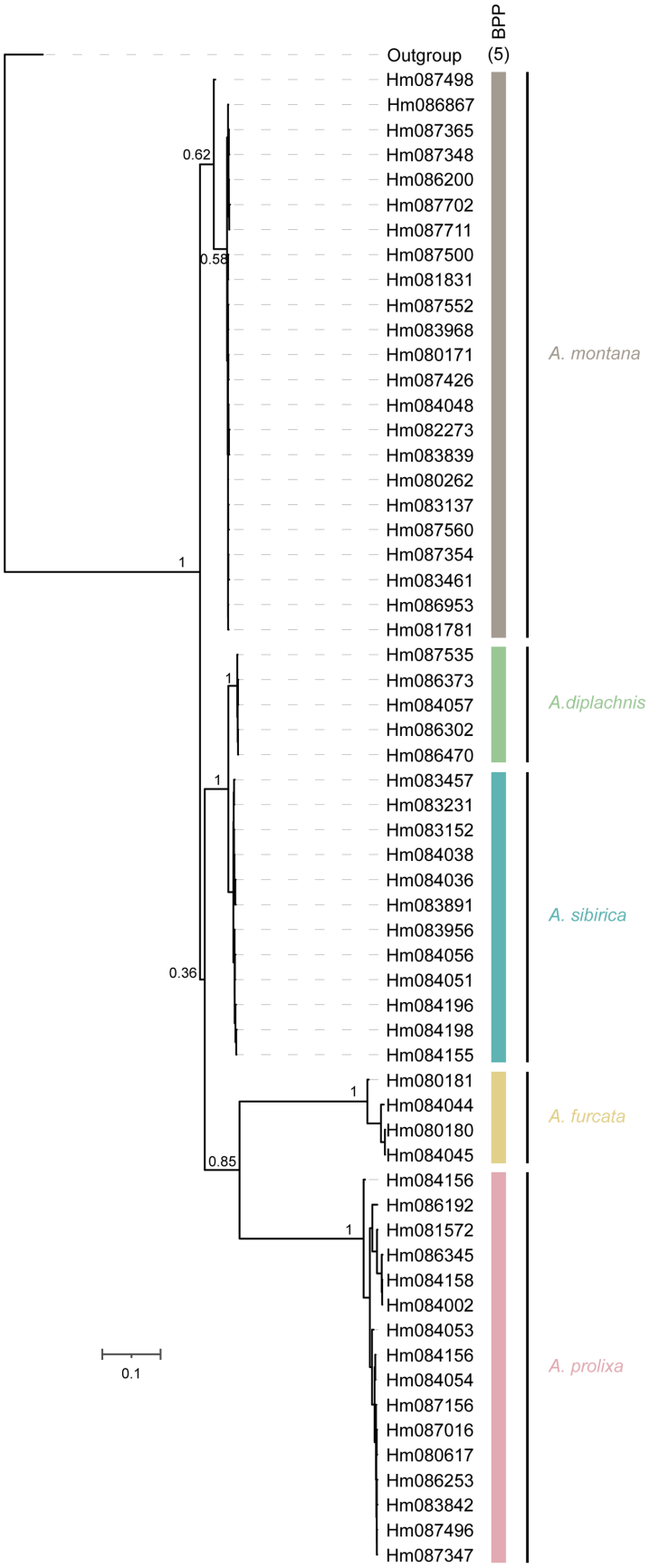

FIGURE 2. Bayesian consensus phylogenetic tree for *Aconurella* based on three-gene data set (COI, 16S and ITS2). Numbers on the node represents the posterior probabilities. The right vertical bars indicate the putative species using BPP. The scale bar shows the number of substitutions per site. Morphological species are uniquely coloured.

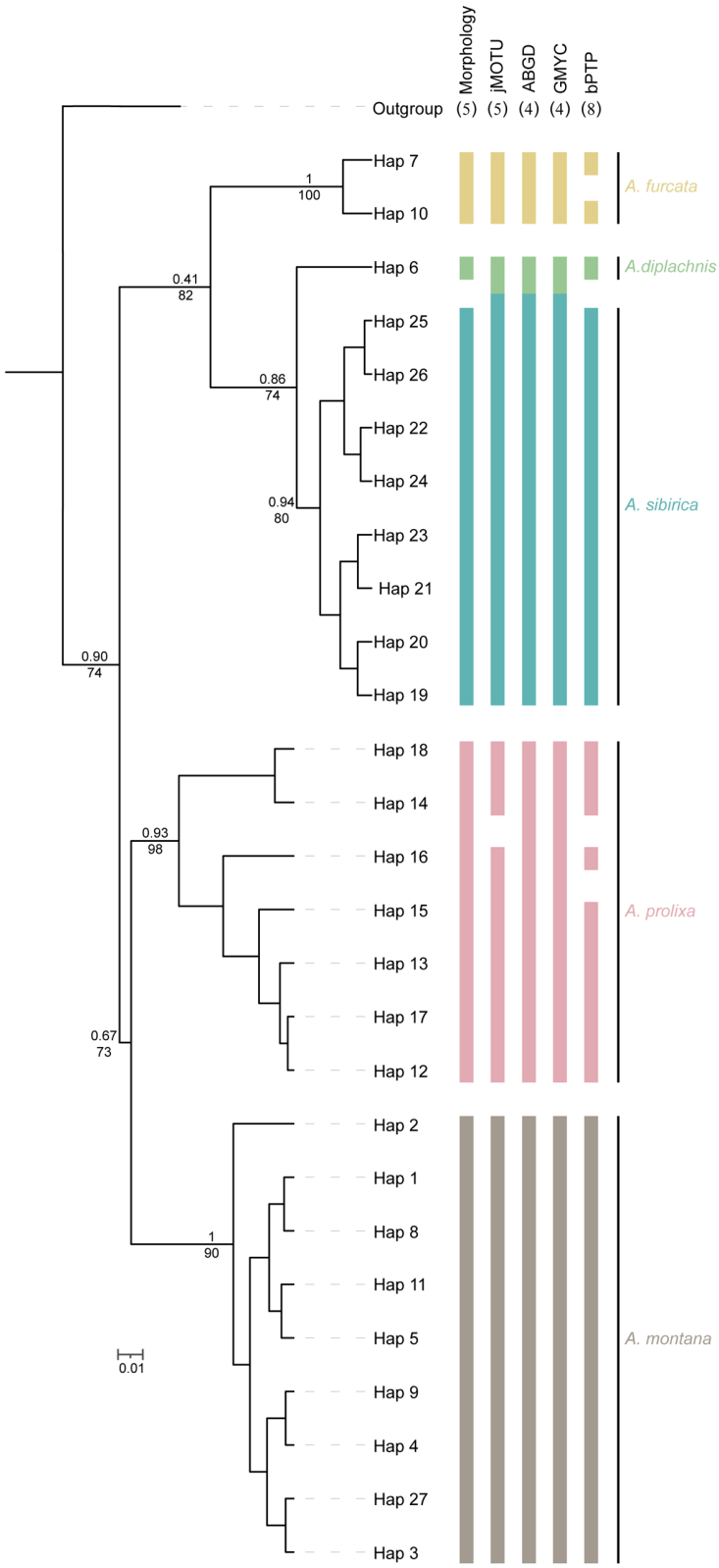

FIGURE 3. Phylogenetic tree for *Aconurella* based on mitochondrial COI haplotypes from BEAST. Bootstrap support and posterior probabilities of nodes are indicated above and below the branches, respectively. The right vertical bars indicate the number of putative species using various methods as indicated at the top. The scale bar shows the number of substitutions per site. Morphological species are uniquely coloured.

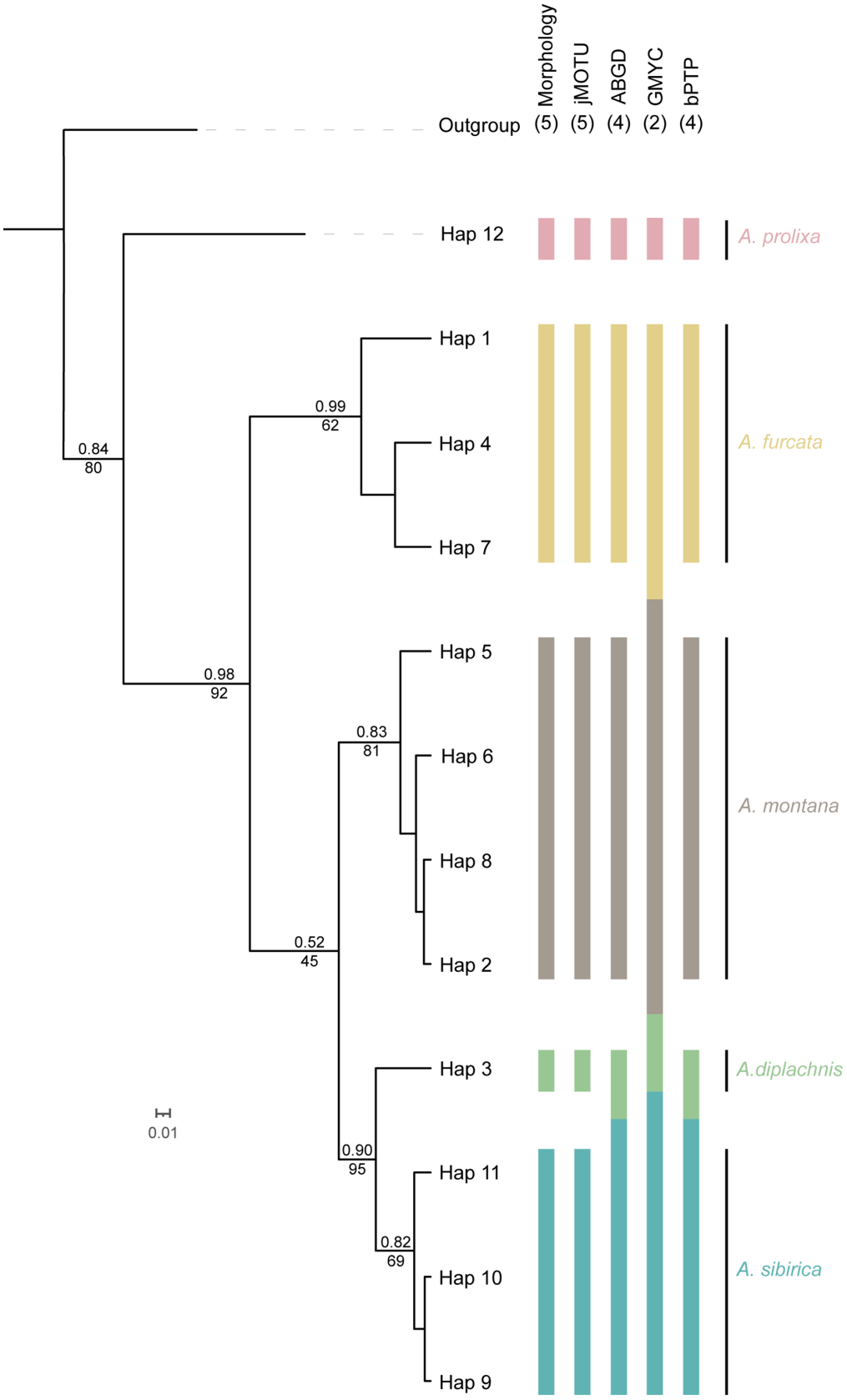

FIGURE 4. Phylogenetic tree for *Aconurella* based on mitochondrial 16S haplotypes from BEAST. Bootstrap support and posterior probabilities of nodes are indicated above and below the branches, respectively. The right vertical bars indicate the number of putative species using various methods as indicated at the top. The scale bar shows the number of substitutions per site. Morphological species are uniquely coloured.

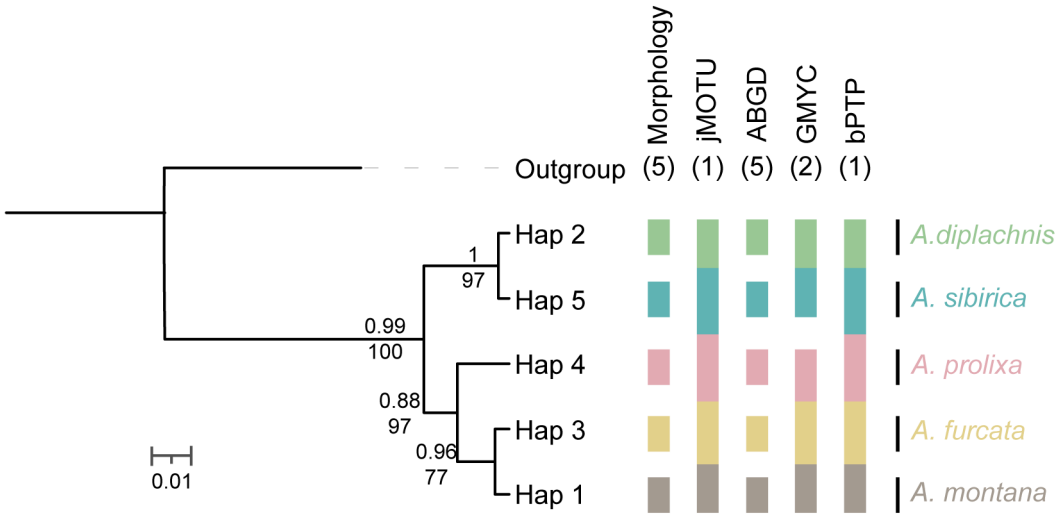

FIGURE 5. Phylogenetic tree for *Aconurella* based on mitochondrial ITS2 haplotypes from BEAST. Bootstrap support and posterior probabilities of nodes are indicated above and below the branches, respectively. The right vertical bars indicate the number of putative species using various methods as indicated at the top. The scale bar shows the number of substitutions per site. Morphological species are uniquely coloured.

ABGD

For COI, this method divided the samples into 4 groups for intraspecific divergence (P value) of 0.037927 to 0.078476, with *A. sibirica* and *A. diplachnis* into one group, and the remaining morphological species recognized as 3 separate groups. The success rate of identification was 75%. For the 16S data, the number of groups decreased gradually with an increase in P value, with the same 4 groups delimited by the COI data recognized when P value) was 0.018330 to 0.061585. For ITS2, this method divided the samples into 5 groups corresponding to the morphological species when the P value was 0.002069 to 0.006952 (Figs. 3–5).

GMYC

For COI, GMYC recognized 4 and 6 MOTUs, respectively, under the single-threshold model and multiple-threshold model, with confidence intervals of 1–8 and 1–9. The same analysis of 16S yielded 2 MOTUs with confidence intervals of 2 and 2–4. ITS2 recognized 2 and 3 MOTUs with confidence intervals 1–2 and 1–3 (Figs. 3–5).

bPTP

The bPTP analysis gives two results based on Bayesian and ML support. The ML method result corresponds to the PTP analysis result, and the Bayesian method corresponds to the bPTP analysis result. Both methods gave the same results for the COI gene recognizing 8 MOTUs. The 16S recognized 4 MOTUs. ITS2 recognized a single MOTU (Figs. 3–5).

BPP

With *D. gravis*, *D. homophyla* and *D. stylata* as outgroups, a phylogenetic tree was constructed by combining two mitochondrial genes (COI, 16S) and one nuclear gene (ITS2) of *Aconurella* as a guide tree for BPP input. Every analysis was run twice. All results recognized eight species, corresponding to the morphological species, with strong support, including the outgroup (Table 4 and Fig. 2).

TABLE 4. Posterior probabilities for the number of delimited species using different priors for model parameters in Bayesian phylogenetics and phylogeography on concatenated data sets of mitochondria markers and all genetic markers.

Prior	Posterior probability for the number of delimited species (all mitochondrial genes)	Posterior probability for the number of delimited species (all genes)
θ: G (2: 1000), τ: G (2: 2000)	P 8=1.00	P 8=1.00
θ: G (2: 100), τ: G (2: 200)	P 8=0.98653	P 8=0.98653
θ: G (2: 100), τ: G (2: 2000)	P 8=1.00	P 8=1.00
θ: G (2: 1000), τ: G (2: 200)	P 8=1.00	P 8=1.00

Discussion

Previous taxonomic studies of *Aconurella* focused only on morphology-based classification (Duan & Zhang 2012) and prior phylogenetic analyses have not included a sample of taxa sufficient to explore relationships among species of this genus or assess species boundaries. In this study, we reconstructed the phylogenetic relationships among *Aconurella* species using ML and BI methods based on data from three genes for multiple individuals of five species occurring in the Oriental region. BI and ML methods yielded identical tree topologies with most branches receiving strong support, and supported the monophyly of these five morphologically delimited species. Both ML and BI analyses generally supported the relationship (*A. montana* + ((*A. diplachnis* + *A. sibirica*) + (*A. furcata* + *A. prolixa*))). Our results suggest that the morphological characters previously used to identify these species are reliable and adequately reflect boundaries between genetically distinct taxa.

We used a variety of methods previously proposed to facilitate species delimitation using molecular data. Interestingly the number of species delimited varied not only based on the method but also based on the particular gene region used. Our results accentuate the importance of comparing the results of different molecular species delimitation methods rather than relying on a single method or locus. The BPP method, which incorporated data from multiple loci as well as outgroup information may be the most robust method tested (Yang & Rannala 2010; Yang 2015; Leaché et al. 2017). Previous studies indicate that this method tends to yield results more congruent with morphological species definitions (Hurtabo-Burillo et al. 2016; Yang & Rannala 2017). In this study, the BPP method strongly supported recognition of the five *Aconurella* species, with all nodes having maximum posterior probabilities for all four combinations of ancestral population size (θ) and degree of divergence among species (τ). BPP generally yields high posterior probabilities for correct species delimitations when appropriate priors are chosen (Yang 2015; Yang & Rannala 2017; Luo et al. 2018). Our results demonstrate that species delimitation analyses based on multiple loci give a more credible and consistent result than methods using a single locus. Nevertheless, the additional MOTUs within the morphological species *A. furcata* and *A. prolixa* delimited by the jMOTU and bPTP methods using COI data may merit further analysis incorporating additional samples of these species. It is possible that these additional MOTUs represent cryptic or incipient species.

Conclusion

Our analysis of multi-gene sequence data consistently resolve the phylogenetic relationships among five species of *Aconurella* and support recognition of these species previously defined based on morphological criteria alone. The analysis divided the included haplotypes into five well-supported subclades. Data from three genes usually lumped the haplotypes of *A. diplachni*s and *A. sibirica* into a single species. Considering the variable results yielded by various methods employing single loci, the BPP method which combines data from multiple loci may be more reliable in *Aconurella*. However, this needs to be further tested by including a broader sample of *Aconurella* populations occurring outside of China.

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