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Molecular Phylogeny of the Barwings (Aves: Timaliidae: *Actinodura*), a Paraphyletic Group, and Its Taxonomic Implications

Feng Dong[1,2], Fei Wu[1], Lu-Ming Liu[1], and Xiao-Jun Yang[1,*]

[1]*State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China*

[2]*Graduate Univ. of the Chinese Academy of Sciences, Beijing, China*

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Feng Dong, Fei Wu, Lu-Ming Liu, and Xiao-Jun Yang (2010) Molecular phylogeny of the barwings (Aves: Timaliidae: *Actinodura*), a paraphyletic group, and its taxonomic implications. *Zoological Studies* **49**(5): 703-709. The barwings (Aves: Timaliidae: genus *Actinodura*) are a small group of poorly studied babblers that inhabit mountains from the Himalayas to continental China and Taiwan. To infer the phylogeny among members of the *Actinodura*, we examined variations of 3 mitochondrial fragments (the entire cytochrome *b* gene, and portions of the NADH dehydrogenase subunit 2 and cytochrome *c* oxidase I genes, with 2,725 bp in total) in multiple samples representing six of the 7 recognized barwing species. Results of both the maximum-likelihood and Bayesian-based analyses indicated that *Actinodura* is composed of 2 major clades; however, there were 2 *Minla* species nested within the clade that otherwise contained all barwing species. Due to the nature of the paraphyly, we propose that the 2 clades of *Actinodura* be split into 2 genera, *Actinodura* and *Ixops*, which can be diagnosed by the tail length, the presence/absence of a striped breast, and other morphological traits. Our results also revealed that *Minla*, an ally of *Actinodura*, is also a paraphyletic group. Our analysis supported the recent taxonomic recommendation to delimit traditional minlas into 3 monotypic genera. We also present evidence suggesting that the speciation of barwings might have partially been promoted by ecological niche differentiation along with geographical isolation. http://zoolstud.sinica.edu.tw/Journals/49.5/703.pdf

Key words: Paraphyly, *Actinodura*, *Minla*.

The barwings (genus *Actinodura* Gould, 1836) are a small group of little-studied babblers defined by the presence of transverse barring on the remiges and rectrices and a crest (Gould 1836). The genus is composed of 7 recognized species: the Rusty-fronted Barwing *A. egertoni*, Spectacled Barwing *A. ramsayi*, Black-crowned Barwing *A. sodangorum*, Hoary-throated Barwing *A. nipalensis*, Streak-throated Barwing *A. waldeni*, Streaked Barwing *A. souliei*, and Taiwan Barwing *A. morrisoniana* (Dickinson 2003, Collar and Robson 2007). The distribution of these species ranges from southwestern China to southern Myanmar and from the Central Himalayas to western Tokin and Taiwan (Fig. 1).

Although Delacour (1946) merged *A. egertoni* and *A. ramsayi* as a superspecies and considered *A. waldeni* to be conspecific with *A. nipalensis*, taxonomists later supported the delimitation of the 6 previously recognized species (including Deignan 1964, Sibley and Monroe 1990) until Eames et al. (1999) described a new member, *A. sodangorum*. Nevertheless, phylogenetic relationships among *Actinodura* species are not well understood.

This study attempted to infer phylogenetic relations among *Actinodura* species with a dataset of 3 mitochondrial genes including the complete cytochrome (Cyt) *b* gene, part of the NADH dehydrogenase subunit 2 (ND2), and part of cytochrome *c* oxidase I (COI). Results of this

*To whom correspondence and reprint requests should be addressed. Tel: 86-871-5192023. Fax: 86-871-5192023. E-mail:yangxj@mail.kiz.ac.cn

study should provide information on the evolution of these babblers and improve our understanding of the diversification of avian species in East Asia.

MATERIALS AND METHODS

In this study, we examined 6 of the 7 *Actinodura* species, with the exception of *A. sodangorum*. Three minlas (*Minla cyanouroptera*, *M. strigula*, and *M. ignotincta*), 1 liocichla (*Liocichla steerii*), 1 leiothrix (*Leiothrix lutea*), and 1 sibia (*Heterophasia auricularis*) were also included as proximate outgroups, while 3 laughingthrushes (*Garrulax erythrocephalus*, *G. affinis*, and *G. morrisonianus*) were used as rooting outgroups. Outgroup selection was based on molecular phylogenies for the Timaliidae constructed by Cibois (2003) and Luo et al. (2009). The sampling details of the present study are presented in table 1.

Total genomic DNA was isolated from muscle or blood samples preserved in 95% alcohol, using a combination of proteinase K digestion and phenol/chloroform extraction (Sambrook et al. 1989). We used primers listed in table 2 to amplify and sequence the mitochondrial full-length Cyt *b*, partial ND2, and partial COI genes. The polymerase chain reactions (PCRs) were conducted on a T3000 Thermocycler (Biomatra, Leipzig, Germany) in 50 μl volumes containing 38.5 μl of double-distilled water, 5 μl of 10x PCR buffer (1% sodium dodecylsulfate, 50% glycerol, and 0.05% bromophenol blue), 3 μl of 2.5mM of the dNTP mixture, 2 μl of bovine serum albumin, 1 μl of a 10 mM solution of each primer, 1.25 units of Taq polymerase (Sangon, Shanghai, China), and about 50 ng of the DNA template. PCR protocols were as follows: 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, extension at 72°C for 50 s, and a final extension step of 7 min at 72°C. The PCR products were electrophoresed in 1.5% agarose and recovered using a DNA purification kit (Sangon). Sequencing of the purified PCR

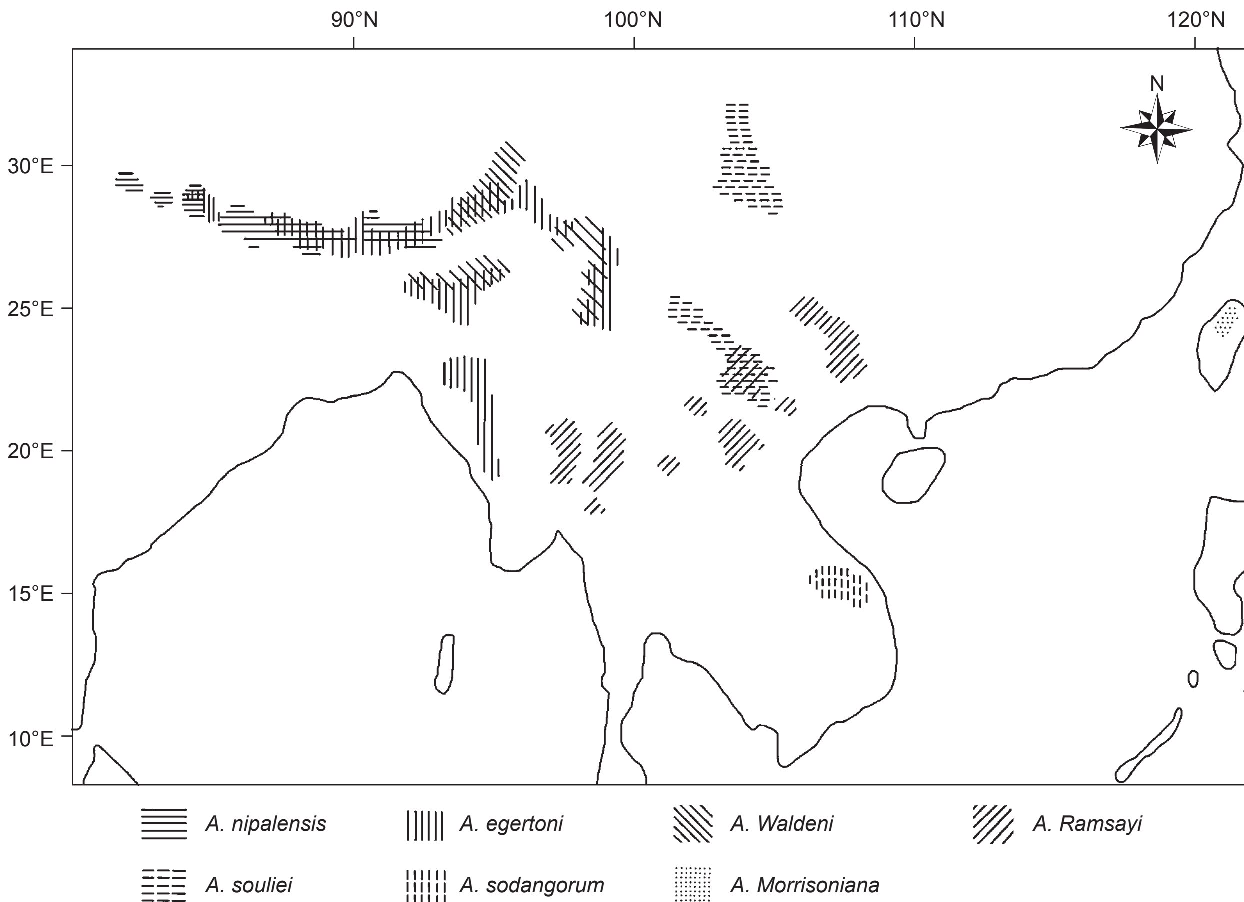

Fig. 1. Distribution ranges of seven species of *Actinodura* (modified from Collar and Robson 2007).

products was performed with BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) using the same primers as those used for the PCRs. To improve the accuracy, multiple individuals and DNA strands from both directions were sequenced when possible. DNA sequences were proofread and assembled with the aid of the SeqMan 7.1.0 program included in

Table 1. Sampling of this study

Taxon	Tissue number	Collection locality	GenBank accession no.		
			Cytb	ND2	COI
Ingroup					
Actinodura egertoni	KIZ, GLGS5797	Yunnan	GU139509	GU139523	GU139495
	KIZ, GLGS5798	Yunnan	GU139511	GU139525	GU139497
	KIZ, GLGS5828	Yunnan	GU139510	GU139524	GU139496
Actinodura nipalensis[a]	GenBank	Nepal	AF484840	-	-
Actinodura waldeni	KIZ, 04500	Yunnan	GU139504	GU139518	GU139490
	KIZ, 04501	Yunnan	GU139505	GU139519	GU139491
Actinodura ramsayi	KIZ, PB001	Yunnan	GU139513	GU139527	GU139499
	KIZ, PB002	Yunnan	GU139512	GU139526	GU139498
Actinodura souliei	KIZ, ALS300	Yunnan	GU139502	GU139516	GU139488
	KIZ, ALS301	Yunnan	GU139503	GU139517	GU139489
Actinodura morrisoniana	NTNU, T0166	Taiwan	GU139506	GU139520	GU139492
	NTNU, T0197	Taiwan	GU139507	GU139521	GU139493
Outgroup					
Garrulax erythrocephalus[b]	GenBank	Yunnan	EU447077	-	EU447123
Garrulax affinis[b]	GenBank	Tibet	EU447074	-	EU447120
Garrulax morrisioniana[b]	GenBank	Taiwan	EU447069	-	EU447115
Heterophasia auricularis[b]	GenBank	Taiwan	EU447093	-	EU447140
Leiothrix lutea[b]	GenBank	Yunnan	EU447094	-	EU447049
Liocichla steerii[b]	GenBank	Taiwan	EU447100	-	EU447147
Minla cyanouroptera	KIZ, GLGS6486	Yunnan	GU139515	GU139529	GU139501
	GenBank[b]	Sichuan	EU447096	-	EU447051
Minla ignotincta	KIZ, 04669	Yunnan	GU139508	GU139522	GU139494
	GenBank[b]	Yunnan	EU447097	-	EU447052
Minla strigula	KIZ, YL07124	Yunnan	GU139514	GU139528	GU139500
	GenBank[b]	Yunnan	EU447098	-	EU447053

Taxonomy follows Dickinson (2003). [a]sequence from Cibois (2003); [b]sequence from Luo et al. (2009). Abbeviation: KIZ, Kunming Institute of Zoology, the Chinese Academy of Sciences; NTNU, Department of Life Science, National Taiwan Normal University.

Table 2. Primers used in this study to amplify and sequence 306 mitochondrial cytochrome *b* (Cytb) gene in full length, and NADH dehydrogenase subunit 2 (ND2) and cytochrome *c* oxidase I (COI) genes in part

Gene	Primer name	Primer sequence (5'-3')	References
Cyt*b*	L14833	CAGGCCTAATAAAGCCTA	Designed in this study
	L15407	TGAGGTGGATTCTCAGTAGAC	Zhang et al. 2007
	H15487	GATCCTGTTTCGTGGAGGAAGGT	Cibois et al. 1999
	H16070	GGAGTCTTCAGTCTTTGGTTTAC	Helm-Bychowski and Cracraft 1993, modified
ND2	L5219	CCCATACCCCGAAAATGATG	Sorenson et al. 1999, modified
	L5809	GCCTTCTCATCCATCTCCCACCTAGGATGAAT	Cicero and Johnson 2001
	H5850	ATTAGGGCGTATAGGTAGAAGTT	Designed in the study
	H6312	CTTATTTAAGGCTTTGAAGGCC	Cicero and Johnson 2001, modified
COI	BirdF1	TTCTCCAACCACAAAGACATTGGCAC	Hebert et al. 2004
	BirdR1	ACGTGGGAGATAATTCCAAATCCTG	Hebert et al. 2004

the DNA analysis package, DNAStar (Madison, WI, USA). All sequences were translated into amino acid residues using MEGA 4.0 (Tamura et al. 2007) to verify the absence of premature stop codons or indels, which would be indicative of nuclear copies of mitochondrial genes (or numts). Assembled sequences were aligned using Clustal X 1.83 (Thompson et al. 1997).

We used the maximum-likelihood (ML) and Bayesian-inference (BI) approaches to reconstruct the phylogenetic relationship among barwings. DNA sequences of all 3 mitochondrial fragments were concatenated for the phylogenetic analysis. The model of molecular evolution that best fit the concatenated datasets for the ML method was determined using Modeltest 3.7 (Posada and Crandall 1998) with the Akaike information criterion (AIC). The ML analysis was performed in PAUP* 4.10b (Swofford 2002) with the model selected by Modeltest. The ML tree was searched using a heuristic method, employing tree bisection-reconnection branch-swapping starting from the stepwise addition with 100 random-addition-sequence replicates. The robustness of the hypothesized clades was assessed using bootstrap analyses with heuristic searches for 100 replicates. We used the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) to reconstruct the phylogeny. For the BI analysis, we used the mixed model tested in Modeltest on the combined data partitioned by genes and codon positions. Clade credibility values were provided using Bayesian posterior probabilities. We simultaneously ran 2 independent Metropolis-coupled Markov Chain Monte Carlo processes. In each process, we ran 5×10^6 generations, sampled once every 100 generations, and diagnosed once every 1,000 generations with 1 cold simultaneous chain and 3 incrementally heated chains under default heating values.

RESULTS

We obtained sequences of the 3 mitochondrial fragments (Cyt *b*, ND2, and COI) for all taxa except several which we downloaded from GenBank (Table 1). In total, 2,725 bp (1,143 bp of Cyt *b*, 967 bp of ND2, and 615 bp of COI) of the sequences was aligned. For all sequences, no premature stop codon, indel, or ambiguous base call was found, suggesting that the sequences were authentic mitochondrial (mt)DNA.

The best-fitting evolutionary model selected by Modeltest on the concatenated data was GTR+I+G (Posada and Buckley 2004), with prior parameters for the substitution rates, Rmatrix = 1.0443, 8.9894, 1.7548, 0.1698, 16.5519, and 1.0000, proportions of invariable sites, I = 0.6415, gamma distribution shape, α = 2.6460, and base frequencies, Fmatrix = 0.3082, 0.3764, 0.1193, and 0.1962. The chains of the BI analysis appeared stationary as the average standard deviation of the split frequencies became < 0.01 by about 85,000 generations; we discarded the foregoing 850 trees as “burn-in”. The inference of the BI consensus tree was based on the remaining sampled trees.

The 2 resulting trees (the ML and BI consensus trees) were congruent in terms of topologies; therefore, only the phylogram inferred from the BI analysis is presented in figure 2. The reconstructed molecular phylogeny indicated that all 6 barwing species were clustered into 2 well-supported clades, clades A and B (Bayesian posterior probability = 1.0 and bootstrap value = 100 for both clades). However the barwings did not form a monophyletic group in our analysis: clade A and 2 minlas formed a well-supported clade (Bayesian posterior probability = 1.0 and bootstrap value = 70), with *M. strigula* appearing as a sister taxon of clade A barwings, while *M. cyanouroptera* was located at the base of the clade. Clade A was composed of 2 barwings (*A. egertoni* and *A. ramsayi*) with longer tails (> 100 mm, Table 3). Clade B was also a well-supported clade (Bayesian posterior probability = 1.0 and bootstrap value = 100) that consisted of 4 barwing species: *A. nipalensis*, *A. souliei*, *A. waldeni*, and *A. morrisoniana*. *Actinodura nipalensis* was at the basal position of clade B, while *A. morrisoniana* shared a common ancestor with the *A. souliei*/ *A. waldeni* clade. All members of clade B possess shorter tails (< 100 mm, Table 3). Our phylogeny indicates that the genus *Minla* is not monophyletic, with *M. ignotincta* is more closely related to *Liocichla steerii*, than to other *Minla* species (Bayesian posterior probability = 1.0 and bootstrap value = 55).

DISCUSSION

Although the barwings have long been considered a homogeneous group (Delacour 1946, Deignan 1964, Sibley and Monroe 1990, Dickinson 2003, Collar and Robson 2007), our results suggest that the group is not monophyletic, but is actually split into 2 well-supported clades separated by 2 minla species (Fig. 2). We found

that each major barwing group possesses shared distinctive morphological features: members of clade A generally have a plain breast, light plumage, weakly barred rectrices, and especially, a greater tail length; while species of clade B possess a streaked breast, dark plumage, strongly darkly barred rectrices, and a shorter tail length. In light of the paraphyly suggested by this study, a taxonomic revision is urgently needed. As provided by Gill et al. (2005), there are 3 criteria

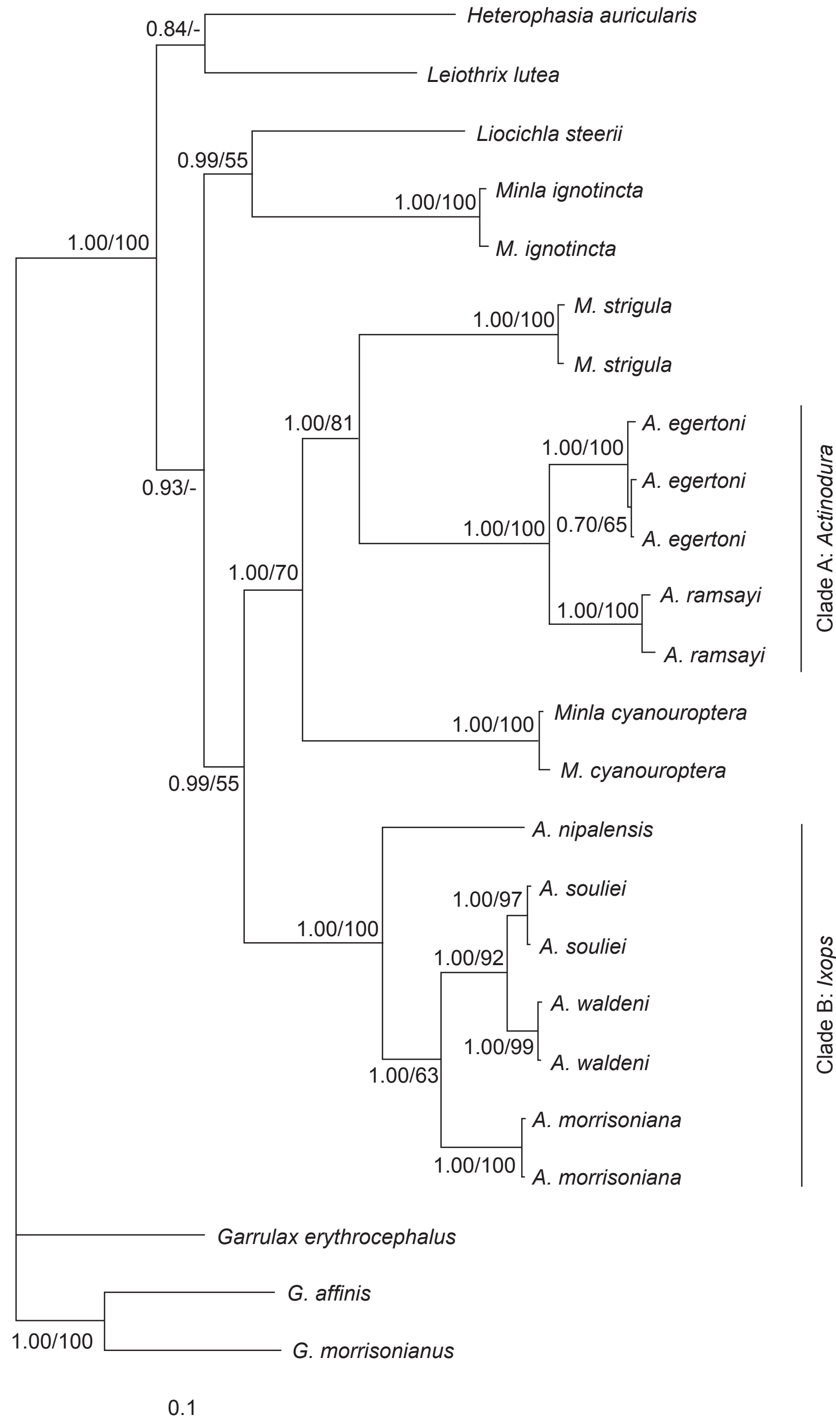

Fig. 2. The phylogram of traditional *Actinodura* inferred by BI analysis. The numbers represent Bayesian posterior probability/ ML bootstrap values and are only shown for nodes with both values larger than 50%.

for determining taxonomic usefulness: generic monophyly, reasonable compactness, and ecological and morphological or biogeographical distinctness. Given the diagnosable morphological traits between traditional barwings and minlas, and also between the 2 barwing clades, we propose that the genus *Actinodura* be split into 2 genera: the genus name *Actinodura* should be reserved for clade A, and the genus *Ixops* "Hodgson" Blyth 1843, which was treated as a synonym of *Actinodura*, should be reinstated for clade B. We suggest that they respectively be called the Long-tailed and Short-tailed Barwings in English. We also note that the ranges of *Actinodura* and *Ixops* overlap from the Himalayas to Myanmar, an area in which the greatest number of species also occurs; however *Actinodura* is distributed at lower latitudes while *Ixops* is found at higher latitudes (Collar and Robson 2007). In addition, where the 2 species, *I. nipalensis* and *A. egertoni* occur syntopically, *I. nipalensis* keeps to bushes and undergrowth while *A. egertoni* is more often found in the canopy (Collar and Robson 2007). We therefore speculated that ecological niche differentiation may have first promoted the divergence of the ancestral group of these 2 closely related genera followed by a geographical displacement pattern.

Although the traditional barwing *A. sodangorum* was not included in the present study, it is very morphologically similar to *A. ramsayi*, as both have a bold, prominent eyering and similar morphological measurements (Eames et al. 1999). Therefore, we provisionally placed this species in the genus *Actinodura*. Since Blyth (1843) did not describe the genus *Ixops*, we depict it as follows: *Ixops* "Hodgson" Blyth 1843. gen. rev. *Type species*: *Ixops nipalensis* Hodgson 1836. *Diagnosis*: Distinguished from other genera (except *Actinodura*) within the Leiothrichinae (Aves: Timaliidae, see Gelang et al. 2009) due to transverse barring on the wings and tail and the presence of a crest. It is very similar to *Actinodura*, but with a shorter tail (< 100 mm, Table 3), more streaking on the body, darker plumage, and more strongly darkly barred rectrices. The members of this genus (*I. nipalensis*, *I. waldeni*, *I. souliei*, and *I. morrisoniana*) inhabit broadleaf evergreen and mixed forests. Sexes are alike.

The traditional genus, *Minla*, has long been treated as a single group by most authors (Delacour 1946, Deignan 1964, Sibley and Monroe 1990, Dickinson 2003). The minlas are all gregarious arboreal birds of mountain areas (Smythies 1986) and are distributed throughout the Himalayas to continental southeastern Asia and southern China (Collar and Robson 2007). However, recent molecular phylogenies suggested that this group is polyphyletic, although these phylogenetic arrangements are considered controversial (Cibois 2003, Luo et al. 2009, Gelang et al. 2009). The most recent world checklist of birds granted each traditional *Minla* species the status of a monotypic genus: *Siva cyanouroptera*, *Chrysominla strigula*, and *Minla ignotincta* (Collar and Robson 2007). Our study shows that the positions of *M. cyanouroptera* and *M. strigula* are embedded within the traditional barwing clade, whereas *M. ignotincta* was more closely related to *L. steerii* (Fig. 2). The former 2 minlas obviously differ from the last in the absence of sexual dichromatism (Zhao 2001, Collar and Robson 2007). Nevertheless, *M. cyanouroptera* and *M. strigula* do not form a monophyletic group and are located within clade A (Fig. 2). Thus, according to the criteria provided by Gill et al. (2005), our results are in agreement with the taxonomic treatment of *Minla* suggested by

Table 3. Tail length for traditional genus *Actinodura*. Only measures of males were used

Species	Subspecies	Length of tail (mm)	No. specimens examined	Referrences
A. nipalensis	-	82-88	not provided	Ali and Ripley 1971
A. waldeni	*waldeni*	79	1	Ali and Ripley 1971
	saturatior	77-93	10	Yang et al. 2004
A. souliei	*souliei*	96	2	Yang et al. 2004
	griseinucha	86	1	Yang et al. 2004
A. morrisoniana	-	70-82	not provided	Zhao 2001
A. egertoni	*ripponi*	101-122	14	Yang et al. 2004
	lewisi	104-116	4	Ali and Ripley 1971
A. ramsayi	*yunnanensis*	111-118	8	Yang et al. 2004
A. sodangorum	-	130, 137	2	Eames et al. 1999

Collar and Robson (2007), to split the genus *Minla* into 3 monotypic genera.

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