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Unexpected divergence and lack of divergence revealed in continental Asian *Cyornis* flycatchers (Aves: Muscicapidae) ☆

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ABSTRACT

The flycatcher genus *Cyornis* (Aves: Muscicapidae) comprises 25 species with Oriental distributions. Their relationships are poorly known. We analyzed the phylogenetic relationships of 70 individuals from 12 species and several subspecies of *Cyornis* based on three mitochondrial genes and five nuclear introns, with special focus on Chinese and Vietnamese populations of the monotypic *C. hainanus* and polytypic *C. rubeculoides*. We found no support for inclusion of *C. concretus* in *Cyornis*. Deep divergences were observed among different subspecies of *C. banyumas* and *C. rubeculoides*. *C. rubeculoides glaucicomans* was also shown to have a highly distinctive song, and we propose that it is treated as a distinctive Chinese endemic species, *C. glaucicomans*. In contrast, the south Vietnamese *C. rubeculoides klossi*, which has a disjunct distribution from the other subspecies of *C. rubeculoides*, along with a recently discovered population in Guangdong Province (China) with several plumage reminiscent of *C. r. klossi*, were indistinguishable in all loci analyzed from the phenotypically markedly different *C. hainanus*. More research is needed to elucidate the reasons for this unexpected pattern.

1. Introduction

The Old World flycatchers in the family Muscicapidae is a diverse group of birds with mainly similar flycatching behavior and associated morphology (del Hoyo et al., 2006). Molecular analyses have recently shown that the Old World flycatchers do not form a monophyletic group, and that the similarities between distantly related clades are due to convergence (Sangster et al., 2010; Zuccon and Ericson, 2010). One flycatcher clade, referred to as Niltavinae by Sangster et al. (2010), comprises the genera *Eumyias*, *Cyanoptila*, *Niltava*, *Anthipes* and *Cyornis* (Sangster et al., 2010; Zuccon and Ericson, 2010). Previously, *Rhinomyias* was also recognized, but Sangster et al. (2010) moved the species of this genus into *Cyornis*, *Eumyias* and *Vauriella*.

The genus *Cyornis* is the largest of the genera mentioned above, comprising 25 currently recognized species (Dickinson and Christidis, 2014), including some species that were previously placed in the genus *Rhinomyias*. The genus is distributed throughout southern Asia, from the Indian subcontinent to Southeast Asia, the Philippines and Indonesia (del Hoyo et al., 2006; Dickinson and Christidis, 2014). Most species are sexually dimorphic in plumage, with males being blue above and mainly blue and white or orange and white below, although a few species (including the ones previously placed in *Rhinomyias*) are sexually monomorphic and lack bright colors.

The phylogeny and taxonomy of the different species of *Cyornis* have not been much studied. Sangster et al. (2010), which analyzed the phylogeny of single samples of seven species, is the only phylogenetic study of multiple species. Rasmussen and Anderton (2005) suggested that the species status of *C. poliogenys* and *C. tickelliae* was in need of re-evaluation, as they intergrade in the northeastern part of the Indian subcontinent. Renner et al. (2009) studied the morphology of the different subspecies of *C. banyumas*, and suggested that *C. b. magnirostris* should be recognized as a separate species based on morphological differences and sympatry

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with *C. b. whitei*. The same authors also suggested that *C. b. lemprieri* should probably be treated as a full species based on its morphological distinctness.

The main objective of this study is to infer the phylogenetic relationships of all except three of the continental Asian *Cyornis* species and a number of different subspecies using sequence data from three mitochondrial genes and five nuclear loci. Special attention is paid to Vietnamese and Chinese populations of *C. rubeculoides* and *C. hainanus*.

2. Methods

2.1. Study group and sampling

Taxonomy follows Dickinson and Christidis (2014). We analyzed a total of 70 individuals from 12 species of *Cyornis* (*C. concretus*, *C. hainanus*, *C. rubeculoides*, *C. banyumas*, *C. poliogenys*, *C. tickelliae*, *C. turcosus*, *C. caerulatus*, *C. rufigastra*, *C. unicolor*, *C. umbratilis* and *C. olivaceus*) (42 individuals in concatenated dataset and another 28 individuals in single-locus analyses). We also included nine individuals from five species of *Niltava* (*N. grandis*, *N. macgrigoriae*, *N. davidi*, *N. sundara*, *N. vivida*) and one species of *Cyanoptila* (*C. cumatilis*), which were shown to be part of the same clade as *Cyornis* (referred to as Niltavinae) by Sangster et al. (2010). *Myiomela leucura* was used to root the tree based on Sangster et al. (2010). A number of different subspecies were also studied, notably four of the five subspecies of *C. rubeculoides*, and special emphasis was placed on Chinese and Vietnamese populations of *C. rubeculoides* and *C. hainanus*. Of the samples analyzed, 46 individuals from 13 species were sequenced specifically for this study, whereas the others were downloaded from GenBank (Table 1).

DNA was extracted from muscle, blood or feathers using QIA Quick DNEasy Kit (Qiagen.Inc.) following the manufacturer's protocol. The three mtDNA genes cytochrome c oxidase I (COI), cytochrome b (cytb) and NADH dehydrogenase subunit 2 (ND2) were amplified and sequenced using the following primer pairs: COI, H7956/L6615; cytb, H16064/L14770; ND2, H6313/L5219 or H6313/L5143 (Sorenson et al., 1999). To reduce the risk of amplifying nuclear paralogs, "numts" (Sorenson and Quinn, 1998), COI, cytb and ND2 were amplified as one fragment separately. The 5 nuclear loci including the Z-linked chromo-helicase-DNA binding protein 1 intron (CHD1Z), Z-linked brama protein gene (BRM), myoglobin intron 2 (myo), muscle-specific tyrosine kinase (MUSK), and Z-185 (similar to transient receptor potential cation channel, subfamily M, member 3). PCR primer information is given in Supplementary Table 1. Sequences were assembled manually with the Staden Package (Bonfield et al., 1995). All new sequences have been deposited in GenBank (Table 1).

2.2. Phylogenetic analyses

Sequences were aligned using the Clustal W algorithm in MEGA 5.0; some manual adjustment was necessary for the non-coding sequences. Trees were estimated by Bayesian inference (BI) using MrBayes 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The choice of model was calculated in PartitionFinder 1.1.1 (Lanfear et al., 2012) based on the Bayesian Information Criterion. The coding sequences were run under the HKY model (Hasegawa et al., 1985) assuming rate variation across sites according to a discrete gamma distribution with four rate categories (Γ; Yang, 1994) and an estimated proportion of invariant sites (I; Gu et al., 1995). The mitochondrial loci were partitioned by codon: cytb position (pos.) 1 HKY + I; cytb pos. 2, ND2 pos. 1 + 3, COI pos 1 + 3 HKY + Γ + I; and cytb pos. 3, ND2 pos. 2 and COI pos. 3 GTR + Γ + I. This resulted in 14 partitions. Analyses were also run in 8 partitions, with each mitochondrial locus analyzed under the GTR + Γ + I model (suboptimal according to PartitionFinder and comparisons of marginal likelihoods calculated in Tracer 1.6.0 [Rambaut et al., 2014]). The loci were analyzed separately as well as concatenated. Concatenated datasets were analyzed including 42 samples: our own samples for which 6–8 (in four cases five) loci were available plus additional taxa for which only one or two markers were available (downloaded from GenBank) (Table 1). Separate 14-partitions analyses were also run including only our own samples with 6–8 (in four cases five) loci. Single-locus analyses were run including all available sequences. Four Metropolis-coupled MCMC chains with incremental heating temperature 0.05 were run for 5×10^6 generations, and sampled every 1000 generations. Convergence to the stationary distribution of the single chains was inspected in Tracer 1.6.0 (Rambaut et al., 2014) using a minimum threshold for the effective sample size. The joint likelihood and other parameter values indicated large effective sample sizes (>1000). Good mixing of the MCMC and search reproducibility was established by multiple runs from independent starting points. Topological convergence was examined by eye and by the average standard deviation of split frequencies (<0.005). We discarded the first 25% of the generations and calculated the posterior probabilities (PPs) from the remaining generations.

The data were also analyzed by maximum likelihood bootstrapping (MLBS). For the full and smaller concatenated datasets in 8 partitions (see above), one thousand replicates were run in RAxML-HPC2 version 8.1.11 (Stamatakis, 2006; Stamatakis et al., 2008) on the Cipres portal (Miller et al., 2010). GTRGAMMA was used both for the bootstrapping phase and for the final tree inference. Single-locus analyses for which the HKY model was selected were analyzed by PhyML3.0 (Guindon et al., 2010).

Molecular dating based on the cytb data set was conducted with BEAST 1.7.4 (Drummond and Rambaut, 2007, 2012) using a GTR + Γ model, a molecular clock rate of 2.1% divergence/million years (cf. Weir and Schluter, 2008), a "birth–death incomplete sampling" prior, and assuming constant population size. 1×10^6 generations were run, sampled every 1000 generations. Every analysis was run twice. The first 25% of the generations were discarded. Trees were summarized using TreeAnnotator version 1.7.4 (Rambaut and Drummond, 2012), choosing "Maximum clade credibility tree" and "Mean heights", and displayed in FigTree version 1.4.0 (Rambaut, 2012). Tracer 1.6.0 was used to evaluate the performance of the MCMC (see above).

2.3. Sound analysis

To illustrate previously noted but as yet undocumented differences in song between *Cyornis rubeculoides glaucicomans* and other *Cyornis* taxa, sound recordings of several taxa were obtained and sonograms produced in Raven Pro 1.5 (Bioacoustics Research Program, 2011). The sound recordings have been deposited in Xeno-canto (www.xeno-canto.org), with the registration numbers given in the caption to Fig. 3.

3. Results

3.1. Sequence characteristics

Not all loci were sequenced for all samples, and for some of the ones downloaded from GenBank only cytb or ND2 was available (see Table 1). For mitochondrial loci, we obtained up to 1125 bp of COI, 990 bp of ND2 and 974 bp of cytb. No stop codons that would indicate the presence of nuclear pseudogenes were found in these sequences. For nuclear sequences we obtained 349–

Table 1
Information about samples used in this study.

Taxon	Sample number	Collection number	Locality	COI	cytb	ND2
C. banyumas whitei	19		Dulongjiang, Yunnan Province, China	KP336984	KP337019	_
C. banyumas whitei	4186	IOZ 4186	Pingchuan, Sichuan, China	KP336985	KP337020	KP337117
C. banyumas whitei[a]	TP		Chiangmai, Thailand	_	KJ456246	KJ455398
C. banyumas whitei	U672	DZUG U672	Thailand	_	_	_
C. banyumas whitei	4953	IOZ 4953	Yunnan, China	_	KP337021	_
C. banyumas banyumas/ligus	U799	DZUG U799	Java, Indonesia	_	_	_
C. caerulatus caerulatus		AMNH BDM978	Tawau Hills, Borneo, Indonesia	_	KP337023	_
C. concretus	VNM107	NRM 20047093 (=NRM 2004-345)	Vietnam	_	HM633288	_
C. concretus	7068 VNM2004-320	NRM 20047068	Dakrong, Quang Tri province, Vietnam	KP336987	KP337024	KP337119
Cyanoptila cumatilis	16356	IOZ 16356	Zhouzhi, Shaanxi, China	KP336988	KP337025	KP337120
C. hainanus	JM000	IOZ JM000	Yangxi, Sanjia Shan, Guangdong, China	KP336966	KP336997	KP337129
C. hainanus	JM001	IOZ JM001	Yangxi Sanjia Shan, Guangdong, China	KP336967	KP336998	KP337099
C. hainanus[b]	JM002	IOZ JM002	Yangxi Sanjia Shan, Guangdong, China	KP336968	KP336999	KP337130
C. hainanus	JM005	IOZ JM005	Yunfu, Yunwu Shan, Guangdong, China	KP336969	KP337000	KP337131
C. hainanus	JM006	IOZ JM006	Hewei Shan, Bajia, Guangdong, China	KP336970	KP337001	KP337100
C. hainanus	6094	IOZ 6094	Guangxi, China	KP336971	KP337002	KP337101
C. hainanus	6257	IOZ 6257	Hainan Island, China	KP336972	KP337003	KP337102
C. hainanus	6624	NRM 20056624	Vu Quang National Park, Ha Tinh province, Vietnam	KP336973	KP337004	KP337103
C. hainanus	6638	NRM 20056638	Vu Quang National Park, Ha Tinh province, Vietnam	KP336974	KP337005	KP337104
C. hainanus	6652	NRM 20026652	Ho Ke Go, Ha Tinh province, Vietnam	KP336975	KP337006	KP337105
C. hainanus	6671	NRM 20026691	Vietnam, captive	_	KP337007	KP337106
C. hainanus	6964	NRM 20046964	Dakrong, Quang Tri province, Vietnam	KP336976	KP337008	KP337107
C. hainanus	7057	NRM 20047057	Dakrong, Quang Tri province, Vietnam	KP336977	KP337009	KP337108
C. hainanus/C. rubeculoides klossi[b]	7077	NRM 20047077	Dakrong, Quang Tri province, Vietnam	KP685717	KP685723	KP685741
C. hainanus/C. rubeculoides klossi[b]	7080	NRM 20047080	Dakrong, Quang Tri province, Vietnam	KP685718	KP685724	KP685742
C. hainanus/C. rubeculoides klossi[b]	JM003	IOZ JM003	Yangxi, Sanjia Shan, Guangdong, China	KP336981	KP337015	KP337113
C. hainanus/C. rubeculoides klossi[b]	6674	NRM 20056674	Vu Quang National Park, Ha Tinh province, Vietnam	KP685714	KP685720	KP685738
C. hainanus/C. rubeculoides klossi[b]	6755	NRM 20056755	Vu Quang National Park, Ha Tinh province, Vietnam	KP685715	KP685721	KP685739
C. hainanus/C. rubeculoides klossi[c]	6673	NRM 20056673	Vu Quang National Park, Ha Tinh province, Vietnam	KP685713	KP685719	KP685737
C. hainanus/C. rubeculoides klossi[c]	6949	NRM 20046949	Dakrong, Quang Tri province, Vietnam	KP685716	KP685722	KP685740
C. hainanus/rubeculoides klossi[c]	7075	NRM 20047075	Dakrong, Quang Tri province, Vietnam	KP336980	KP337014	KP337112
C. hainanus/C. rubeculoides klossi[c]	JM004	IOZ JM004	Yangxi, Sanjia Shan, Guangdong, China	KP336982	KP337016	KP337114
C. olivaceus	AMNH-FHS163	AMNH FHS163	Sabah, Borneo, Malaysia	_	HM633369	_
C. poliogenys	TP		Arunachal Pradesh, India	_	KJ456247	KJ455399
C. poliogenys	U719	DZUG U719	Nepal	_	HM633289	_
C. rubeculoides klossi	6863	NRM 20046863	Dakrong, Quang Tri province, Vietnam	KP336978	KP337010	KP337109
C. rubeculoides klossi	6907	NRM 20046907	Dakrong, Quang Tri province, Vietnam	KP336979	KP337011	KP337110
C. rubeculoides klossi	6963	NRM 20046963	Dakrong, Quang Tri province, Vietnam	_	KP337012	KP337111
C. rubeculoides klossi	7003	NRM 20047003	Dakrong, Quang Tri province, Vietnam	_	KP337013	_
C. rubeculoides rogersi	UO	DZUG 114	Mt Victoria, Chin hills, Myanmar	_	KP739427	
C. rubeculoides rubeculoides	JM45		Nepal	_	KP739426	KP739428
C. rubeculoides glaucicomans	PA20130526-2	IOZ 20224	Longcangguo, Sichuan, China	KP336983	KP337017	KP337115
C. rubeculoides glaucicomans	PA20140528-1	IOZ 20234	Laojun Shan, Sichuan, China	_	KP337018	KP337116
C. rubeculoides glaucicomans[d]	sch562		Yanyuan Pingchuan, Sichuan, China	_	EF081352	_
C. rufigastra simplex	UAM 34043	UAM 34043	Luzon, Philippines	_	_	KF819355
C. rufigastra simplex	UAM 29368	UAM 29368	Luzon, Philippines	_	_	KF819287
C. rufigastra philippensis	UAM 29636	UAM 29636	Mindanao, Philippines	_	_	KF819279
C. rufigastra philippensis	UAM 29635	UAM 29635	Mindanao, Philippines	_	_	KF819278
C. tickelliae ssp.	LSUMZ 148745 (=B-20551)	LSUMZ 148745 (=B-20551)	Captivity (probably SE Asia based on plumage)	_	KJ456248	KJ455400
C. tickelliae (?)[d]	MNHN 04.9F	MNHN 04.9F	Nakhon Ratchasima (Korat), Thailand	_	_	JX256054
C. turcosus turcosus		AMNH RGM571	Kinabalu, Borneo, Indonesia	_	xx	_
C. umbratilis	AMNH-FHS199	AMNH FHS199	Sabah, Borneo, Malaysia	_	HM633370	_
C. unicolor diaoluoensis	6268	IOZ 6268	Wuzhi Shan, Hainan, China	KP336986	KP337022	KP337118
C. unicolor ssp.	AMNH-PRS2252	AMNH PRS2252	Vietnam	_	HM633291	KJ455401
Myiomela leucura	6687	NRM 20056687	Vietnam	KP336965	KP336996	KP337098
Niltava davidi	2582	IOZ 2582	Foping, Shanxi, China	KP336993	KP337030	KP337125
Niltava davidi	2583	IOZ 2583	Foping, Shanxi, China	KP336994	KP337031	KP337126
Niltava davidi	DZUG U2198	AMNH RTC573	Quang Nam, Vietnam	_	HM633352	_
Niltava grandis	5320	IOZ 5320	Gaoligong shan, Yunnan, China	KP336989	KP337026	KP337121
Niltava grandis	12783	IOZ 12783	Longlin, Guangxi, China	KP336990	KP337027	KP337122
Niltava grandis	BMNH A2000.8.34	BMNH A2000.8.34	Chin state, Myanmar	_	HM633353	_

Table 1 (*continued*)

Taxon	Sample number	Collection number	Locality	COI	cytb	ND2
Niltava macgrigoriae	6093	IOZ 6093	Daxin, Guangxi, China	KP336991	KP337028	KP337123
Niltava macgrigoriae	6409	IOZ 6409	Baoshan, Yunnan, China	KP336992	KP337029	KP337124
Niltava macgrigoriae	U1411	DZUG U1411	Nepal	_	HM633354	_
Niltava sundara	4163	IOZ 4163	Baipo Shan, Sichuan, China	_	KP337032	KP337127
Niltava sundara	3822	IOZ 3822	Yanbian, Sichuan, China	KP336995	KP337033	KP337128
Niltava sundara	14360	IOZ 14360	Xizang, China	_	KP337034	KP337132
Niltava sundara	U2199	AMNH JGG1002	Malde, Nepal	_	HM633355	_
Niltava sundara	KJ456364-TP		Nepal	_	KJ456364	_
Niltava vivida	AMNH-GFB3287	AMNH GFB3287	Taiwan	_	HM633356	_

Taxon	Sample number	BRM	MUSK	Z185	myo	CHD1Z
C. banyumas whitei	19	_	KP337052	KP337152	KP337088	_
C. banyumas whitei	4186	KP336923	KP337053	KP337151	_	_
C. banyumas whitei[a]	TP	_	_	_	KJ454785	_
C. banyumas whitei	U672	_	_	_	HM633570	_
C. banyumas whitei	4953	KP336924	KP337054	KP337153	KP337089	_
C. banyumas/ligus	U799	_	_	_	_	_
C. caerulatus	UO	KP336927	_	KP337156	KP337091	KP336957
C. concretus	VNM107	_	_	_	HM633571	_
C. concretus	7068 VNM2004-320	KP336928	KP337057	KP337157	KP337092	KP336958
Cyanoptila cumatilis	16356	_	KP337035	_	KP337093	_
C. hainanus	JM000	KP336902	KP337036	_	KP337067	KP336940
C. hainanus	JM001	KP336903	KP337037	KP337134	KP337068	_
C. hainanus[b]	JM002	KP336904	KP337038	KP337135	KP337069	KP336941
C. hainanus	JM005	KP336905	KP337039	KP337136	KP337070	KP336942
C. hainanus	JM006	KP336906	KP337040	KP337137	KP337071	KP336943
C. hainanus	6094	KP336907	KP337041	KP337138	KP337073	KP336944
C. hainanus	6257	KP336908	KP337042	_	_	KP336945
C. hainanus	6624	KP336909	KP337043	KP337139	KP337074	_
C. hainanus	6638	KP336910	KP337044	KP337140	KP337075	KP336946
C. hainanus	6652	KP336911	KP337045	KP337141	KP337076	KP336947
C. hainanus	6671	KP336912	_	KP337142	KP337077	KP336948
C. hainanus	6964	KP336913	_	KP337143	KP337078	KP336949
C. hainanus	7057	KP336914	KP337046	KP337144	KP337079	KP336950
C. hainanus/C. rubeculoides klossi[b]	7077	KP685706	KP685729	KP685747	KP685735	_
C. hainanus/C. rubeculoides klossi[b]	7080	KP685707	KP685730	_	KP685736	KP685710
C. hainanus/C. rubeculoides klossi[b]	JM003	KP336920	KP337049	KP337148	KP337084	KP336953
C. hainanus/C. rubeculoides klossi[b]	6674	KP685703	KP685726	KP685744	KP685732	KP685712
C. hainanus/C. rubeculoides klossi[b]	6755	KP685704	KP685727	KP685745	KP685733	KP685709
C. hainanus/C. rubeculoides klossi[c]	6673	KP685702	KP685725	KP685743	KP685731	KP685708
C. hainanus/C. rubeculoides klossi[c]	6949	KP685705	KP685728	KP685746	KP685734	KP685711
C. hainanus/C. rubeculoides klossi[c]	7075	KP336919	KP337048	KP337147	KP337083	KP336952
C. hainanus/C. rubeculoides klossi[c]	JM004	KP336921	KP337050	KP337149	KP337085	KP336954
C. olivaceus	AMNH-FHS163	_	_	_	HM633651	_
C. poliogenys	TP	_	_	_	KJ454786	_
C. poliogenys	U719	_	_	_	HM633572	_
C. rubeculoides klossi	6863	KP336915	_	KP337145	KP337080	_
C. rubeculoides klossi	6907	KP336916	_	KP337146	KP337081	KP336951
C. rubeculoides klossi	6963	KP336917	KP337047	_	KP337082	_
C. rubeculoides klossi	7003	KP336918	_	_	_	_
C. rubeculoides rogersi	UO	_	_	_	_	_
C. rubeculoides rubeculoides	JM45	_	_	_	_	_
C. rubeculoides glaucicomans	[PA20130526-2]	_	_	KP337150	_	_
C. rubeculoides glaucicomans	PA 20140528-1]	KP336922	KP337051	_	KP337086	KP336955
C. rubeculoides glaucicomans[d]	EF081352	_	_	_	_	_
C. rufigastra simplex	UAM34043	_	_	_	_	_
C. rufigastra simplex	UAM29368	_	_	_	_	_
C. rufigastra philippensis	UAM29636	_	_	_	_	_
C. rufigastra philippensis	UAM29635	_	_	_	_	_
C. tickelliae ssp.	LSUMZ 148745 (=B-20551)	_	_	_	_	_
C. tickelliae (?)[e]	MNHN 04.9F	_	_	_	_	_
C. turcosus	UO	KP336925	KP337055	KP337154	_	_
C. umbratilis	AMNH-FHS199	_	_	_	HM633652	_
C. unicolor	6268	KP336926	KP337056	KP337155	KP337090	KP336956
C. unicolor	AMNH-PRS2252	_	_	_	HM633574	_
Myiomela leucura	6687	KP336901	_	KP337133	_	KP336937
Niltava davidi	2582	KP336933	KP337062	KP337161	_	KP336962
Niltava davidi	2583	KP336934	KP337063	KP337162	_	KP336963
Niltava davidi	DZUG-2198	_	_	_	HM633634	_
Niltava grandis	5320	KP336929	KP337058	KP337158	KP337094	KP336959
Niltava grandis	12783	KP336930	KP337059	_	_	KP336960
Niltava grandis	BMNHA2000.8.34	_	_	_	_	_
Niltava macgrigoriae	6093	KP336931	KP337060	KP337159	_	KP336961
Niltava macgrigoriae	6409	KP336932	KP337061	KP337160	KP337095	_
Niltava macgrigoriae	U1411	_	_	_	KJ454846	_

(*continued on next page*)

Table 1 (*continued*)

Taxon	Sample number	BRM	MUSK	Z185	myo	CHD1Z
Niltava sundara	4163	KP336935	KP337064	KP337163	KP337096	_
Niltava sundara	3822	_	_	_	_	_
Niltava sundara	14360	KP336936	KP337066	_	KP337097	_
Niltava sundara	DZUG-2199	_	_	_	HM633637	_
Niltava sundara	KJ456364-TP	_	_	_	_	_
Niltava vivida	AMNH-GFB3287	_	_	_	HM633638	_

Abbreviations: AMNH – American Museum of Natural History, New York, USA; DZUG – Department of Zoology, University of Gothenburg, Gothenburg, Sweden; IOZ – Institute of Zoology, Chinese Academy of Sciences, Beijing, China; LSUMZ – Louisiana State University Museum of Natural Science, Baton Rouge, USA; MNHN – Muséum National d'Histoire Naturelle, Paris, France; NRM – Swedish Museum of Natural History, Stockholm, Sweden; UAM – University of Alaska Museum. Sequences downloaded from GenBank are in italics.

[a] Mistakenly reported as *Cyornis magnirostris* in GenBank.
[b] Individual with intermediate plumage, of suspected hybrid origin.
[c] Female (all others males).
[d] Mistakenly reported as *Niltava banyumas* (=*Cyornis banyumas*) in GenBank.
[e] Identified as *C. banyumas*, but probably misidentified (cf. text).

366 bp of BRM, 551–609 bp of MUSK, 603–652 of CHD1Z, 501–609 bp of myo and 898 bp of Z-185. The concatenated data (mitochondrial and nuclear loci) contained 6726 bp.

3.2. Phylogenetic relationships

The BI tree based on the concatenated sequences of all loci is shown in Fig. 1, with PPs and MLBS values indicated. Three major clades (A, B and C) were identified. Clade A, which was sister to the others with high PP but low MLBS, contained *Cyornis concretus*. Clade B, which was sister to clade C with moderate PP and low MLBS, comprised the five species of *Niltava* and the single *Cyanoptila*. The genus *Niltava* was recovered as monophyletic with high PP but low MLBS, but the relationships among the five *Niltava* species received various support in different analyses. In the analysis of all sequences concatenated there was only moderate support for a sister relationship between *N. sundara* and *N. davidi* and none for the other interspecific relationships. However, when the same data were analyzed with *N. vivida* (for which only cytb and myo were available) removed, the sister relationship between *N. sundara* and *N. davidi* received high (PP 0.99, MLBS 72%) support and *N. grandis* and *N. macgrigoriae* came out as sisters with moderate PP (0.87) but <50% MLBS (not shown), and when only one individual of each species for which sequences from 7 to 8 loci were available (i.e. all except *N. vivida*) were analyzed the latter sister relationship grew to PP 0.94/MLBS <50% (Supplementary Fig. 1).

Clade C included all *Cyornis* except *C. concretus*, and was separated into seven subclades, most of which were strongly

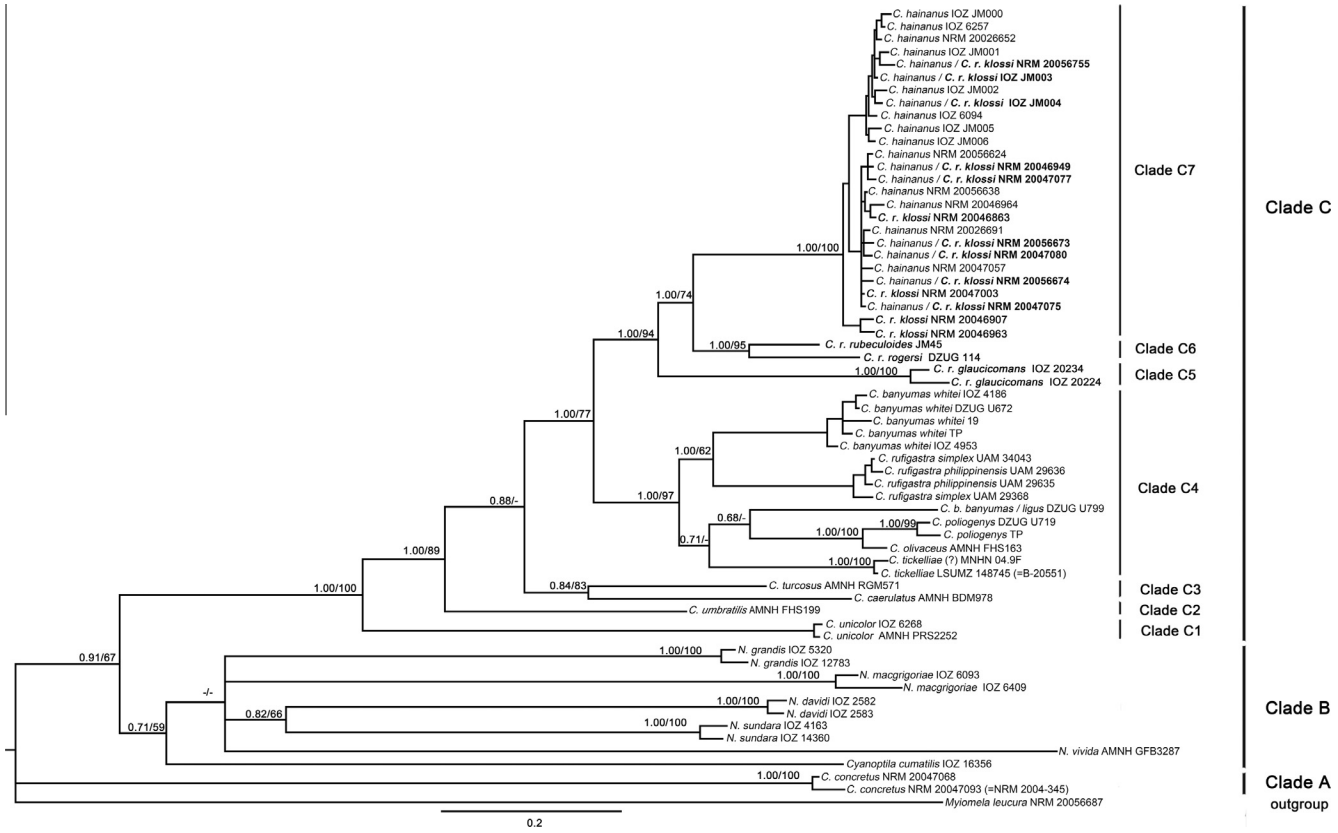

Fig. 1. Relationships of *Cyornis* flycatchers based on eight concatenated loci (COI, cytb, ND2, myo, BRM, CHD1Z, MUSK, Z-185) analyzed in 14 partitions. Values at the nodes are posterior probabilities and maximum likelihood bootstrap values, in this order. All *Cyornis rubeculoides* samples are in bold.

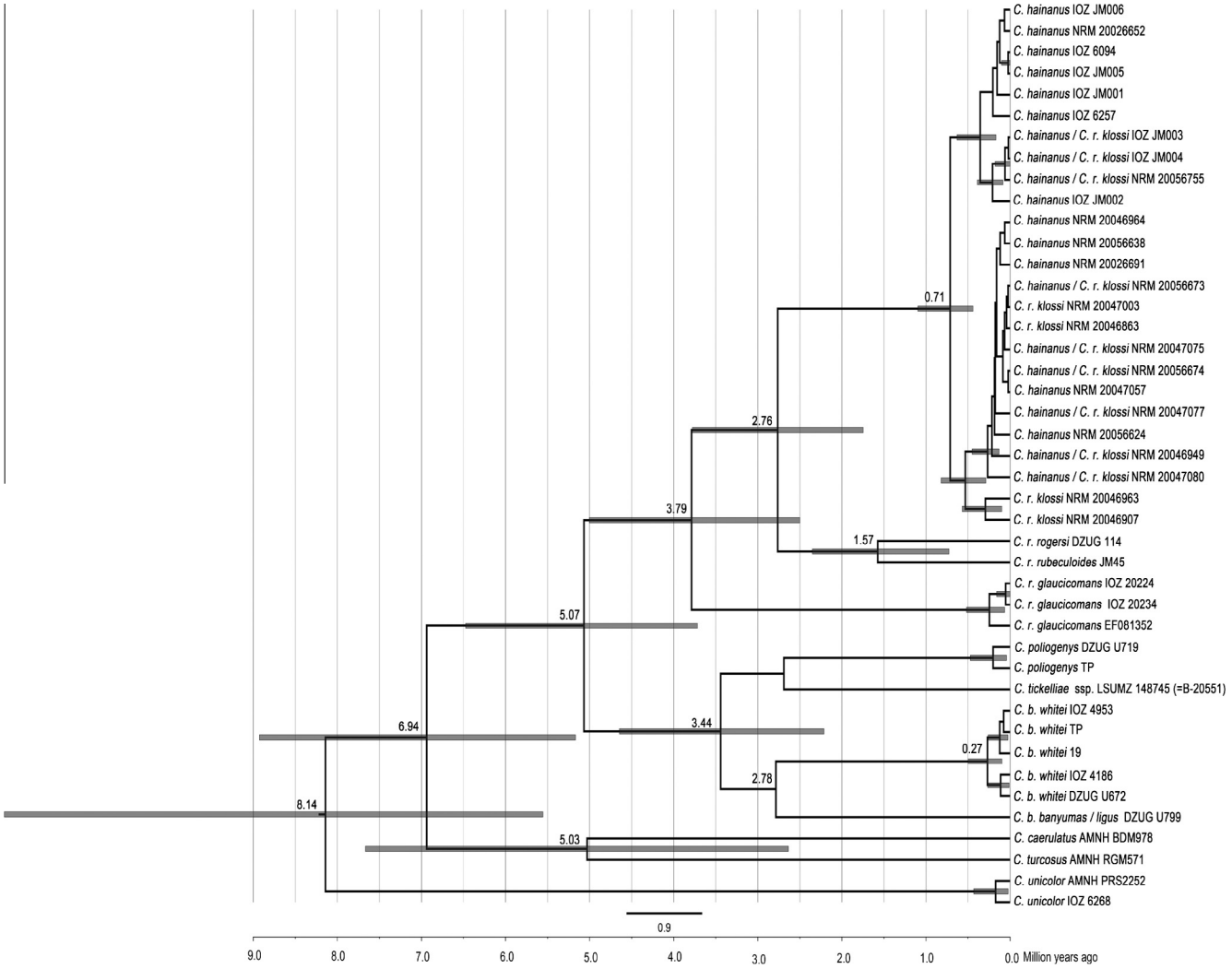

Fig. 2. Chronogram based on cytochrome b sequences. Gray bars at nodes are 95% highest posterior density intervals for the node ages.

supported. Clade C1, containing *C. unicolor*, was sister to the others, with clades C2 (*C. umbratilis*) and C3 (*C. caerulatus* and *C. turcosus*) next in sequence, though with poor support for the relationships among them. Clade C4 included *C. banyumas*, *C. rufigastra*, *C. poliogenys*, *C. olivaceus* and *C. tickelliae*. *C. banyumas* was not monophyletic, with Chinese and two of the three Thai birds sister to *C. rufigastra*, the single Javan bird sister to *C. poliogenys* and *C. olivaceus* and the third Thai bird (from GenBank: JX256054) sister to *C. tickelliae*, although only the latter of these relationships was strongly supported. *C. rubeculoides* was recovered in three clades in a well-supported sister position to clade 4: clade C5 comprised *C. r. glaucicomans* (central China) in a strongly supported sister position to clades 6 and 7; clade C6 contained *C. r. rubeculoides* (Nepal) and *C. r. rogersi* (west Myanmar); and clade 7, which had high PP but no MLBS, comprised an unresolved mix of *C. r. klossi* (east Thailand, south Laos, south Vietnam), birds reminiscent of *C. r. klossi* from Guangdong Province, China, and *C. hainanus*.

Single-locus analyses were generally less well resolved and supported, in particular the nuclear loci, which were generally poorly resolved (Supplementary Fig. 2). The CHD1Z tree showed two incongruent relationships with PP $\geqslant$0.95 and/or MLBS $\geqslant$75% compared to the tree based on the concatenated data: (1) *C. r. glaucicomans* IOZ20224 (but not *C. r. glaucicomans* IOZ20234) and *C. caerulatus* were outside the main *Cyornis* clade (C), which was in a well supported sister position to the *Niltava* clade (1.00/74%); and (2) *Cyornis concretus* NRM20047068 and *Cyanoptila cumatilis* were sisters (0.91/83%) (Supplementary Fig. 2).

3.3. Chronogram

The chronogram based on cytb and the molecular clock is shown in Fig. 2. The deepest split, between *C. unicolor* and the rest, was dated to 8.1 million years ago (MYA) (95% highest posterior distribution [HPD]: 5.6–12.0 MYA). The split between *C. r. glaucicomans* and the other *C. rubeculoides* subspecies was estimated at 3.8 MYA (95% HPD: 2.5–5.0 MYA; between *C. r. rubeculoides* and *C. r. rogersi* at 1.6 MYA (95% HPD: 0.7–2.4 MYA; and between these two and *C. r. klossi* plus *C. hainanus* at 2.8 MYA (95% HPD: 1.8–3.8 MYA).

4. Discussion

4.1. Circumscription of Cyornis

Our data support those of Sangster et al. (2010) that *C. unicolor*, *C. umbratilis* and *C. olivaceus* are part of the *Cyornis* clade (*C. umbratilis* and *C. olivaceus* were previously placed in *Rhinomyias*; e.g. Sibley and Monroe, 1990; Dickinson, 2003), although the *C. umbratilis* and *C. olivaceus* sequences were the same as used in Sangster et al. (2010). Also in agreement with Sangster et al. (2010), *C. concretus* was more distantly related to *Cyornis*. Although the precise

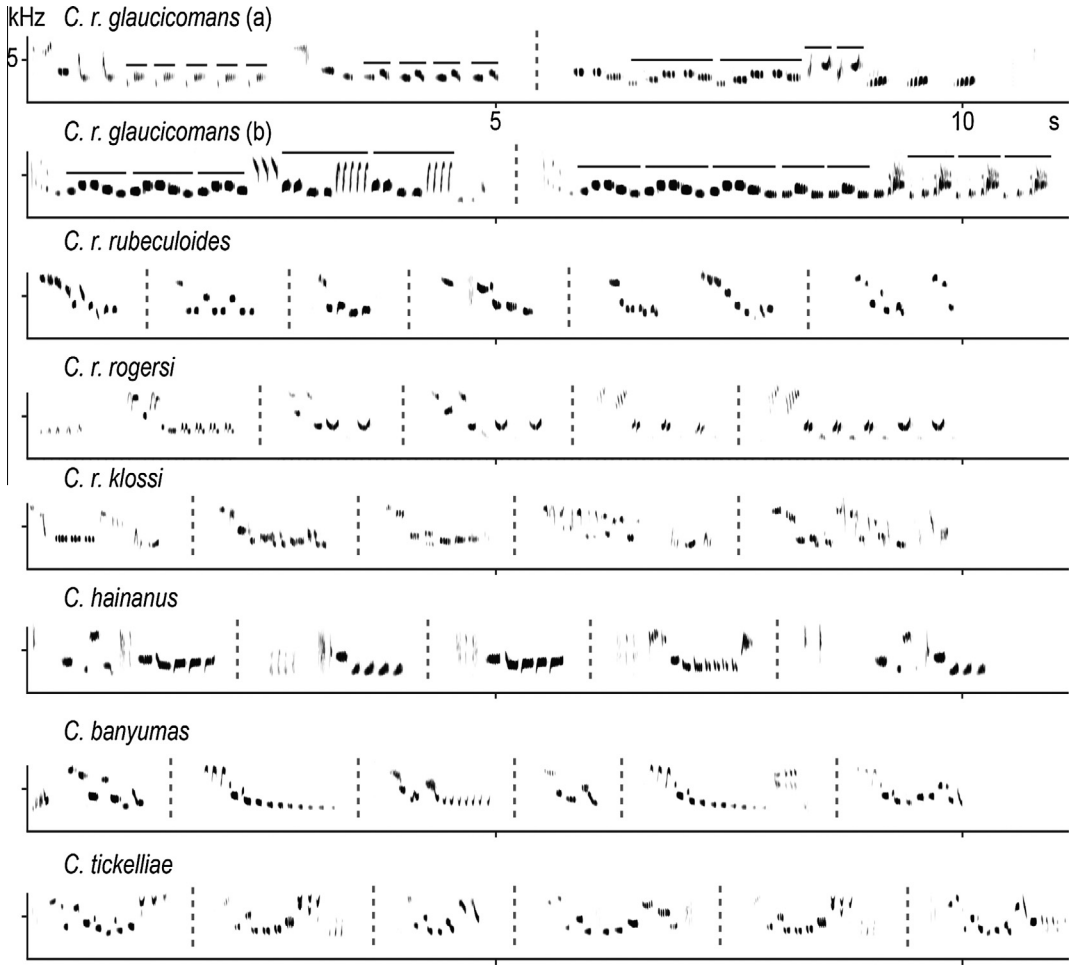

Fig. 3. Sonograms of *Cyornis* taxa. *C. rubeculoides glaucicomans*, A, Longcangguo, Sichuan, May 2013 (XC212318); B, Emei Shan, Sichuan, May 1989 (XC212320). *C. rubeculoides rubeculoides* Corbett national park, Uttarakhand, India, June 1997 (XC212322). *C. rubeculoides rogersi* Mt Victoria, Myanmar, April 2000 (XC212323). *C. rubeculoides klossi* Quang Tri district, Vietnam, April 2004 (XC212324). *C. hainanus* Hainan island, China, March 1987 (XC21232). *C. banyumas lekhakuni* Khao Yai, Thailand, April 1991 (XC212325). *C. tickelliae tickelliae* Ranthambore, Rajasthan, India, June 1999 (XC212326). Pauses between strophes have been artificially shortened (indicated by vertical dashed lines). Repeated phrases typical of *C. r. glaucicomans* have been indicated in this species by horizontal bars. All recordings by Per Alström. The XC numbers refer to the registration numbers in the xeno-canto database (www.xeno-canto.org).

position of *C. concretus* was not unanimously strongly supported by our data, we suggest that it should be removed from the genus *Cyornis*. However, we are not aware of any available name that could be applied, and suggest that before a new name is proposed a more comprehensive analysis of Niltavinae *sensu* Sangster et al. (2010) be undertaken.

4.2. Cyornis rubeculoides and C. hainanus

C. rubeculoides glaucicomans has been treated as specifically distinct from *C. rubeculoides* without any published justification (Viney et al., 1994; Gill and Donsker, 2014). This is supported by the deep divergence between *C. r. glaucicomans* (Clade C5) and other *C. rubeculoides* subspecies (Clades C6 and C7). Although it has been suggested that the song of *C. r. glaucicomans* is distinct (P. Alström and B. King, pers. comm. in Inskipp et al., 1996; Clement, 2006), this has never been described in detail. We show in Fig. 3 that the song of *C. r. glaucicomans* is markedly different from closely related mainland Asian *Cyornis*, whose songs are basically more similar to each other. In particular, the much longer strophes, with repetitions of complex phrases, and the deeper, richer voice are typical of *C. r. glaucicomans* compared to the others. We support treatment *C. r. glaucicomans* as a distinct species, named Chinese Blue Flycatcher *C. glaucicomans*. In addition, the sample with GenBank number EF081352 identified as *C. banyumas* (Lei et al., 2007) matches *C. glaucicomans* (not shown).

Our data strongly suggest that *C. r. rubeculoides* and *C. r. rogersi* (Clade C6) are sisters, and that they form the sister clade to Clade C7. There was a deep divergence, dated to 2.8 MYA, between Clades C6 and C7 and also a substantial divergence, dated to 1.6 MYA, between *C. r. rubeculoides* and *C. r. rogersi*. These two taxa, which are diagnosably different by plumage, as well as geographically separated (Rasmussen and Anderton, 2005; Clement, 2006), may be better treated as separate species. As we have only one sample from each taxon, and only mitochondrial sequences, more research is needed.

Totally unexpectedly, *C. rubeculoides klossi* from southern Vietnam, birds reminiscent of *C. r. klossi* from Guangdong Province, south China and *C. hainanus* were inseparable by the loci we analyzed (Clade C7). These results may question the status of *C. hainanus* as a distinct species. However, as *C. hainanus* was well differentiated from *C. r. rubeculoides* and *C. r. rogersi* at least by mitochondrial markers (see above), and breeds sympatrically with *C. r. dialilaemus* over a fairly large area without any known interbreeding (Fig. 4), *C. hainanus* should continually be treated as a separate species.

There are at least three possible explanations for the lack of genetic divergence between *C. r. klossi* (including birds reminiscent of *C. r. klossi* from Guangdong Province) and *C. hainanus*, which have

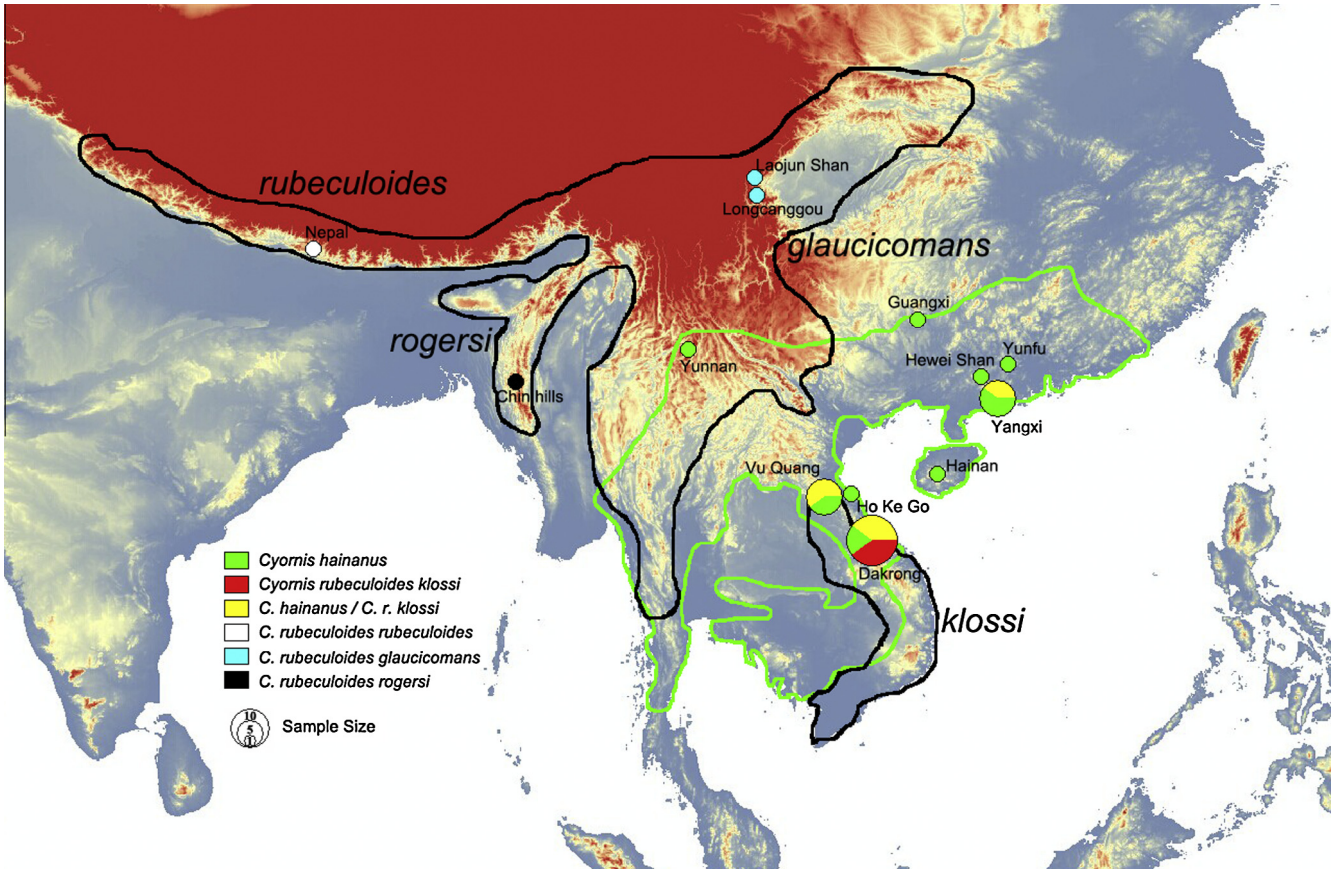

Fig. 4. Distributions of *C. rubeculoides* (black line) and *C. hainanus* (green line), with sampling localities indicated by colored circles. Ranges based mainly on Clement (2006). The exact ranges of the different taxa are incompletely known, and the ranges are probably not as continuous as indicated here, as both *C. hainanus* and *C. rubeculoides* occur at fairly low to mid elevations (the latter up to c. 2000 m in the Himalayan part of the range, lower elsewhere; Clement, 2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

strongly different-looking adult male plumages (Fig. 5): (i) *C. r. klossi* and *C. hainanus* interbreed to the extent that the markers that we have sequenced have become homogenized; (ii) *C. r. klossi* and *C. hainanus* are so recently diverged that incomplete lineage sorting is still prevalent; or (iii) *C. r. klossi* and *C. hainanus* are merely color morphs of the same species. The first hypothesis is supported by

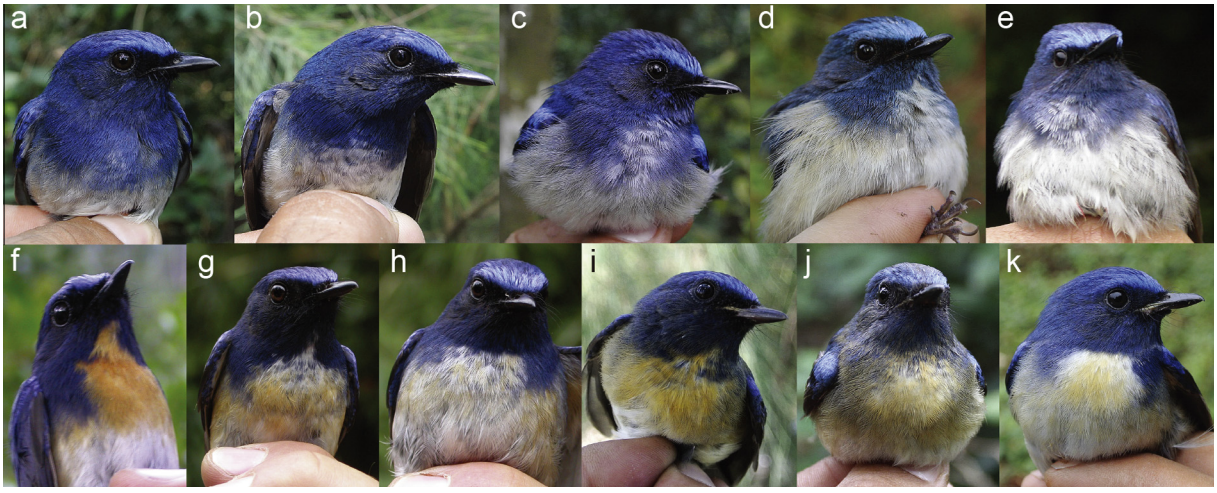

Fig. 5. Photos of males of *Cyornis hainanus* (a–e), *C. rubeculoides klossi* (f) and birds showing intermediate plumage between these two taxa (g–k). (a) adult, Leizhou Beitan, Guangdong, China, 27 October 2014; (b) 1st-calendar year, Xitou, Guangdong, China, 27 September 2014; (c) adult, Sanjia Shan, Guangdong, China, 19 April 2014 (IOZ JM001); (d) 2nd-calendar year, Weizhou Island, Guangxi, China, 15 April 2013; (e) 2nd calendar-year, Vu Quang National Park, Ha Tinh province, Vietnam, 13 March 2005 (NRM 20056624); (f) 2nd calendar-year, Dakrong, Quang Tri province, Vietnam, 25 March 2004 (NRM 20046963); (g) 2nd calendar-year, Dakrong, Quang Tri province, Vietnam, 4 April 2004 (NRM 20047077); (h) Dakrong, Quang Tri province, Vietnam, 4 April 2004 (NRM 20047080); (i) 1st calendar-year, Xitou, Guangdong, China, 29 September 2014; (j) 1st calendar-year, Leizhou Beitan, Guangdong, China, 24 October 2014; (k) 1st calendar-year, Leizhou Beitan, Guangdong, China, 13 November 2013. Photos: (a–d), (i–k) Jonathan Martinez, (f–h) Peter Nilsson/Swedish Museum of Natural History, and (e) Ingrid Cederholm/Swedish Museum of Natural History.

the fact that males with intermediate plumages between *C. r. klossi* and *C. hainanus* have been observed in Quang Tri province, central Vietnam, where both taxa breed in sympatry (P.A. pers. obs.; Figs. 4 and 5). Hybridization is known to occur between two other *Cyornis* flycatchers, *C. tickelliae* and *C. poliogenys*, in northeastern India, despite that males of these species have even more strikingly different plumages than *C. r. klossi* and *C. hainanus* (the former looks like a "classic" *Cyornis* male, while the latter is sexually monomorphic in plumage, with males looking like a typical female *Cyornis*) (Rasmussen and Anderton, 2005; Clement, 2006).

The second hypothesis is supported by our result that all loci showed the same lack of differentiation. Moreover, there was no evidence of markedly divergent haplotypes as would have been expected if the gene pool had originated from two long separated populations. As further evidence of recent divergence, *C. r. klossi* and *C. hainanus* have only slightly overlapping distributions (Fig. 4), indicating that they have been geographically separated and later come into secondary contact. Both hybridization and incomplete lineage sorting may have contributed to the observed pattern, as both these processes are more likely to occur in more recently diverged taxa than in more anciently separated ones (Price, 2008). The pattern resembles that in the two crows *Corvus corone* and *C. cornix*, which are variously treated as separate species or as conspecific. These are easily separable by plumage, meet in a hybrid zone in Western Europe, and exhibit extremely few genetic differences (Wolf et al., 2010; Poelstra et al., 2014). Remarkably, out of 8.4 million single-nucleotide polymorphisms only 82 fixed differences were found in these crows, all except one in a region linking genes involved in pigmentation and visual perception (Poelstra et al., 2014), showing that the genetic foundation for morphological differences that may contribute to reproductive isolation between species may comprise just a tiny proportion of the genome.

The third hypothesis may be supported by the fact that the birds with some plumage characters reminiscent of *C. r. klossi* that have recently been found to breed in Guangdong (by J.M.) are geographically widely separated from the south Vietnamese *C. r. klossi* as well as from other *C. rubeculoides* subspecies.

In conclusion, *C. r. klossi* is better classified as a subspecies of *C. hainanus* or possibly as a color morph of *C. hainanus*. More research on the causes of the intricate pattern is needed, including field work to evaluate whether there exists a phenotypically stable population with features reminiscent of *C. r. klossi* in and around Guangdong Province, China.

4.3. Cyornis banyumas

Our samples of *C. banyumas* were from three geographically different areas representing two different subspecies: south China and Thailand (*C. b. whitei*) and Java (*C. b. banyumas/ligus*). The Chinese and two of the Thai samples formed a clade, whereas the Javan and third Thai (from GenBank: JX256054) samples were markedly different both from the Chinese/two other Thai samples and from each other. The estimated divergences among these three "clades" were 0.3–2.8 MYA. The precise positions of the *C. banyumas* subspecies within Clade 4 were unresolved. However, the Thai ND2 sequence downloaded from GenBank (JX256054) was in a strongly supported sister position with our only sample identified as *C. tickelliae* (LSUMZ 148745), whose sequence was also downloaded from GenBank. As these two sequences differ in only three bp, it seems likely that one of them was misidentified to species. We have only examined photos of the voucher of the *C. tickelliae* sample (LSUMZ 148745), which unfortunately originated from a bird in captivity with unknown provenance (Steve Cardiff in litt.), but its plumage matches a Southeast Asian *C. tickelliae* (also the opinion of Pamela C. Rasmussen, in litt.). For the putative Thai *C. banyumas* downloaded from GenBank no voucher is available (Jérôme Fuchs, in litt.), but it seems likely that it was in fact a *C. tickelliae*.

Based on a detailed morphological analysis, Renner et al. (2009) suggested that the two Javan subspecies, *C. b. banyumas* and *C. b. ligus*, were similar to each other, but distinct from other subspecies of *C. banyumas*. This, in combination with our results, suggest that the name *C. banyumas* should apply to the Javan birds (and possibly others, not analyzed here), whereas Chinese/Thai birds should be treated as *C. whitei*. More research is needed on this complex.

4.4. Other Cyornis

Although we only had ND2 sequences (from GenBank) of *C. rufigastra* (Philippines), this was strongly supported to belong in Clade C4 together with *C. banyumas*, *C. tickelliae*, *C. poliogenys* and *C. olivaceus*. The two subspecies of *C. rufigastra*, *C. r. simplex* (northern Philippines) and *C. r. philippensis* (central, western and southern Philippines), were intermixed in the tree. It should be noted that the *C. olivaceus* and one of the two *C. poliogenys* (DZUG U719) were represented by the same sequences as in Sangster et al. (2010). Except for a strongly supported sister relationship between *C. olivaceus* and *C. poliogenys*, all relationship among taxa in clade 4 were poorly supported.

The precise positions of *C. umbratilis* (same sequences as in Sangster et al., 2010), *C. turcosus* and *C. caerulatus* were poorly supported, although the two latter were sisters with moderate PP and fairly high MLBS in the concatenated analysis.

4.5. Cyanoptila and Niltava

Despite the addition of sequence data compared to Sangster et al. (2010), the position of *Cyanoptila* as sister to *Niltava* was poorly supported. Also the relationships among the different species of *Niltava* remained uncertain. Although plumage similarity is a very unreliable indicator of relatedness, the two sister pairs indicated in these analyses are the ones that are most similar to each other in plumage (del Hoyo et al., 2006).

5. Conclusion

The main findings of the present study were the complex pattern within *C. rubeculoides–C. hainanus*, with both deep divergences among *C. rubeculoides* subspecies, supporting species status of at least *C. r. glaucicomans* (supported by latter's distinctive song), and a complete lack of divergence between *C. r. klossi* and *C. hainanus*. Deep divergences were also found between the two analyzed subspecies of *C. banyumas*. Despite the brilliant plumage colorations of most adult male *Cyornis* flycatchers, the plumage variation within the genus is fairly slight (except for some species with female-like males), and we predict that future molecular analyses will reveal multiple cases of deep splits within what is currently treated as polytypic species.

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sequences of *C. tickelliae*, and Pamela C. Rasmussen for comments on photos of the same; Jérôme Fuchs (MNHN) for information on a published sequence of *C. banyumas*; and Phil Round for comments on a sound recording and subspecific name of a *C. banyumas*. This work was supported by a grant from the Ministry of Science and Technology of China (2014FY210200; to F.L.); the Laboratory of molecular evolution of Shaanxi Normal University; Jornvall Foundation and the Chinese Academy of Sciences Visiting Professorship for Senior International Scientists (No. 2011T2S04) (both to P.A.); and the Sound Approach (to P.A. and U.O.).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.08.024.

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