



Short Communication

Clarifying the systematics of an enigmatic avian lineage: What is a bombycillid?

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1. Introduction

Avian Tree of Life (TOL) projects using multilocus sequence data have begun to reweave Sibley and Ahlquist's (1990) "tapestry" of the systematic relationships among birds, which was based on DNA–DNA hybridization. Many of these studies have focused on relationships among members of the most species-rich avian order, Passeriformes, or the perching birds (e.g., Barker et al., 2002, 2004; Cibois and Cracraft, 2004; Voelker and Spellman, 2004; Alström et al., 2006). Bringing direct sequence-based character evidence to bear on the systematic relationships among passerine birds has demonstrated that monophyly cannot be assumed for many lineages (Barker et al., 2004), from suborder to tribe, proposed by Sibley and Ahlquist (1990) and Sibley and Monroe (1990). However, many of the paraphyletic passerine lineages identified by modern sequence-based studies represent taxa identified as difficult to place or otherwise problematic by Sibley and Ahlquist (1990).

One of the consistently problematic groups has been the Bombycillidae (waxwings and allies). Sibley and Ahlquist (1990, p. 630) ultimately placed the Bombycillidae within the superfamily

Muscipapoidea, but they were not confident of this placement. Recent studies have only confirmed the ambiguous placement of Bombycillidae, removing the family from the "core Muscipapoidea" because of poorly supported (low Bayesian credibility and bootstrap values) relationships with the core Muscipapoidea (Barker et al., 2002, 2004; Cibois and Cracraft, 2004; Voelker and Spellman, 2004), but keeping it in the superfamily.

A potentially confounding factor for placing the Bombycillidae within the passerine phylogeny is that membership in and relationships among members of the family have long been controversial; therefore, a more thorough sampling of the group is desirable. Many early taxonomies recognized the morphological similarities among waxwings (*Bombycilla*, 3 spp.), silky-flycatchers (*Phainopepla*, *Phainoptila*, and *Ptilogonys*, 4 spp.), and the palmchat (*Dulus*, 1 sp.) and proposed them as members of a single family Bombycillidae (Arvey, 1951; Beecher, 1953). However, studies of egg-white proteins in the waxwings, silky-flycatchers, and palmchat indicated a close relationship between the former two, but not the latter (Sibley, 1970). The DNA–DNA hybridization analyses of Sibley and Ahlquist (1990) supported the close association among these three groups as indicated by morphology, leading these authors and Sibley and Monroe (1990) to maintain these species as members of the Bombycillidae, while also recognizing their differences by labeling them as distinct tribes (Bombycillini, Ptilogonatini, and Dulini; Table 1). Other modern taxonomies of the bombycillids (Voous, 1977; Cramp, 1988; AOU, 1998) have followed Wetmore

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Table 1
Specimen information

Superfamily	Family	Subfamily	Tribe	Representative taxon	Voucher Data
Corvoidea	Corvidae	Pachycephalinae		<i>Hylocitrea bonensis</i>	AMNH12574
	Petroicidae			<i>Tregellasia leucops</i>	KUMNH 4701 (AM819), voucher BPBM
Muscicapoidea	Bombycillidae		Dulini	<i>Dulus dominicus</i>	AMNH 25478 (NKK1035)
			Ptilogonatini	<i>Ptilogonys cinereus</i>	FMNH (Tissue 6132)
			Ptilogonatini	<i>Ptilogonys caudatus</i> (1)	FMNH 398067
			Ptilogonatini	<i>Ptilogonys caudatus</i> (2)	FMNH 393064
			Ptilogonatini	<i>Phainopepla nitens</i>	FMNH 343270 (MEX145)
			Ptilogonatini	<i>Phainoptila melanoxantha</i>	AMNH (PEP2205)
			Bombycillini	<i>Bombycilla garrulus</i>	AMNH Unvouchered (PRS1417)
			Bombycillini	<i>Bombycilla japonica</i>	Mus. Nat. d'Histoire Naturelle, Paris
			Bombycillini	<i>Bombycilla cedrorum</i>	AMNH (PAC631)
	Hypocoliidae			<i>Hypocolius ampelinus</i>	AMNH
	Cinclidae			<i>Cinclus cinclus</i>	AMNH NA (PRS2328)
	Muscicapidae	Turdinae		<i>Turdus falklandii</i>	AMNH NA (PRS1825)
	Sturnidae		Sturnini	<i>Sturnus vulgaris</i>	FMNH 389606 (MCP96-437)
Sylvioidea	Certhiidae	Certhiinae		<i>Certhia familiaris</i>	FMNH 351158 (S87-0026)
	Cisticolidae			<i>Cisticola anonymus</i>	AMNH 832156 (PB159)
Passeroidea	Fringillidae	Emberizinae	Thraupini	<i>Thraupis cyanocephala</i>	AMNH 24097 (GFB3133)

(1930) in emphasizing skeletal differences among the three groups and elevating each to the family level (Bombycillidae, Ptilogonidae, and Dulidae). If the waxwings, silky-flycatchers, and palmchat are considered the “core” bombycillids (but relationships among the core groups are still unknown), then the remaining ambiguity lies in placement of the only other species ever to be considered among the bombycillids, the grey hypocolius (*Hypocolius ampelinus*). The grey *Hypocolius* is generally considered to belong to a monotypic family, Hypocoliidae; however, at various points in its taxonomic history it has been considered closely allied with shrikes of the families Prionopidae and Laniidae (morphological characters; Mayr and Amadon, 1951), cuckoo-shrikes (jaw musculature; Campephagidae, Beecher, 1953), bulbuls (morphology; Pycnonotidae, Sibley and Ahlquist, 1990; Sibley and Monroe, 1990; Clements, 1991), and the Bombycillidae (plumage and morphological characters; Delacour and Amadon, 1949). The most recent taxonomic treatment of waxwings, silky-flycatchers, palmchat, and grey hypocolius listed each as a separate but closely related family (Bombycillidae, Ptilogonidae, Dulidae, and Hypocoliidae), reflecting the continued ambiguity of relationships among the groups (del Hoyo et al., 2005).

In this study, we attempted to identify a monophyletic bombycillid lineage and to clarify the phylogenetic relationships among the members of this group. To identify membership within the bombycillid lineage, we first compared nuclear RAG-1 sequences from each putative bombycillid with the extensive RAG-1 dataset available for passerines (Barker et al., 2002, 2004). Then, to clarify relationships among bombycillid species, we analyzed sequence variation at three loci (RAG-1, RAG-2, and mtDNA).

2. Materials and methods

2.1. Taxon sampling, DNA isolation and sequencing

Because the purpose of this study was to identify taxa that belong to a monophyletic bombycillid lineage and to elucidate relationships within this lineage, we conducted phylogenetic analyses with all the putative members of the family, as described by Cramp (1988), Sibley and Monroe (1990), and Clements (1991). This list included: *Bombycilla cedrorum*, *B. garrulus*, *B. japonica*, *Phainopepla nitens*, *Phainoptila melanoxantha*, *Ptilogonys cinereus*, *P. caudatus*, *Dulus dominicensis*, and *H. ampelinus* (Table 1). Also included in the analysis was the Indonesian endemic and enigmatic olive-flanked whistler, *Hylocitrea bonensis*, which in an exploratory molecular analysis had demonstrated affinities with members of

the Bombycillidae (Moyle, unpublished). In addition, we gathered new data from a number of non-bombycillid outgroups, representing all of the superfamily groups and most of the major lineages in Sibley and Monroe's (1990) Passerida, to match RAG-1 and RAG-2 sequences already available from these taxa (Table 1). Genomic DNA was extracted from cryopreserved muscle tissue for all samples except *H. ampelinus* using a Qiagen DNeasy Tissue Kit following the manufacturer's protocol. Our sample of *H. ampelinus* came from a cut toepad from a museum specimen; therefore, genomic DNA was extracted from this sample using a modified Qiagen extraction protocol described by Nishiguchi et al. (2002). We amplified and sequenced two nuclear loci (partial single exons of the RAG-1 and RAG-2 genes, 2887 and 1164 bp, respectively), and two mitochondrial genes (ND2 and *cyt b*, 1041 and 1000 bp, respectively), for all samples except *H. ampelinus*, for which we were only able to sequence a portion of the RAG-1 gene and the nearly complete ND2 gene. Methods of amplification, sequencing, and sequence alignment followed Barker et al. (2002) for the nuclear loci and Klicka et al. (2005) for the mitochondrial genes. All newly-derived sequences have been deposited in GenBank (Accession Nos. FJ177315–FJ177361).

2.2. Phylogenetic analyses

To identify all the members of a monophyletic bombycillid lineage, we conducted preliminary analyses with previously published RAG-1 sequences. RAG-1 sequences (2887 bp, for all taxa except *H. ampelinus* for which only a portion of the RAG-1 gene was amplifiable; see above) for all putative ingroup taxa plus sequences for several other enigmatic avian taxa were aligned by eye with RAG-1 data from Barker et al. (2004). This dataset included 153 taxa and 2947 bp of aligned sequence data. Maximum likelihood (ML) phylogenetic analysis (Multiphyl; Keane et al., 2007; SPR branch swapping on an initial neighbour-joining tree) using the GTR + I + Γ model of sequence evolution was performed using this alignment to determine the taxa that had affinity with the bombycillid lineage.

Bayesian and ML phylogenetic analyses were conducted on the expanded molecular dataset, which included RAG-1, RAG-2, and the mitochondrial genes. The fit of 56 nested nucleotide substitution models was evaluated separately for each gene region using the Akaike Information Criterion (AIC1) option in the program Multiphyl (Keane et al., 2007). Bayesian phylogenetic analysis was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and the best-fit nucleotide substitution models selected by

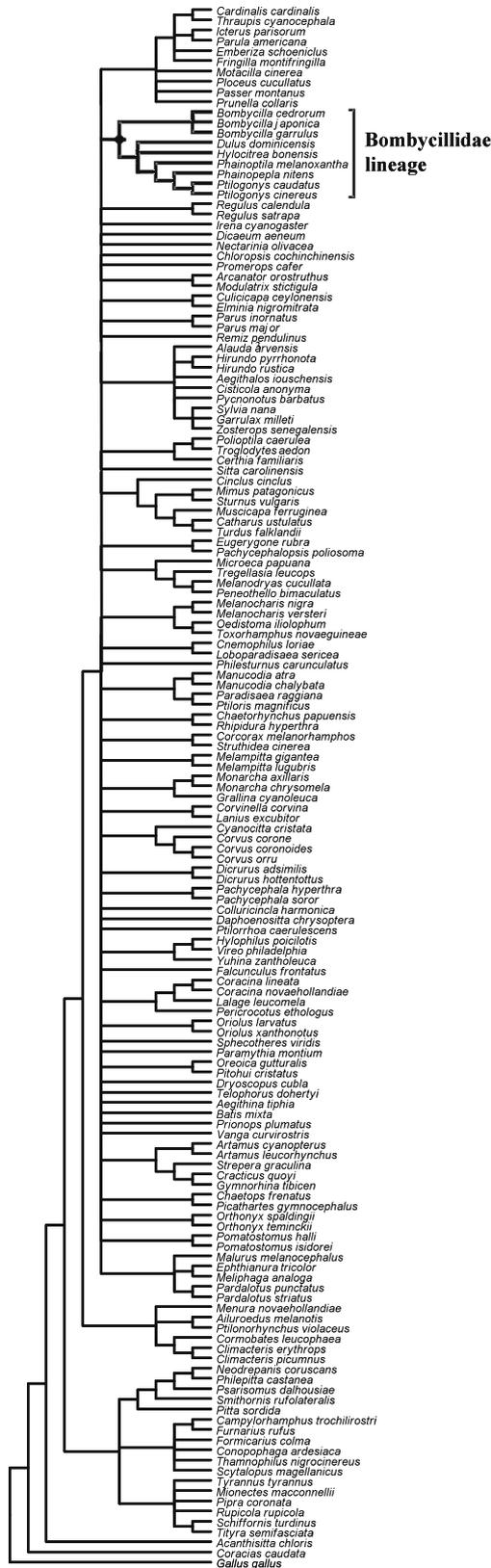


Fig. 1. Maximum likelihood phylogeny estimated from the expanded RAG-1 dataset (Barker et al., 2004) showing the position and membership of taxa within the Bombycillidae lineage. All branches with less than 70% bootstrap support were collapsed.

Multiphy for each of the separate gene regions (RAG-1, GTR+I+Γ; RAG-2, HKY+I+Γ; mtDNA, GTR+I+Γ). Two inde-

pendent runs of four (one cold and three heated) Metropolis-coupled Markov chains were run for 20,000,000 generations, and trees were saved every 1000th generation. Both runs were checked by eye for stationarity, and the first 5000 saved trees from each run were discarded as burn-in. Consensus trees were constructed from the remaining 15,000 trees for each run to confirm that the independent runs converged upon the same topology and posterior estimates, and then were combined into a single consensus tree (made up of 30,000 trees total). All ML analyses were performed in Multiphy. Multiphy was used to evaluate nucleotide substitution model fit, construct a ML phylogeny, and test the robustness of the inferred phylogeny (100 nonparametric bootstrap replicates) for the complete concatenated dataset.

3. Results

The preliminary analysis of the RAG-1 alignment suggested that all the putative Bombycillidae taxa listed above plus a single enigmatic SE Asian bird, *Hylocitrea bonensis*, belong to the bombycillid lineage (Fig. 1). Inclusion of these taxa in the bombycillid lineage was supported by 100% of 100 bootstrapped datasets and a synapomorphic insertion of a single codon in the RAG-1 reading frame (data not shown). Because only a partial RAG-1 sequence was available for *Hypocolius*, it was not included in the global tree search; however, the portion of the RAG-1 gene sequenced for *Hypocolius* contained the previously described and uniquely derived synapomorphic one-codon insertion possessed by all other taxa in the Bombycillid lineage, and *Hypocolius* was included in all subsequent analyses.

Sequence features and selected nucleotide substitution models for the three locus dataset (to clarify relationships among the bombycillids) are detailed in Table 2. Both Bayesian and ML phylogenetic analysis of this dataset yielded identical tree topologies (Fig. 2). The monophyly of the bombycillid clade was strongly supported (100% bootstrap and 1.00 posterior probability), and the members of this clade included *B. cedrorum*, *B. garrulus*, *B. japonica*, *Phainopepla nitens*, *Phainoptila melanoxantha*, *Ptilogonys cinereus*, *P. caudatus*, *Dulus dominicensis*, *H. ampelinus*, and *Hylocitrea bonensis*. Within the bombycillid clade there was strong support for the monophyly of the waxwings (*B. cedrorum*, *B. garrulus*, *B. japonica*) and silky-flycatchers (*Phainopepla nitens*, *Phainoptila melanoxantha*, *Ptilogonys cinereus*, *P. caudatus*) and evidence that each of the other members belonging to monotypic genera (*Dulus dominicensis*, *H. ampelinus*, and *Hylocitrea bonensis*) was genetically very distinct (each on long branches; Fig. 2). Support for higher-level relationships among members of the clade were generally poorly supported; however, there was substantial bootstrap support (but not significant posterior probability) for an early divergence of *Dulus* from the rest of the members of the clade.

Table 2
Sequence features for the 17 taxon, 3 locus (6093 bp) dataset

	Locus		
	mtDNA (ND2 & cyt b)	RAG-1	RAG-2
Number of characters	2042	2887	1164
Phylogenetically informative (%)	927 (45.4)	235 (8.14)	118 (10.14)
<i>Nucleotide frequencies (%)</i>			
A	28.43	31.28	29.23
C	32.53	20.49	21.64
G	12.07	23.91	22.88
T	23.7	23.75	25.92
Average pairwise distance	0.3846	0.0433	0.0486
Model selected	GTR+I+Γ	GTR+I+Γ	HKY+I+Γ

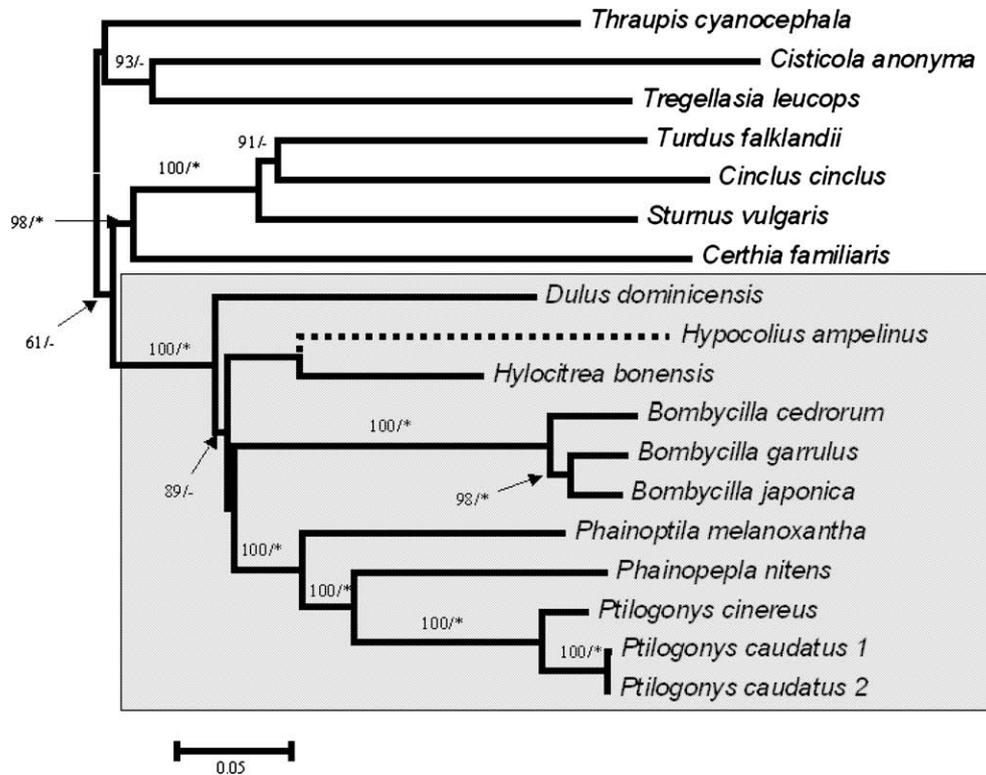


Fig. 2. Relationships among members of the Bombycillidae lineage. The phylogeny presented is the best maximum likelihood estimate rooted with *Tregellasia*, *Thraupis*, and *Cisticola*. The numbers above or beside the branches indicate maximum likelihood bootstrap support (100 replicates) and the asterisk (≥ 0.95) and dash (< 0.95) indicate Bayesian posterior probabilities.

4. Discussion

The analysis of nuclear exon data (RAG-1) and combined nuclear exon (RAG-1 and RAG-2) and mtDNA data resolved the persistent ambiguity concerning the systematic affinities of the waxwings and their proposed allies. High bootstrap support and statistically significant posterior probability combined with a synapomorphic insertion in the reading frame of the RAG-1 gene identified membership in a monophyletic bombycillid clade. The members of this clade include all the previously hypothesized members of the bombycillids, *H. ampelinus*, and the enigmatic monotypic genus *Hylocitrea bonensis*. However, well-resolved phylogenetic relationships among members of the clade remain elusive.

To our knowledge, this is the first molecular phylogenetic treatment of the bombycillids to include sequence data from *H. ampelinus*, and this inclusion appears to resolve the longstanding controversy over this taxon's association with the other members of the clade. Although we were unable to obtain complete sequences from the museum skin sample of *Hypocolius*, the data obtained were sufficient to confidently place it in the bombycillid clade. Specifically, the occurrence of a one-codon insertion in RAG-1 also present in all other bombycillids strongly supports this contention. In addition, combined analysis of nuclear and mtDNA data with an array of outgroups representing most major lineages of Passerida yielded strong support for its placement (Fig. 2).

More surprising was the inclusion of *Hylocitrea bonensis* (olive-flanked whistler) in the bombycillid clade. *Hylocitrea bonensis* has an extremely convoluted taxonomic history and under various taxonomic treatments is currently placed within the Pachycephalidae (Morony et al., 1975), Corvidae (Sibley and Monroe, 1990), and Muscicapidae (Peters, 1987). A recent systematic assessment of the crown Corvida (Jönsson et al., 2008) included *Hylocitrea bonen-*

sis and was the first to recognize that the species is not a member of the avian parvorder Corvida, but instead groups with parvorder Passerida. The placement of *Hylocitrea* within the bombycillid clade, a systematic relationship never previously hypothesized, further demonstrates the need to reconsider the systematics of many Wallacean taxa using molecular techniques.

The lack of statistically significant support for the basal nodes within the bombycillid lineage makes a biogeographic hypothesis for the evolution of the group difficult. If all poorly supported nodes are collapsed, then a large polytomy defines the relationships among the major lineages within the bombycillid clade. Two of the lineages stemming from the polytomy have multiple species and broad distributions: waxwings (*Bombycilla*; Holarctic) and silky-flycatchers (Ptilogonatinae; New World temperate and tropical regions). The remaining three lineages each include only a single species with restricted geographic distributions: Palmchat (*Dulus*; Hispaniola), grey *Hypocolius* (Iraq and Iran), and the olive-flanked whistler (*Hylocitrea*; SE Asia). The restricted geographic distributions and long branches of these monotypic taxa suggest that extinction has likely been important in the evolution of this group (Novacek and Wheeler, 1992). However, the inclusion of *Hylocitrea* in the bombycillid clade suggests that additional taxonomically enigmatic avian taxa might belong to this clade, and that future taxonomic studies of such taxa may help resolve systematic relationships within recognized groups.

While a thorough taxonomic revision of the bombycillid lineage is needed and should wait until a well-resolved phylogeny is available, we recommend the following taxonomic changes in the interim. The common ancestry of all the species within the clade should be recognized at the family level, Bombycillidae. The well-supported clades within the Bombycillidae (waxwings and silky-flycatchers) along with the monotypic lineages should all receive subfamily recognition: Bombycillinae (*Bombycilla*), Ptilogonatinae

(*Phainopepla*, *Phainoptila*, *Ptilogonys*), Dulinae (*Dulus*), Hypocoliinae (*Hypocolius*), and Hylocitreinae (*Hylocitrea*). All generic and species nomenclature should remain the same.

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