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Plumage based classification of the bowerbird genus *Sericulus* evaluated using a multi-gene, multi-genome analysis

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Abstract

Past classifications of taxa within the bowerbird genus *Sericulus* (family: Ptilonorhynchidae) conflict since the discovery of hybrids identified though male plumage characteristics. We use molecular data to help define species within this genus, and by estimating a phylogeny, test for lability in the evolution of male plumage patterns. Because this genus includes the most brightly colored bowerbird species, and is hypothesized to be the basal genus of the avenue building bowerbird clade, the organization of the four taxa within this genus is especially important in understanding how bowerbird plumage coloration evolved. Analyses of two mitochondrial and six nuclear gene regions confirm the basal placement of *Sericulus* in the avenue building bowerbirds and *Sericulus* monophyly, and suggests the Australian *S. chrysocephalus* is the basal *Sericulus* species. Our analysis additionally supports the existence of three New Guinea *Sericulus* species, contrary to some previous plumage based classifications, as they are genetically equidistant from each other. Molecular and geographic data of New Guinea are consistent suggesting a series of speciation events starting approximately 3.7–4.3 MYA leading to four extant *Sericulus* species. The absence of resolution within the New Guinea species precludes any statements of trait lability, but does suggest that traits under high selection pressures may not accurately indicate species level distinctions within this genus.
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Keywords: Bowerbird; *Sericulus*; Trait evolution; Nuclear intron; RAG-1; Bayesian analysis; Phylogeography; Species concept

1. Introduction

The use of phylogenetic analyses based on molecular data has become increasingly important as evolutionary biologists test hypotheses of trait evolution, and in defining species/subspecies limits. While much of the trait evolution research focuses on the effects of different types and degrees of selection intensity, many by mapping trait variation onto a phylogeny, little work has described the effects of selection on traits with large learned versus large genetically determined components. While other studies have found that traits with large learned components can be highly labile, here we use molecular data to test for evidence of labile evolution in a sexually selected trait with a large genetically determined component. In addition, a species concept relying on reproductive isolation can be complicated by the discovery of hybrids. Here, we use molecular data to define species boundaries in a genus where taxa originally ranked as species were considered conspecifics after the discovery of hybrids. In studying the bowerbird (Ptilonorhynchidae) genus *Sericulus* from Australia and New Guinea, we address both of these issues. By mapping plumage pattern on to our phylogeny, we test whether sexual selection can result in lability in a trait with a large genetic component. In addition, using genetic distances and a phylogeographic analysis, we address whether classifying *S. ardens* and *S. aureus* as conspecifics due to the discovery of hybrids based on plumage characteristics is appropriate.

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Taxa in the genus *Sericulus* exhibit highly elaborate mating displays involving decorated courts and bowers, bright plumage, vocalizations, and physical acrobatics, traits that are well known among many of the bowerbird species (Lenz, 1994, 1999; Marshall, 1954), and have been shown to be under sexual selection pressures in related species (Borgia, 1985, 1995; Coleman et al., 2004; Patricelli et al., 2002; Uy and Borgia, 2000). Males in this genus exhibit the most colorful plumage found in the bowerbirds, expressing vivid yellow and orange in the crown, crest, mantle, and wing in contrast to the black plumage found on the remainder of the body (Fig. 1, Cooper and Forshaw, 1977; Frith and Frith, 2004). Evidence from other bowerbird species, and considerable variation among *Sericulus* species in the ratio of brightly colored to black plumage suggests that the pattern of plumage coloration is highly evolutionarily labile and that classifications based on this trait may be misleading. However, in defining *Sericulus* taxa, male plumage has been routinely used (Bell, 1970; Coates, 1990; Cooper and Forshaw, 1977; Frith and Frith, 2004; Ogilvie-Grant, 1915).

Originally, four *Sericulus* species were defined, *S. chrysocephalus*, *S. ardens*, *S. aureus*, and *S. bakeri* (see review in Bell, 1970). The discovery of a presumed *S. ardens* and *S. aureus* hybrid (Ogilvie-Grant, 1915) based on intermediate plumage characteristics, in the Wataikwa River Valley of New Guinea, resulted in these taxa being considered subspecies, *S. aureus aureus* and *S. a. ardens*, rather than full species (Beehler et al., 1986; Coates, 1990; but see Iredale, 1956). Not until Lenz (1999) reported significant differences in four size-related morphological characteristics where these taxa again designated as separate species. Absent from these analyses though are molecular data and any estimates of genetic divergence.

Molecular phylogenetic analyses including the bowerbirds are few. The most recent and complete bowerbird molecular phylogeny includes 14 bowerbird species (Kusmierski et al., 1997). This topology, including only

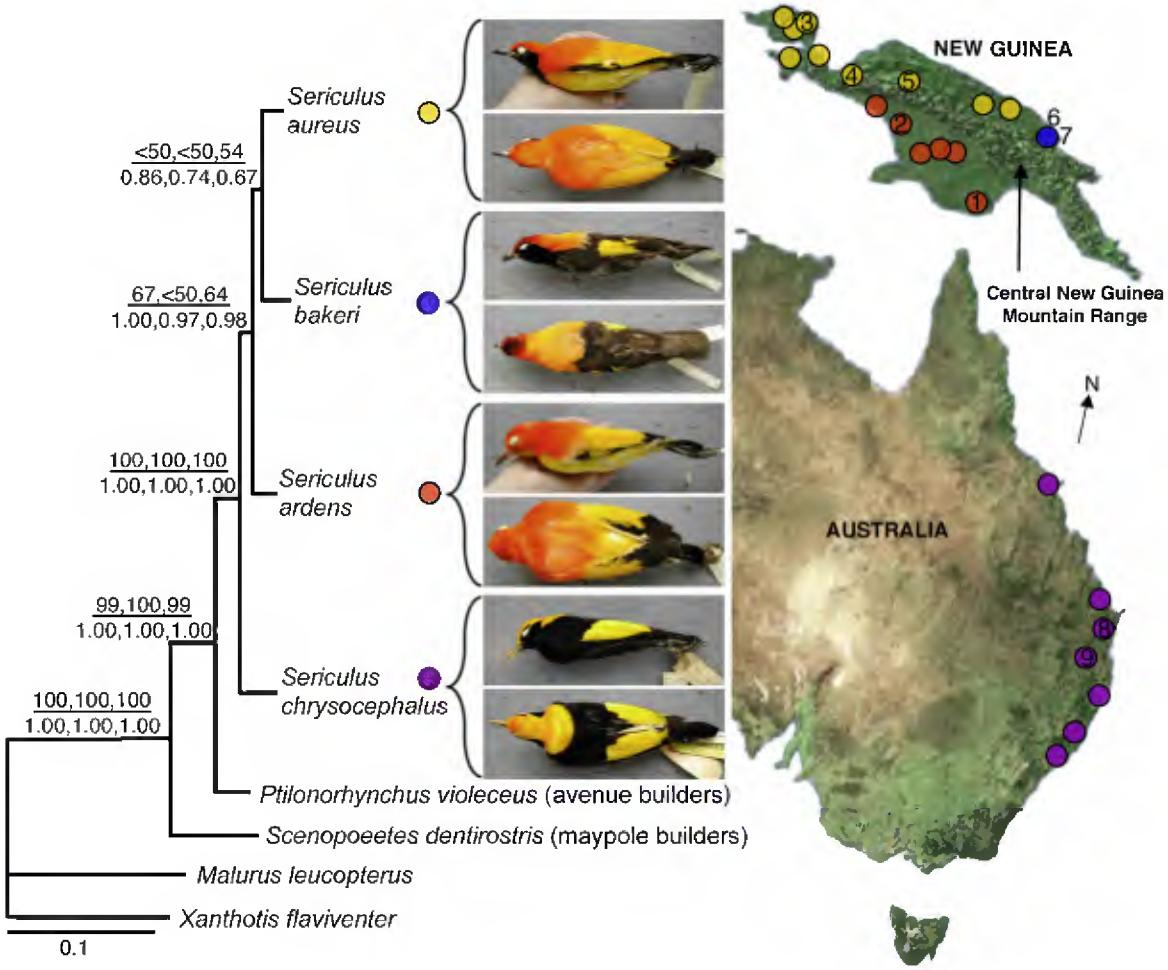

Fig. 1. Most likely topology based on 2474 bps of mitochondrial and nuclear data. At each node, above line—heuristic maximum likelihood bootstrap (100 replicates), exhaustive maximum parsimony bootstrap (1000 replicates), neighbor joining bootstrap (1000 replicates), below line—posterior probabilities: non-partitioned dataset (6 million generations), genome partitioned dataset (8 million generations), gene partitioned dataset (15 million generations). Markers on map identify museum specimen location reviewed by Bell (1970) and Lenz (1999), and are used to represent the range of species. Numbers on map coincide with samples used in this paper and referred to in Appendix A.

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S. chrysocephalus from the genus *Sericulus*, does estimate that this genus is the most basal taxon in the avenue building bowerbirds, an important node when testing the evolution of colorful displays among the bowerbird species. Previous work provides evidence for the labile evolution of some sexually selected bowerbird traits (bower design—Kusmierski et al., 1997; and bower decorations—Uy and Borgia, 2000), and observations of juvenile males visiting and inspecting adult male bowers suggests the possibility that these behaviors are learned (Borgia, 1986). Results from this paper, using a multi-locus, multi-genome dataset, would be the first to show that a sexually selected bowerbird trait with a large genetic component may also evolve in a labile manner. In addition, hybridization between *S. aureus* and *S. ardens* has resulted in these two taxa being considered conspecifics. Here we use divergence estimates, and a phylogeographic analysis, to help define species within this genus.

2. Methods

2.1. Tissue collection and DNA extraction

DNA for this study came from both field and museum samples. Blood samples were collected from three *S. chrysocephalus* and three *Ptilonorhynchus violaceus* adult males from New South Wales, Australia, and two *Scenopoeetes dentirostris* adult males from Queensland, Australia. DNA was extracted using Qiagen extraction protocols. *P. violaceus* is the next most closely related species to *Sericulus* and represents where the remaining avenue building species would fall. *Scenopoeetes dentirostris* is the basal species of the maypole builders, a sister clade to the avenue bower building clade, and represents where the remaining maypole building species would fall (Kusmierski et al., 1997). In addition, *Malurus leucopterus* and *Xanthotis flaviventer* were chosen as outgroups (Barker et al., 2004). Toe pad samples from museum specimens of *S. ardens*, *S. aureus*, *S. bakeri*, and *S. chrysocephalus* were collected (Appendix A), and extracted in an ancient DNA Lab using a phenol–chloroform and centrifugal dialysis protocol detailed in Fleischer et al. (2000).

2.2. Polymerase chain reaction (PCR) and sequencing

Initial amplification and sequencing was done using standard primers for *S. chrysocephalus* and the outgroups for two mitochondrial genes regions, cytochrome *b* (Cytb) (Kocher et al., 1989) and NADH dehydrogenase subunit 2 (ND2) (Sorenson et al., 1999), and five nuclear regions, β-fibrinogen intron 7 (Fib), glyceraldehyde-3-phosphate dehydrogenase exon 11 and intron 11 (Gapdh), ornithine decarboxylase intron 7 (ODC), ribosomal protein 40 exons 5 and 6 and intron 5 (RP40), transforming growth factor β-2 intron 5 (TGFβ2) (Primmer et al., 2002), and recombination activating gene exon 1 (RAG-1) (Barker et al., 2002; Groth and Barrowclough, 1999). Additional primers were designed from sequences obtained from *S. chrysocephalus* in an attempt to sequence smaller, overlapping fragments from the museum specimen extractions (Appendix B). PCRs and conditions for museum samples followed protocols for amplifying 'ancient' DNA (Fleischer et al., 2000) in 25 μL reactions. For field samples, a similar protocol was used excluding BSA and using annealing temperatures of 55–58 °C. Amplification was confirmed using gel electrophoreses, after which samples were purified by centrifugation following Qiagen manufacturer protocols. Cycle sequencing of both strands occurred in 20 μL reactions using BigDye Terminator v 3.1 according to ABI protocols, and reactions were analyzed using an ABI 3100 Automated Sequencer. Sequencher (Genecodes, Ann Arbor) was used to automatically align sequences, after which they were corrected by sight to ensure proper base calling and gap alignment. A list of museum samples, museum accession numbers, collection locations, and associated GenBank (NCBI) accession numbers are provided in Appendix A.

Difficulty in amplifying all regions from ancient samples resulted in regions of missing data for one or more of the museum specimens of each species. While indels were included in the alignments, regions containing ambiguous or missing data, found particularly at the edges of the datasets, were removed before concatenation. Individual ambiguous sites found within the dataset were also removed. In total, 349 base pairs (bps) of Cytb, 533 bps of ND2, 405 bps of Fib, 267 bps of Gapdh, 169 bps of ODC, 215 bps of LRPP40, 143 bps of TGFB2, and 393 bps of RAG-1 remained in the final dataset, making for 882 bps of mitochondrial DNA and 1592 bps of nuclear DNA, totaling 2474 bps. Of these sites, 327 are parsimony informative. To confirm DNA sequence stereotypy among the New Guinea taxa and identify additional variation in *S. chrysocephalus*, five additional museum samples were extracted using similar procedures, PCR amplified, and sequenced for Cytb and ND2 (Appendix A).

2.3. Phylogenetic analyses

Thorough phylogenetic analyses were performed on the original dataset, which does not include the additional museums specimens used to verify the original museum sequences. Non-significant partition homogeneity tests when treating all gene regions as separate partitions ($P = 1.00$), treating genomes separately ($P = 0.91$), Cytb and ND2 only and separately ($P = 0.46$), and the nuclear loci only and separately ($P = 0.15$), supported concatenating the individual gene data sets into a total evidence dataset. Afterwards, the Bayesian Inference Criterion (BIC), as implemented in Modeltest (v3.7: Posada and Crandall, 1998), was used to estimate nucleotide substitution models for use in the distance, likelihood, and Bayesian analyses. Models were estimated for a non-partitioned total evidence dataset (TrN+I+G), a genome partitioned total evidence dataset (HKY+I+G for mitochondrial data, HKY for nuclear intron data), and for individual gene regions

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(HKY+I for CytB; HKY+G for ND2; HKY for Fib, GAPDH, ODC, and LRRP40; K80 for TGFβ2 and RAG-1). Corrected genetic distances were estimated from the non-partitioned total evidence topology using PAUP* (v4.0b10 (Altivec): Swofford, 2001), as were relationships estimated using the likelihood and parsimony criteria. The neighbor joining distance tree was estimated with support from 1000 bootstrap replicates, the maximum parsimony topology was estimated through an exhaustive search with support from 1000 branch and bound bootstrap replicates, and the maximum likelihood topology was estimated through a heuristic (tree bisection and reconnection with 10 random starts) search with support from 100 heuristic bootstrap replicates.

Posterior probabilities for branches were estimated using a parallel version of MrBayes (v3.1.2p: Altekar et al., 2004; Ronquist and Huelsenbeck, 2003). An analysis was run on each of three datasets, a non-partitioned total evidence dataset, a genome partitioned total evidence dataset, and a gene partitioned total evidence dataset, to identify possible differences in topology resulting from alternative partitionings of the data. The analyses utilized four chains set over two simultaneous runs and, using Pooch (v1.7; Dauger Research, Inc.), were run across a cluster of four Dual G5 Macintosh computers. The analyses ran for 6, 8 and 15 million generations for the non-partitioned, genome partitioned, and gene partitioned datasets, respectively, to ensure proper exploration of the parameters. A "temperature" of 0.05 ensured adequate chain swapping and a 10% "burnin" was used for each analysis.

The retained data were used to estimate parameters, their variance, and posterior probabilities of nodes within the topology for each analysis. Upon termination, convergence diagnostics were used to determine whether topologies agreed among each run and whether each run had come close to stationarity. The average standard deviation of the split frequencies was <0.001 for each analysis. The standard deviation for each partition that constructs the backbone of the phylogeny, when compared between the two runs, in each of the three analyses, was the optimal value of 0.00. Only standard deviations of within clade bipartitions deviated from zero, being <0.003 in the New Guinea *Sericulus* clade. The potential scale reduction factor (PSRF) for all branches, on average, varied less than 0.001 from the optimal value of 1.00, and PSRF values for parameters used to determine stationarity were all 1.00, in each dataset. While the reliability of PSRF values is contingent on sample size, support for these bipartitions results from each bipartition being observed in both runs.

2.4. Tree dating

We utilized a calibration combining two genes, Cytb and ND2, to date nodes within the *Sericulus* phylogeny, necessary for the phylogeographic analyses. To estimate within taxa divergence estimates, we used a dataset containing all samples, including those used to verify original museum samples. Since the expected topology could not be estimated from either 195 bps of Cytb or 346 bps of ND2 alone, we concatenated these data into a single 541 bps dataset from which the expected topology was estimated. Non-significant differences between topologies with and without an enforced molecular clock, as determined using the likelihood ratio test ($p = 0.22$), suggests these data evolve in a clock-like fashion. Homologous regions of known Kauai creeper (*Oreomystis bairdii*) and Maui creeper (*Paroreomyza mana*) Cytb and ND2 sequences (Fleischer et al., 1998, unpublished data) were concatenated, a model of sequence evolution (HKY+I+G) was estimated (BIC in Modeltest v3.7: Posada and Crandall, 1998), and pairwise distances were estimated. We used the estimated Oahu subaerial (3.5 MYA) and shield building (3.0 MYA) dates (Price and Clague, 2002) to calculate mutation rates of 3.1%/MY and 3.6%/MY (see Fleischer et al., 1998 for the rationale and caveats of the approach). These calibration rates were then applied to divergence values corrected by removal of intraspecific variation to account for lineage sorting (Edwards and Beerli, 2000) to roughly estimate dates at nodes within the *Sericulus* phylogeny.

3. Results

3.1. Phylogenetic analyses

To verify museum sequences, we compared each sample to at least one additional sample from each species at two mitochondrial loci. For each *Sericulus* species, intraspecific variation was <1%. Topologies estimated using the original dataset under all phylogenetic criteria, rooting with *M. leucopterus* and *X. flaviventer* and using *Scenopoeetes dentirostris* to represent the maypole builders and *P. violaceus* to represent the avenue builders, place the non-*Sericulus* ingroup taxa as expected (see Kusmierski et al., 1997) with bootstrap support values >70 and significant posterior probabilities. This supports a monophyletic *Sericulus* clade with *S. chrysocephalus* as the basal *Sericulus* species (Fig. 1). In addition, it is possible that some gene regions contribute little phylogenetic signal while others contribute substantially more, resulting in a gene or genome biased topology. Yet, by comparing a non-partitioned analysis to both the gene and genome partitioned analyses using appropriate models of sequence evolution, we found no difference in topology and no support for gene or genome bias (Likelihood ratio test, $p = 1.00$).

The most likely topology places *S. aureus* sister to *S. bakeri*, as do all Bayesian and distance analyses (Fig. 1). While there was no significant difference in likelihood values when comparing constrained topologies of *S. aureus* and *S. ardens* to *S. ardens* and *S. bakeri* as sister taxa (Likelihood ratio test, $p = 0.92$), finding *S. ardens* sister to *S. bakeri* was 3.5 times more likely when considering posterior probabilities (average among the three Bayesian analyses). Posterior probabilities support the divergence of the New Guinea taxa, but bootstrap and posterior prob-

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ability values can only support a polytomy among the New Guinea *Sericulus* species. The most parsimonious analysis places *S. ardens* sister to *S. aureus*, but again without statistical support. In addition, topologies estimated from independent mitochondrial and nuclear analyses agree, suggesting that even with large divergence estimates, the mitochondrial data was able to infer appropriate relationships.

3.2. Dating of nodes

Divergence values used to estimate dates were estimated from concatenated Cytb and ND2 mitochondrial sequences and included all museum samples. Interspecific divergence estimates among the New Guinea species were similar, ranging from 7.1 to 10.8%. Using rates of 3.1 and 3.6% per million years estimated from homologous Drepanidine sequence, we estimate that the New Guinea *Sericulus* ancestor split from *S. chrysocephalus* 3.7–4.3 MYA, and the New Guinea *Sericulus* taxa diverged from each other between 1.9 and 3.5 MYA (Table 1). These data show the *Sericulus* species likely diverged before the Pleistocene.

4. Discussion

4.1. Phylogeny and species limits

Species and subspecies limits in the *Sericulus* genus have been defined on the basis of male plumage color patterns and the discovery of hybrids. We used a dataset of nearly 2500 bps of mitochondrial and nuclear DNA sequence data from eight loci to estimate divergences among the *Sericulus* species. This allowed us to define species limits, estimate the first phylogeny to include all *Sericulus* taxa, and to test the lability of plumage pattern evolution. While the most likely topology suggests *S. aureus* and *S. bakeri* are sister taxa, our dataset cannot statistically support this resolution. Divergence estimates among the three New Guinea species are relatively similar, and since there are species level distinctions between *S. bakeri* to each *S. aureus* and *S. ardens*, the similar level of divergence supports ranking *S. aureus* and *S. ardens* each as separate species (see also Fleischer et al., 2006). These data also suggest these species may have evolved from a common ancestor nearly simultaneously, but an analysis containing additional sequence data would be needed to confirm this.

Table 1
Divergence estimates within and among species based on 195 bps of Cytb and 346 bps of ND2

	Within species divergence
Sericulus ardens	0.002
Sericulus aureus	0.001
Sericulus bakeri	0.000
Sericulus chrysocephalus	0.001
Ptilonorhynchus violaceus	0.007

	Among species divergence	3.6% per million years	3.1% per million years
S. aureus, S. ardens	0.071	1.89	2.19
S. ardens, S. bakeri	0.103	2.81	3.26
S. aureus, S. bakeri	0.108	2.97	3.45
NG Polytomy	0.094	2.53	2.94
S. chrysocephalus, S. ardens	0.143	3.89	4.52
S. chrysocephalus, S. aureus	0.134	3.67	4.26
S. chrysocephalus, S. bakeri	0.135	3.72	4.32
S. chrysocephalus, NG Polytomy	0.137	3.69	4.29
Ptilonorhynchus, Sericulus	0.201	5.28	6.13

Mutation rates calculated from homologous Kauai and Maui creeper sequences using Oahu subaerial and shield building dates. NG-New Guinea.

4.2. Evolution of color patterns in male plumage

The bowerbirds are an important group with which to test hypotheses addressing the evolution of male sexual display traits. Kusmierski et al. (1997) show that while the two types of bower, avenue and maypole, are found exclusively in one of two bower building clades, variation within these clades show little phylogenetic pattern. This is true even among recently diverged sister species, suggesting this complex trait is highly labile. Bowers, though, likely have a large learned component, as juvenile males observe adult males at their bowers (Borgia, 1986), possibly explaining the labile evolution of this trait. The pattern of plumage coloration on the other hand, is likely to be highly genetically determined, and possibly less likely to evolve in a labile manner. Contradictory evidence though suggests plumage coloration is suggested to evolve rapidly (Haavie et al., 2000; Lande, 1981; Omland and Lanyon, 2000). We therefore tested whether general patterns of plumage coloration, i.e. black versus yellow and orange, in the *Sericulus* genus are labile by mapping this trait onto the newly estimated phylogeny, predicting a lack of phylogenetic pattern if patterns of plumage coloration are labile. Also, it is important to note that we distinguish between rapid and labile evolution with rapid referring to the rate at which a trait evolves along a lineage, and labile referring to the amount of change among lineages.

The lack of resolution within the New Guinea *Sericulus* species precludes us from directly stating that male plumage patterns evolved in a labile manner. *S. ardens* and *S. aureus* share a similar color pattern to each other where much of the body is brightly colored, leaving only the tail and the outer primaries, and the *S. aureus* face and throat, black, but we show no support for or against referring to these species as sister taxa (Fig. 1). Our data suggest two possible reconstructions. First, a single evolution of brightly colored plumage from black, if *S. aureus* and *S. ardens* are sister taxa, and second, two separate evolutions of either brightly colored plumage from black, or black from brightly colored plumage depending on the ancestral

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state, if *S. bakeri* is sister to either *S. aureus* or *S. ardens*. If further analyses show *S. bakeri* is sister with either *S. ardens* or *S. aureus*, this would suggest that *Sericulus* male plumage patterns do evolve in a labile manner. Our results suggest that exclusive use of male plumage coloration, a trait that is likely under high sexual selection pressures, in defining species within this genus is unreliable.

4.3. Phylogeography

The New Guinea orogeny, which has been suggested to have started as early as the mid Miocene (Cloos et al., 2005) or as late as the early Pliocene (Dow and Sukamto, 1984; Pigram and Davies, 1987; Pigram and Symonds, 1991) has been suggested to have greatly influenced the biodiversity of New Guinea (Dumbacher and Fleischer, 2001; Heads, 2001a, 2002; Joseph et al., 2001), and may have played a part in the New Guinea *Sericulus* radiation. *S. chrysocephalus* is found along the Eastern coast of Australia (0–900 m), *S. ardens* in the Fly watershed of southern New Guinea (0–900 m), *S. aureus* in Northern and Western New Guinea (900–1500 m), and *S. bakeri* isolated in the Adelbert Mountains (900–1200 m) in Northeastern New Guinea (Cooper and Forshaw, 1977; Gilliard, 1969; Lenz, 1999) (Fig. 1). This radiation of the New Guinea *Sericulus* species may have resulted from isolation caused by the uplift of the central New Guinea mountain range. A phylogeographic analysis showing consistency between dates for the isolation of northern and southern New Guinea populations and inferred dates from divergence estimates would provide evidence that the central New Guinea orogeny contributed New Guinea *Sericulus* radiation.

Results of our phylogeographic analysis suggest a likely sequence of events leading to the extant *Sericulus* taxa. As *S. chrysocephalus* is located in Eastern Australia and is the basal *Sericulus* species, and the fact that the vast majority of the remaining non-*Sericulus* avenue builders and outgroups are found in Australia (Gilliard, 1969; Simpson and Day, 2004), it is reasonable to assume an Australian *Sericulus* ancestor. This suggests, most parsimoniously, that ancestors of the modern New Guinea *Sericulus* species split from *S. chrysocephalus*, approximately 3.7–4.3 MYA, and dispersed northward into southern New Guinea. Dates for the orogeny of the central New Guinea Mountains differ, beginning as early as 12 MYA (Cloos et al., 2005) up until 5 MYA (Dow and Sukamto, 1984; Pigram and Davies, 1987; Pigram and Symonds, 1991), with mountains becoming a barrier to gene flow approximately 3.5–4.5 MYA (Heads, 2001b, 2002). This range of dates coincides with the divergence of the New Guinea species from *S. chrysocephalus*, suggesting the New Guinea orogeny is likely to have played an important role in the radiation of the New Guinea *Sericulus* species. These results support a vicariance model for the New Guinea *Sericulus* species radiation. Based on the unresolved polytomy of the three New Guinea *Sericulus* taxa and nearly simultaneous separation, the vicariance model suggests that the New Guinea *Sericulus* ancestor, once in southern New Guinea, spread across New Guinea before the mountains became a barrier to gene flow. Then the uplift occurred, isolating one population in Southern New Guinea, and two populations along the northern coast.

In addition to the phylogeographic analysis are observations of the altitudinal limits of the four *Sericulus* species. Altitudinal limits have been suggested to play an important role in speciation events in New Guinea, as it is likely that shifts to higher ranges occurred with contemporaneous geological uplift leading to population isolation (Heads, 2001a, 2002). *S. chrysocephalus* and *S. ardens* both reside in lowland areas with an altitudinal limit of up to 900 m. In comparison, *S. bakeri* resides between 900 and 1200 m and *S. aureus* resides between 900 and 1500 m. This evidence, and predictions of Heads (2001a, 2002), suggests *S. aureus* and *S. ardens* are not subspecies because they do not share similar ranges in altitude.

4.4. Sericulus classification

A study of *Sericulus* wing length, tail length, and two measures of bill size support the existence of four *Sericulus* species, i.e. that *S. ardens* and *S. aureus* are separate species (Lenz, 1999), but these data do not describe species divergences. Results from our molecular and phylogeographic analyses support the classification of four distinct *Sericulus* species. Our data suggest *S. chrysocephalus* is the basal *Sericulus* species, and that the three New Guinea *Sericulus* taxa are similarly diverged from each other, suggesting *S. ardens* and *S. aureus* are separate species. While our data do not suggest one way or another that the evolution of plumage color patterns in the *Sericulus* species is labile, they do suggest that decisions to rank taxa as conspecifics due to hybrids described from highly sexually selected traits should be re-examined using alternative types of characters.

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Appendix A

Specimen identification; number next to specimen represents location on Fig. 1; AMNH, American Museum of Natural History, New York, USA; NHM, The Natural History Museum, Tring, England; NSW- New South Wales; NG- New Guinea; AUST- Australia

Species (Reference No. on Fig. 1)	GenBank Accession numbers EU341380-EU341493	
Sericulus ardens		
(1) Museum: AMNH Accession #: 427633 Location: Tarara, NG	(2) Museum: NHM Accession #: 1916.5.30.1018 Location: Waitakwa River, NG	
Sericulus aureus		
(3) Museum: NHM Accession #: 99.11.3.3 Location: Mt. Moari, Arfak Mts., NG	(4) Museum: AMNH Accession #: 342290 Location: Bernhard Camp, NG	(5) Museum: AMNH Accession #: 303007 Location: Weyland Mts., NG
Sericulus bakeri		
(6) Museum: AMNH Accession #: 791276 Location: Nawawu, Adelbert Mts., NG	(7) Museum: AMNH Accession #: 791268 Location: Memenga, Adelbert Mts., NG	
Sericulus chrysocephalus		
(8) Museum: AMNH Accession #: 703207 Location: Bunya Mts., Queensland, AUST	(9) Location: Wallaby Creek, NSW, AUST	

Appendix B

Primers

Gene	Primer pair	Primers	Sequence	Reference
Cytb	S2H/2k	Cytb2S2H	GAATCTACTACGGCTCATAC	Developed by RCF
	2RC/wow	Cytb2.RC	TGAGGACAAATATCCTTCTGAGG	Developed by RCF
		Cytb2.wow	ATGGGTGGAATGGAATTTTGTC	Developed by RCF
	wow-29/C	Cytb2.wow-29	CCCATTAGGCATCCCATCAGA	This study
		Cytb2.C	AATAGGAAGTATCATTCGGGTTTG	Modified from Kocher et al. (1989)
	Cor3/L	Cor3	GACTCCTCCTAGTTTATTTGGG	Developed by RCF
		CorL	ACTGCGACAAAATCCCATTC	Developed by RCF
ND2	L5219/H5419	Pt_L5219	CCCATACCCCGAAAATGAGWSG	Modified from Sorenson et al. (1999)
		Pt_H5419	AAGTATTTTGTTGCAGCTTCAAT	This study
	L5419/H5578	Pt_L5419	GAAGCTGCAACAAAATACTT	This study
		Pt_H5578a	CCTTGGAGTACTTCTGGGAATCARA	This study
	L5758/H5977	Pt_L5758	GGATGAATRGGVYTMAAYCARAC	Modified from Sorenson et al. (1999)
		Pt_H5977a	GKCCKGCTAGAGAKAGWAGTGTGA	This study
	L5969/H6113	Pt_L5969	AACTATCAACAYTAATAACCTCRTG	This study
		H6113	CAGTATGCAAGTCGGAGGTAGAAG	Developed by A. Baker (personal communication)

(*continued on next page*)

Appendix B (*continued*)

Gene	Primer pair	Primers	Sequence	Reference
Fib	Fib1f/r	Fib1f	TGGATGGTATGTACYTGCACT	This study
		Fib1r	GKCTATGCCAAGAGAACAGC	This study
	Fib2f/r	Fib2f	TGCTGTTCTCTTGGCATAGM	This study
		Fib2r	TTCCTACTCAGTGTCCTCAGCA	This study
	Fib4f/r	Fib4f	CCTTCTGAGTGTRCTCTGTAGC	This study
		Fib4r	TTGGATCTGCAGTTAACCTGAT	This study
Gapdh	Gapdh1f/r	Gapdh1f	TGGCTCCAACTTGAAACAGTC	This study
		Gapdh1r	CAGGGCTRACCCATTTCTTA	This study
ODC	ODC1f/r	ODC1f	TCGTTGGAGTTAGGTGAGCTG	This study
		ODC1r	GGGTCTGTACATCCACTTCCA	This study
	ODC2f/r	ODC2f	TCGTTGGAATTTTTGAGGTC	This study
		ODC2r	CAGCAACACTGTCAAKAAATCA	This study
RP40	RP401f/r	RP401f	AGCACCCATGGGAAGTCAT	This study
		RP401r	ATTGGSAAAACCTGTCCTCA	This study
	RP402f/RP40r	RP402f	CACTTGAATGTGGTGGTTGG	This study
TGFB2	TGFB21f/r	TGFB21f	TTGTTTTAGGTAACTATGCCTCCA	This study
		TGFB21r	CTGTGGGATTGGAGACCACT	This study
	TGFB23f/r	TGFB23f	AGTGGTCTCCAATCCCACAG	This study
		TGFB23r	AGCTYATGGCTTCCCTGGAG	This study
RAG-1	MRAG09f/r	MRAG09f	ACAAACAAGCAGATGAATTGGA	This study
		MRAG09r	CAGGTTACCACCCATTCGAG	This study
	MRAG19f/r	MRAG19f	ACTTCCACAAAACGCTTGCT	This study
		MRAG19r	GTCTTGAAGCATCACTGGCT	This study
	RAG1-anc2L/R	RAG1-anc2L	AGGTTCCACTTACATTTGTACCC	Developed by RCF
		RAG1-anc2R	AAATATGGAGGTCCAACCCA	Developed by RCF
	RAG1-anc7L/R	RAG1-anc7L	GCCTTAAAGGACATGGAGGA	Developed by RCF
		RAG1-and7R	GCTGTTCGCTTTTCTTTCACA	Developed by RCF

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