Comparison of Species Tree Methods for Reconstructing the Phylogeny of Bearded Manakins (Aves: Pipridae, *Manacus*) from Multilocus Sequence Data

ROBB T. BRUMFIELD,^{1,2} LIANG LIU,³ DAVID E. LUM,^{4,5} AND SCOTT V. EDWARDS³

 ¹Museum of Natural Science, Baton Rouge, Louisiana 70803, USA; E-mail: brumfld@lsu.edu
²Department of Biological Sciences, Louisiana State University, Baton Rouge, Lousiana 70803, USA
³Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138, USA; E-mail: Iliu@oeb.harvard.edu (L.L.); sedwards@fas.harvard.edu (S.V.E.)
⁴Department of Biological Sciences, University of Washington, Seattle, Washington 98195, USA
⁵Current address: 95-390 Kuahelani Ave., Suite 3E, Mililani, Hawaii 96789, USA

Abstract.— Although the power of multi-locus data in estimating species trees is apparent, it is also clear that the analytical methodologies for doing so are still maturing. For example, of the methods currently available for estimating species trees from multiocus data, the Bayesian method introduced by Liu and Pearl (2007; BEST) is the only one that provides nodal support values. Using gene sequences from five nuclear loci, we explored two analytical methods (deep coalescence and BEST) to reconstruct the species tree of the five primary *Manacus* OTUs: *M. aurantiacus, M. candei, M. vitellinus*, populations of *M. manacus* from west of the Andes (*M. manacus* (w)), and populations of *M. manacus* from east of the Andes (*M. manacus* (w)). Both BEST and deep coalescence supported a sister relationship between *M. vitellinus* and *M. manacus* (w). A lower probability tree from the BEST analysis and one of the most parsimonious deep coalescence trees also supported a sister relationship between *M. candei* and *M. aurantiacus*. Because hybrid zones connect the distributions of most *Manacus* species, we examined the potential influence of post-divergence gene flow on the sister relationship of parapatrically distributed *M. vitellinus* and *M. manacus* (w). An isolation-with-migration (IM) analysis found relatively high levels of gene flow between *M. vitellinus* and *M. manacus* (w). Whether the gene flow is obscuring a true sister relationship between *M. manacus* (w) and *M. manacus* (w) and *M. manacus* (e) remained unclear, pointing to the need for more detailed models accommodating multispecies, multilocus DNA sequence data. [Gene trees; incomplete lineage sorting; manakin; phylogeny; species tree.]

Phylogenetic reconstruction from gene sequences presents multiple technical and analytical challenges if allelic variation is not reciprocally monophyletic with respect to the terminal taxa (Nei, 1987; Avise, 1994; Edwards, 1997; Maddison and Knowles, 2006; Pollard et al., 2006; Carstens and Knowles, 2007; Carling and Brumfield, 2008). Even when all gene trees are concordant with the species tree and do not exhibit incomplete lineage sorting, the best means of combining such information in a multilocus, multiallelic phylogenetic analysis is controversial (Kubatko and Degnan, 2007). In such cases, phylogenetic inferences must consider the population processes that produced the gene tree. This can be done (1) using mathematical population genetic methods, such as calculating the conditional probabilities of genealogies given different species trees or population histories (Maddison, 1997; Knowles and Maddison, 2002; Degnan and Salter, 2005); (2) by estimating population genetic parameters using methods that incorporate into the analysis both the large variance inherent in the coalescent process as well as uncertainty about the genealogical reconstruction (Kuhner et al., 1995; Beerli and Felsenstein, 1999); or (3) by calculating summary statistics that do not use the gene tree for parameter estimation (Hey and Machado, 2003; Hey and Nielsen, 2004). For example, pairwise divergence time estimates among a group of taxa could be used to infer sister relationships.

Population genetic methods incorporate knowledge of the fact that, following a divergence event, the genealogies of independently segregating loci follow a predictable sequence of shared ancestral polymorphisms (i.e., non-monophyly) initially, followed by polymorphisms unique to one or the other taxon (i.e., paraphyly), and, finally, unique polymorphisms in descendant taxa as well as fixed differences between them (i.e., reciprocal monophyly; Nei, 1987; Pamilo and Nei, 1988; Takahata, 1989; Harrison, 1991). For an independently sorting gene, the expected time to reciprocal monophyly is a positive function of the effective population size $(N_e$; Hudson, 1992). Migration or introgression after divergence can also produce incongruence of gene and species trees (Takahata and Slatkin, 1990), thus analyses of species groups in which migration or introgression could be occurring should incorporate migration parameters (Nielsen and Wakeley, 2001; Hey and Nielsen, 2004). Nonetheless, some of the first species tree or population tree methods were performed on human populations demonstrably exchanging genes, with the effect that populations exchanging migrants tended to cluster together when treated in a pure-isolation framework (Cavalli-Sforza, 1966). Phylogenetic models assuming isolation continue to be commonly used on populations that are demonstrably exchanging genes, such as when pairwise F_{st} is used to infer phylogenetic relationships.

Because estimates of population genetic parameters inferred from a single locus have large errors associated with them (Hudson, 1992; Kuhner et al., 1995), there are good statistical reasons underlying the current shift in population genetics and phylogenetics from single-locus studies of cytoplasmic markers to multi-locus studies of nuclear loci (Brumfield et al., 2003). The variance in population genetic parameter estimates can be reduced by integrating over the information contained in many independent genealogies (Takahata, 1989; Beerli and Felsenstein, 1999; Edwards and Beerli, 2000; Kuhner et al., 2000; Nielsen, 2000; Wakeley et al., 2001; Wilson and Rannala, 2003; Jennings and Edwards, 2005). How many loci are required remains unclear, but simulations (Pluzhnikov and Donnelly, 1996; Felsenstein, 2006; Carling and Brumfield, 2007) and empirical studies (Jennings and Edwards, 2005; Won and Hey, 2005; Won et al., 2005) suggest the number in many cases is on the order of tens to hundreds. A practical limitation in parameter estimation that is more acute with nuclear loci arises from the smaller amount of information available from any one independently segregating locus (Bensch et al., 2006; Carling and Brumfield, 2007). This is especially problematic for taxa that have relatively low genome-wide variation, such as birds. Unfortunately, collecting longer sequences does not remedy the problem, because longer sequences often contain multiple recombining regions, which often can only be included in the analysis as separate but very short loci (Hey and Nielsen, 2004). The remedy, again, is to collect data from more loci (Carling and Brumfield, 2007).

To explore the utility of incompletely sorted nuclear genes in phylogenetic inference, we collected sequence data from five autosomal loci and used several analytical methods to infer the species tree and speciational history of Manacus manakins. Because concatenated data can produce well-supported phylogenies that are inconsistent with the true species tree (Degnan and Rosenberg, 2006; Kubatko and Degnan, 2007; Rosenberg and Tao, 2008), particularly in scenarios spanning a short time period such as we suspect for these birds, we used analytical methods that build a species tree by incorporating the individual gene histories of multiple loci. An advantage of testing these methods in the Manacus system is that we were able to perform exhaustive searches at the species level (five ingroup OTUs were analyzed), even though many more alleles were examined at the gene level.

Manakins (Pipridae) are small, frugivorous inhabitants of forests throughout the Neotropics, remarkable for their lek mating behavior and striking secondary sexual plumage traits. The genus *Manacus* comprises four allospecies (Fig. 1), each readily diagnosable by differences in the color of the definitive male throat and belly plumage. The four forms of *Manacus* have been treated as distinct, but closely related, species (Sibley and Monroe, 1993), but some investigators have considered them members of a single polytypic biological species, *M. manacus* (Snow, 1975; Traylor, 1979; Remsen et al., 2007).

Hybrid zones that could provide a conduit for introgression connect the distributions of three of the four species. In western Panamá, a narrow hybrid zone unites *candei* and *vitellinus*, and unidirectional introgression of *vitellinus* plumage traits has been documented there (Parsons et al., 1993; Brumfield et al., 2001). Two hybrid zones unite *M. vitellinus* and *M. manacus*, one in the Caribbean lowlands of northern Colombia, the other in the Pacific lowlands of southwestern Colombia (Fig. 1); neither has been characterized morphologically or genetically. *M. aurantiacus* is the only species that is not in geographic contact with the other species.

An anomaly of *Manacus* biogeography is the presence of populations of *M. manacus* in the lowlands west of the Andes (Haffer, 1967; Fig. 1). Based on comparative biogeographic studies of lowland birds in northwestern South American, it is unusual for a lowland bird species whose predominate distribution is east of the Andes (*M. manacus*) to have any populations in the lowlands west of the Andes (Brumfield and Capparella, 1996). The Andes, and the associated high elevation habitats, present a barrier to dispersal for lowland birds. To explain the unusual presence of *M. manacus* populations west of the Andes, Brumfield and Braun (2001) proposed a testable hypothesis that the populations are actually conspecific with M. vitellinus but have not yet been impacted by recently derived and geographically spreading vellow plumage traits of *M. vitellinus* that would indicate this relationship.

Efforts to use mitochondrial gene sequences to reconstruct a Manacus phylogeny and test previously proposed phylogenetic hypotheses have been thwarted by PCR co-amplification of nuclear pseudogenes. Two studies have addressed *Manacus* species relationships, one reconstructing an outgroup-rooted phylogeny from isozyme frequency data (Brumfield and Braun, 2001), the other inferring an unrooted network from microsatellite allele frequencies (Höglund and Shorey, 2004). Both studies found shared allelic polymorphisms among taxa and assumed implicitly in their phylogenetic analyses that the shared genetic variation reflected the retention of ancestral alleles. A sister relationship between M. aurantiacus and M. candei received support from both analyses, but the studies differed concerning the phylogenetic relationships of *M. vitellinus* and *M. manacus*. The isozyme study found that populations of *M. manacus* west of the Andes were actually sister to *M. vitellinus* instead of to conspecific populations of *M. manacus* east of the Andes. This controversial relationship, supported largely by a synapomorphic allele at the locus PGM-2 (Brumfield and Braun, 2001), was not corroborated by the unrooted microsatellite network wherein all M. manacus populations clustered together (Höglund and Shorey, 2004). Here, we test both alternatives using divergence population genetics and species tree approaches.

We also test whether the divergence times of Manacus populations on opposite sides of major biogeographic barriers in South America are consistent with a Pleistocene timeframe, as proposed by Haffer (1969). East of the Andes, the distribution of Manacus manacus encompasses both sides of several major biogeographic barriers, occurring in humid lowland forests of the Amazon Basin on both banks of the Amazon and Negro Rivers (Capparella, 1988; 1991) and disjunctly in the Atlantic Forest of Brazil (Fig. 1). All three barriers-the Amazon, the Negro, and the caatinga separating the disjunct Amazon-Atlantic Forest populations-have been linked to a Pleistocene model of diversification (Haffer, 1969; Costa, 2003), but recent molecular diversification studies of humid forest, lowland birds are more consistent with older divergence times in the late Miocene



FIGURE 1. Schematic distribution of the four *Manacus* species. Elevations above 1500 m are in black. Digital maps (Ridgely et al., 2003) for each species were downloaded from InfoNatura (InfoNatura: Birds, 2005).

and Pliocene (Marks et al., 2002; Pereira and Baker, 2004; Cheviron et al., 2005; Ribas et al., 2005).

METHODS

Data Collection

Samples.—We collected genotypic data at five nuclear loci from 20 Manacus individuals, including representatives from each of the five ingroup OTUs: the four currently recognized species (*M. aurantiacus, M. candei, M. vitellinus, M. manacus*), plus populations of *M. manacus* from both sides of the Andes. Most individuals sequenced in this study were the same used to infer phylogenetic relationships in *Manacus* based on isozyme variation (Brumfield and Braun, 2001). Because introgression may be occurring across the narrow hybrid zone between *M. candei* and *M. vitellinus* in western Panama, we included samples collected distant from it (*candei*: 16157, 16282; *vitellinus*: 1858, 1862). To root genealogies we included seven other outgroup piprids (Online Table 1; see online supplementary material at http://www.systematicbiology.org) that represent potential sister genera to *Manacus*.

PCR amplification, cloning, and sequencing.—Genomic DNA was extracted from approximately 25 mg of pectoral muscle tissue using the DNeasy (Qiagen) DNA extraction kit. We amplified five nuclear loci: β -fibrinogen intron 7 (hereafter β f7; Prychitko and Moore,

		Number of chromosomes	Number of segregating				Sequence	
Locus	Species OTU	sampled	sites	θ_w	θ_{π}	Tajima's D	length	
βa3	M. aurantiacus	4	1	0.545	0.50	-0.612	377	
	M. candei	8	0	0	0	0	377	
	M. vitellinus	6	4	1.752	2.13	1.1806	377	
	M. manacus (west)	8	4	1.543	2.04	1.3942	377	
	M. manacus (east)	14	3	0.943	0.89	-0.1728	377	
	All	40	6	1.411	1.73	0.6130	377	
βf7	M. aurantiacus	4	0	0	0	0	635	
	M. candei	8	2	0.771	1.07	1.4488	635	
	M. vitellinus	6	0	0	0	0	635	
	M. manacus (west)	8	0	0	0	0	635	
	M. manacus (east)	14	11	3.459	2.03	-1.6249	635	
	All	40	13	3.056	1.94	-1.1398	635	
ODC67	M. aurantiacus	4	0	0	0	0	485	
	M. candei	8	6	2.314	2.57	0.5185	485	
	M. vitellinus	6	6	2.628	3.20	1.2465	485	
	M. manacus (west)	8	6	2.314	3.21	1.8134	485	
	M. manacus (east)	14	6	1.887	1.20	-1.3108	485	
	All	40	12	2.821	3.14	0.3478	485	
rho2	M. aurantiacus	4	0	0	0	0	315	
	M. candei	8	1	0.386	0.25	-1.0548	315	
	M. vitellinus	6	0	0	0	0	315	
	M. manacus (west)	8	0	0	0	0	315	
	M. manacus (east)	14	3	0.943	0.77	-0.5651	315	
	All	40	3	0.705	0.55	-0.4800	315	
TGFB5	M. aurantiacus	4	0	0	0	0	114	
	M. candei	8	0	0	0	0	114	
	M. vitellinus	6	0	0	0	0	114	
	M. manacus (west)	8	1	0.386	0.25	-1.0548	114	
	M. manacus (east)	14	3	0.943	0.77	-0.5651	114	
	All	40	5	1.175	0.86	-0.6879	114	
Combined	M. aurantiacus	4	1	0.545	0.50	-0.612	1926	
	M. candei	8	9	3.471	3.89	0.5952	1926	
	M. vitellinus	6	10	4.380	5.33	1.3080	1926	
	M. manacus (west)	8	11	4.242	5.50	1.4804	1926	
	M. manacus (east)	14	26	8.176	5.66	-1.3099	1926	
	All	40	39	9.169	8.22	-0.3625	1926	

TABLE 1. Polymorphism data summary from largest independently sorting gene region. Sequence length excludes indels.

1997), β -actin intron 3 and rhodopsin intron 2 (hereafter β a3 and rho2; Waltari and Edwards, 2002), transforming growth factor β 2 intron 5 (hereafter TGFB5; Burt and Paton, 1991), and ornithine decarboxylase introns 6 and 7 (hereafter ODC67; Friesen et al., 1999). Amplification reactions contained approximately 20 ng of genomic DNA, 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, 100 μ M dNTPs, and 0.5 units of Promega Taq DNA Polymerase in a total volume of 25 μ L. After an initial denaturation step of 95°C for 2 min, the PCR proceeded with 35 cycles of a 95°C dentauration step for 30 s, an annealing step for 30 s, and a 72°C extension step for 30 s. To facilitate cloning, a final 5-min 72°C extension step was used to add a template-independent dATP onto the 3' ends of the PCR product. Annealing temperatures were 55°C for rho2, 60° C for $\beta a3$, $\beta f7$, and ODC67, and 65° C for TGFB5. PCR amplicons were precipitated by incubation at 37°C with polyethylene glycol (PEG), washed with cold 80% EtOH, and resuspended in diH₂O. Cycle sequencing reactions with forward and reverse PCR primers were carried out using BigDye Terminator chemistry (PE Applied Biosystems) and visualized on an ABI Prism 377 or 3100 DNA Sequencer. Because of length polymorphisms, PCR products that did not sequence well directly or which were needed for phasing were cloned into the PCR 2.1 TOPO TA cloning vector (Invitrogen) and sequenced in both directions using M13 primers. We aligned sequences for each gene using the programs Sequencher (Gene Codes) and CLUSTAL W (Higgins and Sharp, 1988), with minor adjustments made manually.

Haplotype Reconstruction

We identified single nucleotide polymorphisms (SNPs) by eye initially in the directly sequenced products and further validated them using the program PolyPhred (Nickerson et al., 1997; Brumfield et al., 2003). Heterozygous sites were inferred from the presence of two equal-height peaks in the chromatograms that were approximately half the height of the peaks from flanking sites presumed to be homozygous. To obtain phased haplotypes, we used a three-stage method of (1) inferring haplotypes with the program PHASE (Stephens et al., 2001; Stephens and Donnelly, 2003), initial runs were for 10,000 cycles; (2) sequencing cloned amplicons for individuals having uncertain haplotype assignments (<0.95 posterior probability); and 3) incorporating the known haplotypes into the haplotype inference step (stage 1). We iterated this process until all haplotypes were inferred with a posterior probability of at least 0.95.

Intragenic Recombination and Neutrality Tests

To detect intragenic recombination we used the fourgamete test (Hudson and Kaplan, 1985), which is based on the observation that under an infinite sites model, two variable sites at a locus can produce a maximum of four gametes if recombination between the sites occurs. Even though we used finite sites models for the maximumlikelihood and Bayesian phylogenetic analyses described herein, the dimorphic sequence variation at all polymorphic sites within Manacus is consistent with an infinite sites mutation model. Using the implementation of the four-gamete test in the program SITES (Hey and Wakeley, 1997), we estimated the minimum number of recombination events within each gene and retained for analysis the independently sorting fragment containing the largest number of variable sites. The smaller independently sorting fragments were not analyzed further. Although we tested for and failed to reject neutrality within each species using Tajima's D, we note that the relatively low number of variable sites in the loci greatly

reduced the statistical power of this test and therefore do not discuss this further.

Congruence between Gene Trees and Species Designations

For each locus, we reconstructed unrooted maximumlikelihood (ML) gene trees of *Manacus* haplotypes using the best-fit finite sites substitution model based on AIC tests performed using ModelTest (Online Table 2; Posada and Crandall, 1998). The AIC tests evaluated likelihood scores from a neighbor-joining tree (Saitou and Nei, 1987) reconstructed for each locus from the LogDet distance matrix using PAUP* (Swofford, 2003). Heuristic tree searches were performed using PAUP* (addseq = random, nreps = 10, TBR branch-swapping, maxtrees = 1000), and trees rooted along the midpoint of the longest branch. We performed both an unconstrained search and a constrained search in which haplotypes from each of the five OTUs were constrained to cluster together. Statistically significant ($P \leq 0.05$) incongruence between optimal gene trees and gene trees constrained to be monophyletic within species was assessed with 10,000 resampling estimated log-likelihood (RELL) bootstrap replicates in a Shimodaira-Hasegawa (SH; Shimodaira

TABLE 2. Deep coalescence scores for the 15 unrooted species trees (upper panel) calculated from (A) each of 5 maximum likelihood gene trees and (B) 5000 Bayesian trees for each gene. In the lower panel, scores for the seven alternate rootings of the two best species trees (trees 1 and 3) calculated from a rooted maximum likelihoood gene tree and 1000 rooted Bayesian gene trees. The best scores for each dataset are presented in bold.

	(A) Maximum likelihood					(B) Bayesian						
Unrooted species trees ^a		β f7	rho2	ODC67	TGFB5	Total score	βa3	β f7	rho2	ODC67	TGFB5	Total score
1. (aur,(can,(manE,(manW,vit))))	12	4	4	7	4	31	25	20	39	33	41	158
2. (aur,((can,manE),(manW,vit)))	13	7	3	7	4	34	31	26	37	30	38	162
3. (aur,((can,(manW,vit)),manE))	13	6	4	6	4	33	27	25	41	25	35	153
4. (aur,(can,((manE,manW),vit)))	13	6	5	8	4	36	29	24	39	33	42	167
5. (aur,(can,((manE,vit),manW)))	13	6	5	8	4	36	30	24	42	32	41	169
6. (aur,(((can,manE),manW),vit))	14	10	3	8	4	39	35	32	43	30	40	180
7. (aur,(((can,manE),vit),manW))	14	10	3	8	4	39	37	32	40	32	41	182
8. (aur,(((can,vit),manW),manE))	13	8	5	6	3	35	28	28	43	27	38	164
9. (aur,(((can,manW),vit),manE))	14	8	5	6	4	37	30	28	44	25	39	166
10. (aur,((can,manW),(manE,vit)))	15	8	5	7	4	39	33	31	43	27	40	174
11. (aur,((can,(manE,vit)),manW))	15	9	4	8	4	40	36	33	41	34	40	184
12. (aur,((can,vit),(manE,manW)))	14	8	5	7	3	37	31	30	42	33	38	174
13. (aur,((can,(manE,manW)),vit))	15	9	4	8	4	40	32	32	44	30	39	177
14. (aur,(((can,vit),manE),manW))	14	9	4	7	3	37	34	32	39	34	41	180
15. (aur,(((can,manW),manE),vit))	15	9	4	7	4	39	34	33	43	25	39	174
Rooted species trees												
Tree 1 (see above panel)												
1.1 (aur,(((manŴ,vit),manE),can))	16	16	4	12	11	59	31	33	47	33	47	191
1.2 (can,(((manW,vit),manE),aur))	14	15	6	13	11	59	35	33	48	42	50	208
1.3 (((manE,(manW,vit)),(can,aur))	13	11	6	10	10	50	35	29	47	40	47	198
1.4 (manE,((can,aur),(vit,manW)))	14	8	8	8	10	48	34	26	50	34	50	194
1.5 (((manE,(can,aur)),(vit,manW))	16	7	7	9	7	46	36	24	42	38	49	189
1.6 (manW,(vit,(manE,(can,aur))))	21	8	10	11	7	57	38	26	41	40	52	197
1.7 (vit,(manW,(manE,(can,aur))))	21	7	10	10	5	53	42	24	42	41	46	195
Tree 3 (see above panel)												
3.1 (aur.(manE.(can.(manW.vit))))	17	11	4	8	6	46	33	34	49	27	49	192
3.2 ((can.(manW.vit)).(manE.aur))	15	11	6	8	7	47	36	32	53	29	53	203
3.3 (can.((manE.aur).(manW.vit)))	17	13	8	11	8	57	37	35	49	38	50	209
3.4 ((can,(manE,aur)),(manW,vit))	17	11	7	10	9	54	38	29	47	37	50	201
3.5 (manW.((can.(manE.aur)).vit))		14	10	13	11	70	40	30	46	37	54	207
3.6 (vit,(((manE,aur),can),manW))	23	14	10	12	11	70	44	29	47	38	50	208
3.7 (manE,(((manW,vit),can),aur))	16	8	6	11	7	48	34	30	57	27	54	202

^{*a*} Species name abbreviations: aur = M. aurantiacus; can = M. candei; manW = M. manacus (w); manE = M. manacus (e).

and Hasegawa, 1999) test between most likely trees from the unconstrained and constrained searches.

Species Tree Reconstruction

We explored two analytical methods for reconstructing the species tree from the five incompletely sorted gene trees.

Deep coalescence.—This method was evaluated in two ways: by using the ML point estimate of each gene tree and by using Bayesian methods to sample multiple gene trees from the posterior distribution. In both cases, we used the coalesence module in the program Mesquite (Maddison, 2005; Maddison and Maddison, 2005) to calculate the number of deep coalescence steps for each gene tree (either the ML point estimates or a group of Bayesian trees) on each of the 15 possible unrooted species trees. The genealogies used to calculate deep coalescence steps on the species trees were either the ML gene trees estimated above or a set of 5000 gene trees generated by Bayesian analyses performed individually on each of the five genes using the program MrBayes (version 3.1.2; Huelsenbeck and Ronquist, 2001). When making gene trees, uniform interval priors were assumed for the parameters, except for base frequencies, which were assigned a Dirichlet prior (Huelsenbeck and Ronquist, 2001). The finite sites substitution model parameters used were the same as in the ML analysis (online Table 2). Four heated chains were run for 5.0×10^6 generations and sampled every 1000. This run length produced average deviation of split frequencies less than 0.01. Three independent runs with different random seeds were performed to ensure the posterior probabilities were stable. On the ML tree plus each of the final 5000 trees saved in the Bayesian analysis of each gene, the number of deep coalescence steps on each of the 15 possible unrooted species trees was measured. Deep coalescence scores for each species tree were calculated by adding the deep coalescence steps for each of the genes (Maddison, 1997).

To root the two best species trees, we performed ML and Bayesian analyses as described above except that we included the seven outgroup taxa, constrained *Manacus* to be monophyletic, and constrained interrelationships among outgroup taxa to those supported by a Bayesian analysis of the five concatenated loci. We constrained the outgroup interrelationships so that differences in deep coalescence scores among genealogies would reflect differences in the outgroup rooting and the interrelationships of ingroup gene sequences and not the interrelationships of outgroup taxa. Using the ML gene tree and a sample of 1000 genealogies from the posterior distribution of each gene, we calculated the deep coalescence steps on the seven possible rootings of the two best species trees.

Bayesian estimation of species trees (BEST).—Liu and Pearl (2007) developed a hierarchical Bayesian method for estimating the species tree from the DNA sequences of multiple, unlinked loci. The multi-allele method used here (BEST version 1.6; Liu et al., 2008) is an extension of the single allele per OTU method presented elsewhere (Liu and Pearl, 2007; Edwards et al., 2007). The model considers the fact that all gene trees share some dependence through a common species tree, thus it is appropriate to estimate the gene trees jointly across multiple loci.

As in Liu et al. (2008), we searched for gene trees using a rough approximation of the species tree as discussed in Liu and Pearl (2007), changing both the topology and branch lengths of this initial prior species tree 1000 times in each MCMC cycle to explore a larger space of gene trees; we used a total of 20,000,000 cycles for estimating gene trees, with the first 10,000,000 steps discarded as burn-in. Stationarity of the posterior distribution was determined by visual inspection of the likelihood scores. A gamma distribution was used as a prior for theta at each node, with $\alpha = 1$ and $\beta = 200$. All gene trees were first estimated without a clock and then updated to an ultrametric tree using a simple heuristic (Liu and Pearl 2007). A second Markov chain (BEST part 2) was used to sample the posterior distribution of species trees while evaluating the likelihood of gene tree vectors from part 1, using a birth-death process as a species tree prior (Nee et al., 1994; Nee, 2006), with 200,000 MCMC cycles, discarding the first 100,000 as burn-in. Importance sampling is used in the final step to correct for having used an approximate species tree prior in step 1 (Liu and Pearl, 2007). As per constraints of the BEST software, a single outgroup sequence (Chiroxiphia pareola) was used in the analysis.

Divergence Population Genetics

The above phylogenetic methods assume that migration/introgression since species divergence has not affected the genealogies, but the speciational history of Manacus manakins may have not followed a strict isolation model. We explored this issue by estimating and comparing the divergence time of vitellinus and manacus (w) versus the divergence time of manacus (w) and manacus (e) using an "isolation with migration" model for multilocus data (Hey and Nielsen, 2004). We ran the program a sufficient number of steps so that that the effective sample sizes (ESSs), the program's measure of autocorrelation during the run, were all at least 45 for each parameter. Data were recorded every 100 steps of the chain. The shortest run was for 1.0×10^7 steps and the longest for 1.0×10^8 steps, with the first 5.0×10^5 steps of all runs discarded as burn-in. Using the ML estimate from each run we performed likelihood-ratio tests between two nested models to identify the best-fit IM model that had the fewest number of parameters. Model I had only three estimated parameters, with the population size parameters θ_A , θ_1 , and θ_2 constrained to have the same value and an assumption of symmetric migration rates ($m_{1\rightarrow 2} = m_{2\rightarrow 1}$). In the more parameterrich Model II, these constraints were relaxed. The best-fit model, which was Model I in all cases, was then used in the full IM analysis (online Table 3).

To convert the divergence parameter t to an absolute time estimate in years (τ) we assumed a neutral mutation

TABLE 3. Maximum likelihood estimates (MLEs) and the 90% highest posterior density (HPD) confidence intervals of demographic parameters, inferred from the largest independently sorting gene region of each gene. The geometric mean of the mutation rates was 1.2×10^{-6} , except for the *M. vitellinus* × *M. manacus* (w) comparison, in which the rate was 9.9×10^{-7} . The divergence time parameter estimate for the *M. vitellinus* × *M. manacus* (w) analysis should be considered with caution, because the posterior distribution was flat.

Comparison		θ_1	θ_2	θ_A	m/μ	$t\mu$	N_1	N_2	N_A	4Nm	τ (years)
$M.$ vitellinus $\times M.$ manacus (w)	MLE	0.391	0.391	0.391	90.1	1.05	11,896	11,896	11,896	35.249	1.1×10^{6}
	Lower 90%	0.140	0.140	0.140	23.4	0.99	4,259	4,259	4,259	3.283	1.0×10^{6}
	Upper 90%	0.778	0.778	0.778	99.9	19.99	23,670	23,670	23,670	77.761	2.0×10^{7}
$M.$ manacus (w) \times $M.$ manacus (e)	MLE	0.739	0.739	0.739	0.85	0.55	18,549	18,549	18,549	0.632	4.6×10^{5}
	Lower 90%	0.427	0.427	0.388	0.18	0.23	10,718	10,718	9,739	0.079	1.9×10^{5}
	Upper 90%	1.191	1.191	1.242	2.25	4.99	29,895	29,895	31,175	2.686	4.2×10^{6}
Amazonia × Atlantic Forest	MLE	0.682	0.682	0.682	6.45	0.20	17,118	1,7118	1,7118	4.40	1.7×10^{5}
	Lower 90%	0.387	0.387	0.370	1.83	0.05	9,714	9,714	9,287	0.35	4.2×10^{4}
	Upper 90%	1.210	1.210	1.238	9.89	9.94	30,371	30,371	31,074	11.97	8.3×10^{6}
N. Amazon River × S. Amazon River	MLE	0.232	0.232	0.232	1.61	0.27	5,823	5,823	5,823	0.37	2.2×10^{5}
	Lower 90%	0.077	0.077	0.048	0.54	0.10	1,933	1,933	1,205	0.04	8.4×10^{4}
	Upper 90%	0.661	0.661	0.771	4.99	2.98	16,591	16,591	19,352	3.30	2.5×10^{6}
W. Negro River \times E. Negro River	MLE	0.469	0.469	0.469	2.22	0.20	11,772	11,772	11,772	1.043	1.7×10^{5}
	Lower 90%	0.200	0.200	0.188	0.04	0.02	5,020	5,020	4,719	0.009	1.7×10^{4}
	Upper 90%	1.122	1.122	1.122	9.99	1.61	28,163	28,163	28,163	11.214	1.3×10^{6}
$M.$ candei $\times M.$ vitellinus	MLE	0.186	0.186	0.172	1.84	18.52	4,669	4,669	4,317	0.342	1.5×10^{7}
	Lower 90%	0.060	0.060	0.045	0.56	3.42	1,506	1,506	1,130	0.034	2.8×10^{6}
	Upper 90%	0.089	0.089	0.459	5.97	49.92	9,764	9,764	11,521	2.322	4.2×10^{7}
$M.$ candei $\times M.$ aurantiacus	MLE	0.089	0.089	0.089	1.01	5.04	2,234	2,234	2,234	0.089	4.2×10^{6}
	Lower 90%	0.024	0.024	0.019	0.28	1.52	602	602	477	0.007	1.3×10^{6}
	Upper 90%	0.256	0.256	0.321	3.66	9.99	6,426	6,426	8057	0.937	8.3×10^{6}

rate for noncoding avian DNA of 3.6×10^{-9} substitutions/site/year (Axelsson et al., 2005). To make estimates of the effective population size (N_e) , we assumed a generation time of 8.3 years, the mean of generation time estimates (4.9 and 11.7 years for females and males, respectively) from a long-term study of the piprid Chiroxiphia *linearis* (McDonald, 1993). In addition to testing the sister relationship between *M. vitellinus* and *M. manacus* (w), we also estimated divergence times between M. manacus populations separated by three major biogeographic barriers in South America. These included a comparison between populations from opposite banks of the Amazon River, a comparison between populations from opposite banks of the Negro River, and a comparison between Amazonian populations and those that occur disjunctly in the humid forests of southeastern Brazil.

RESULTS

Genetic Variation

From each individual, we sequenced a total of 2616 bp, representing gene fragments of 315 bp (rho2), 361 bp (TGFB5), 377 bp (β a3), 589 bp (ODC67), and 974 bp (β f7) (TreeBASE submission SN3806; GenBank accession numbers EU522490 to EU522624). Considering all genes, Hudson's four-gamete test detected eight recombination events, ranging from zero in β a3 and rho2 to four in β f7. The recombination-free regions analyzed from each locus ranged in length from 114 bp (TGFB5) to 635 bp (β f7) (Table 1). One β f7 haplotype (B9771b) had an unusually high number of singleton mutations (online Fig. 1); its inclusion or exclusion changed the detection rate at β f7 from one variable site every 48.8 bp to one every 90.7 bp. All of the variable sites in this individual were confirmed in the laboratory by cloning, and we were able to reject

the possibility of a paralogous copy. Because this individual (B9771) did not have a higher number of variable sites at the other genes sequenced, sample degradation seems an unlikely cause for the increased variation and it was retained in all analyses. Averaging over all loci, the %GC content was low (43.9%), but three genes (β a3, rho2, TGFB5) had %GC over 50%.

Congruence between Gene Trees and the Species Tree

For each gene we performed likelihood ratio tests between the ML network from the unconstrained search (Fig. 2) and a constrained network in which each of the five *Manacus* OTUs was constrained to cluster together (online Table 4). We detected significant (P < 0.05) differences between the unconstrained and constrained networks for three (β a3, β f7, ODC67) of the five genes.

Species Tree Reconstruction

Deep coalescence.—The unrooted species tree with the lowest deep coalescence scores differed between the ML (tree 1: 31 steps) and Bayesian (tree 3: 153 steps) gene tree sets (Table 2; Fig. 3a). The large difference in the number of steps between species trees based on either the ML or Bayesian analyses (e.g., 31 versus 153) reflects the fact that the ML gene trees contained many branches of zero length that were collapsed. In contrast, none of the Bayesian gene tree branches were zero length. Because species tree 3 was only two steps longer when evaluated using the ML gene trees, we consider trees 1 and 3 equally parsimonious reconstructions of the species phylogeny.

In both the ML and Bayesian analyses, the most parsimonious deep coalescence rootings of trees 1 and 3 (see rootings 1.5 and 3.1 in Table 2) placed *M. manacus* (w) and *M. vitellinus* as sister taxa (Fig. 3b). The rooted species



FIGURE 2. Maximum-likelihood trees inferred for each gene. Trees are midpoint rooted. Bayesian posterior probabilities presented above branches for values greater than or equal to 0.95.

726





FIGURE 3. Unrooted (a) and rooted (b) deep coalescence species trees and the (c) BEST tree. The source of the gene tree(s) used in the deep coalescence analyses indicated by "ML" (maximum likelihood) and "Bayesian." Posterior probabilities for BEST tree nodes presented above branches. BEST tree rooted using Chiroxiphia.

trees differed in the phylogenetic relationships of candei, aurantiacus, and M. manacus (e) (Fig. 3b). In tree 1.5, aurantiacus and candei occurred as sister species in a clade with *M. manacus* (e), and this clade was sister to the *M*. vitellinus/M. manacus (w) clade. In tree 3.1, M. manacus (e) was sister to the M. vitellinus/M. manacus (w) clade, and candei sister to this clade, and aurantiacus occurring as a basal branch.

BEST.—Both M. vitellinus and M. manacus (w) and M. candei and M. auranticaus were found as sister taxa (Fig. 3c). BEST posterior probabilities for these relationships were 1.0 (*M. vitellinus* and *M. manacus* (w)) and 0.95 (*M.* candei and M. auranticaus). M. manacus (e) appeared as sister to the (M. vitellinus/M. manacus (w)) clade but with weaker support (posterior probability 0.93). Monophyly of Manacus was strongly supported (posterior probability 1.0). The BEST tree was identical to the unrooted ML tree (Fig. 3a) but differed from the rooted ML tree in the placement of M. manacus (e).

Divergence Population Genetics

Test of sister relationship between M. vitellinus and M. manacus (w).—The IM test of the sister relationship between M. vitellinus and M. manacus (w) produced equivocal results. The posterior distribution of the divergence time parameter (μt) remained flat (Fig. 4a) despite multiple runs using different starting parameters and run lengths. Although the divergence time estimates (Table 3) indicated an earlier divergence time of *M. vitellinus* and M. manacus (w) than of M. manacus (w) and M. manacus (e)—a result that would suggest the sister relationship of M. vitellinus and M. manacus (w) is incorrect—the posterior distribution suggests that the opposite could be true (Fig. 4). The earliest divergence times sampled during the run were between M. vitellinus and M. manacus (w) instead of between M. vitellinus and M. manacus (e). We conclude that because the posterior distribution is flat, essentially any value is possible for *M. vitellinus* and *M.* manacus (w), from a very recent to a very old divergence.

ML

vit

aur

manw

can tree 1 - 31 steps

mane

c) BEST



FIGURE 4. Likelihood plots of divergence time (a) and symmetrical migration rate (b) from two IM analyses: (1) *vitellinus* and *manacus* (w), and (2) *manacus* (w) and *manacus* (e).

With regard to migration, the analysis indicated a substantial level (4Nm = 35) of ongoing gene flow between *M. vitellinus* and *M. manacus* (w), with essentially none (4Nm = 0.6) occurring between *M. manacus* (w) and *M. manacus* (e). The posterior distribution of the migration rate in the *M. vitellinus* and *M. manacus* (w) comparison was rising (Fig. 4), suggesting that, if anything, the rate may have been underestimated. To provide a context for the IM estimates above, we estimated migration rates between two species known to hybridize extensively where their distributions meet (*M. candei* and *M. vitellinus*) and two allopatric species (*M. candei* and *M. aurantiacus*). In both cases, the estimates of 4Nm (0.3 and 0.0, respectively) were consistent with a low level and lack of ongoing gene flow, respectively.

Divergence time estimates across biogeographic barriers.— The posterior distributions of parameters in the three biogeographic comparisons across the Amazon River, across the Negro River, and between Amazonia and the Atlantic coastal rain forests were all unimodal. The analyses suggest that populations on opposite banks of the Amazon River diverged 0.22 million years ago (Ma), populations on opposite banks of the Negro River diverged 0.17 Ma, and populations isolated between Amazonia and the Atlantic coastal rain forest diverged 0.17 Ma.

DISCUSSION

None of the five nuclear loci examined in this study were completely sorted with regard to the five *Manacus* OTUs, but both Bayesian and parsimony species tree inference methods supported sister relationships between *M. manacus* (w) and *M. vitellinus* and between *M. candei* and *M. aurantiacus*. These results corroborate the same sister relationships found in a minimum-evolution phylogeny of the genus inferred from allele frequencies at 31 allozyme loci (Brumfield and Braun, 2001). Of the analytical methods we used to reconstruct the species tree, we consider the Bayesian species tree method (Liu

and Pearl, 2007) to represent the best estimate of the species tree based on the current data, if only because it provided nodal support values. Two of the three internal nodes in the Bayesian species tree analysis (BEST) were strongly supported (posterior probability \geq 0.95), but additional sampling will be needed to address the relationships of M. manacus (e). It was supported as the sister taxon to the (M. vitellinus, M. manacus (w)) clade with a posterior probability of only 0.93. Thus, although we estimated the Manacus species tree from five nuclear loci and at least two individuals per OTU, a level of sampling that performed well in simulation studies of the deep coalescence approach (Maddison and Knowles, 2006), additional data are clearly needed to resolve all nodes strongly. A recent study (Edwards et al., 2007) that inferred phylogenetic relationships of eight yeast species from 106 loci using the BEST method found that only eight independently segregating loci were sufficient to resolve the species tree with high confidence, but this result is probably specific to the dataset and may also have depended on a single allele being sampled per species, as well as the level of gene tree discordance.

A key question is whether the two Colombian hybrid zones between *M. vitellinus* and *M. manacus* (w) represent zones of secondary intergradation or simply the southern termini of spreading M. vitellinus plumage traits. Unfortunately, neither of the hybrid zones between M. vitellinus and M. manacus (w) in Colombia has been studied in detail, but the zone in northern Colombia appears to represent a broad zone of intergradation (Hellmayr, 1929; Haffer, 1967). Populations of M. vitellinus immediately west of the contact zone, M. v. milleri (Chapman, 1915), have much paler yellow plumage than populations in eastern Panama, consistent with introgression. Few specimens are available from the hybrid zone in western Colombia, but at leks near the town of Guapí we observed males with straw-colored collar plumage that were probably indicative of introgression (R. T. Brumfield and M. J. Braun, personal observation).

To address the possibility that introgression between *M. manacus* (w) and *M. vitellinus* could account for their sister relationship in the phylogenetic analyses, we used an IM analysis to jointly estimate divergence time and migration rate. Support for a sister relationship of *M*. manacus (w) and M. vitellinus would be evidenced by a more recent divergence between them than between M. manacus (w) and M. manacus (e). The results of the analysis with regard to divergence time were equivocal, but the analysis did suggest substantial gene flow between M. manacus (w) and M. vitellinus is occurring. Whether the inferred gene flow confirms the two are conspecific populations exchanging genes freely, supporting Brumfield and Braun's (2001) hypothesis, or, conversely, is obscuring a true sister relationship between *M. mana*cus (w) and M. manacus (e), remains unclear.

Even with new species tree phylogenetic methods, disentangling the effects of hybridization and lineage sorting on a phylogeny of recently diverged species is and will continue to be difficult (Braun and Brumfield, 1998; Holder et al., 2001; Buckley et al., 2006). In this regard it is noteworthy that *M. candei* and *M. vitellinus*, despite hybridizing extensively where their distributions meet in western Panama, were not resolved as sister taxa in our phylogenetic analyses; in fact the trees that clustered them together had some of the highest deep coalescence scores (Table 2). Moreover, the relatively high level of gene flow across the contact zone between M. vitellinus and *M. manacus* (w) (4Nm = 35) contrasts markedly with the lack of gene flow across the hybrid zone between *M*. candei and M. vitellinus (4Nm = 0.342). These results suggest that limited introgression across a hybrid zone may not translate to high migration values in an IM analysis of multi-locus data, and that the relatively high migration rate between hybridizing *M. vitellinus* and *M. manacus* (w) may indicate a greater level of introgression than is suggested by the marked plumage color differences between them.

Divergence of M. manacus across Major Biogeographic Barriers

The divergence times of M. manacus populations on opposite banks of the Amazon River, opposite banks of the Negro River, and of populations isolated between Amazonia and the Atlantic Forest all fell within the late Pleistocene 0.17 to 0.20 Ma (Table 3). Although these time estimates clearly have large errors associated with them (Graur and Martin, 2004; Ho et al., 2005; Ho and Larson, 2006), they are substantially younger than those reported in mitochondrial studies of other South American birds across the same biogeographic barriers. For example, in a phylogeographic study of the piprid Lepidothrix coronta, Cheviron et al. (2005) estimated a divergence time across the Amazon River of 1.4 Ma and across the Negro River of 2.0 Ma. Populations of the furnariid Glyphorynchus spirurus in southeastern Brazil and southeastern Amazonia were estimated to have diverged 1.1 Ma (Marks et al., 2002). Both of these dates are consistent with the advent of the Amazonian river system that occurred with the final uplift of the Andes 2 to 3 Ma (Gregory-Wodzicki, 2000).

Taken at face value, divergence estimates within *M*. manacus suggest the intraspecific differentiation of some avian taxa within South America may have been relatively recent, postdating the origin of major biogeographic barriers in South America such as the Amazon River. This raises the possibility that late Pleistocene fragmentation of the humid secondary forests in which Manacus occurs could explain the differentiation of these populations. The most widely cited model of Neotropical diversification, Haffer's (1969) Pleistocene refugia model of diversification in the Neotropics posited that dry climatic periods associated with glacial cycles fragmented formerly continuous stands of humid forest into isolated refugia. The differentiation has been invoked to explain nearly all biogeographic patterns shared among Neotropical organisms (Vanzolini, 1970; Brown et al., 1974; Prance, 1978; Simpson and Haffer, 1978; Whitmore and Prance, 1987), including the geographic partitioning of taxa on opposite sides of the Andes (Haffer, 1967), opposite sides of the Amazon and its major tributaries (Haffer, 1974), and in populations of Amazonia and southeastern Brazil that are isolated by intervening dry caatinga scrub (Silva, 1995). In recognition of the effect of Milankovitch cycles on climatic fluctuations during the entire Cenozoic (65 Ma to today), the domain of the refugia model was extended back temporally to include Miocene and Pliocene differentiation times (Haffer and Prance, 2001; Haffer, 2002). M. manacus provides molecular evidence from birds that late Pleistocene dry cycles impacted the differentiation of species in humid Neotropical forests, as envisioned in the original Haffer model. Reconstructions of the distributions of Neotropical bird species during the Last Glacial Maximum 21,000 years ago also showed fragmentation that could have resulted in genetic differentiation (Bonaccorso et al., 2006).

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