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*Received 7 February 2000, accepted 20 January 2001.
Associate Editor: J. L. Dickinson*

The Auk 118(3):799–804, 2001

Phylogenetic Utility of Avian Ovomucoid Intron G: A Comparison of Nuclear and Mitochondrial Phylogenies in Galliformes

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ABSTRACT.—A novel nuclear marker, the avian ovomucoid intron G (OVOG) was sequenced from 19 galliform taxa. Results of the phylogenetic analyses using OVOG were compared to those obtained using the mitochondrial cytochrome *b* (cytb) gene to determine the phylogenetic utility of OVOG. OVOG appeared to have strong phylogenetic signal for reconstructing relationships among genera and families, and the only difference between OVOG and cytb was in the placement of the New World quail (Odontophoridae). Genetic distances estimated using OVOG are approximately half of those estimated using cytb, although that relationship was not linear. OVOG exhibited patterns of nucleotide substitution very different from cytb, with OVOG having little base compositional bias, a relatively low transition–transversion ratio, and little among-site rate heterogeneity.

Mitochondrial DNA (mtDNA) sequences are commonly used to estimate vertebrate phylogenies. MtDNA markers evolve rapidly, making investigation among closely related species possible, yet also contain enough slowly evolving sites to resolve deeper relationships. Although mtDNA phylogenies are likely to be correct in many cases (Moore 1995), use of mtDNA sequences can be problematic. MtDNA rarely undergoes recombination (Wolstenholme 1992), so problems due to lineage sorting or introgression cannot be detected. Nuclear pseudogenes of mtDNA sequences can also confound phylogenetic estimation (Sorenson and Quinn 1998). Therefore, it is useful to compare mtDNA phylogenies with nuclear gene phylogenies to control for such problems.

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TABLE 1. Species examined and accession number of sequence data. Accession numbers available from GenBank.

Group	Species[a]	Common name	cytb	OVOG
Cracids	*Crax pauxi*	Helmeted Currassow	AF068190	AF170973
	Ortalis vetula	Plain Chachalaca	L08384	AF170974
New World quail	*Cyrtonyx montezumae*	Montezuma Quail	AF068192	AF170976
	Oreortyx pictus	Mountain Quail	AF252860	AF170977
Guineafowl	*Numida meleagris*	Helmeted Guineafowl	L08383	AF170975
Turkeys	*Meleagris gallopavo*	Wild Turkey	L08381	AF170984
Grouse	*Falcipennis canadensis*	Spruce Grouse	AF170992	AF170986
	Tympanchus phasianellus	Sharp-tailed Grouse	AF068191	AF170985
	Alectoris chukar	Chukar	L08378	AF170987
Partridges	*Alectoris rufa*	Red-legged Partridge	Z48775	AF170988
	Bambusicola thoracica	Chinese Bamboo Partridge	AF028790	AF170978
	Perdix perdix	Gray Partridge	AF028791	AF170982
Pheasants	*Afropavo congensis*	Congo Peafowl	AF13760	AF170991
	Catreus wallichi	Cheer Pheasant	AF028792	AF170980
	Crossoptilon crossoptilon	White-eared Pheasant	AF028794	AF170981
	Gallus gallus	Red Junglefowl	AF028795	AF170979
	Pavo cristatus	Common Peafowl	L08379	AF170990
	Pavo muticus	Green Peafowl	AF013763	AF170989
	Pucrasia macrolopha	Koklass Pheasant	AF028800	AF170983

[a] Samples were obtained from aviaries and zoos that maintain captive-reared stock of legal and documented origin, except for *Falcipennis* (collected by J. Hagelin in Alaska) and *Tympanchus* (collected by H.B. Tordoff in North Dakota).

Nuclear intron sequences appear to evolve more rapidly than nuclear exons due to the relative lack of constraint upon their sequence (Li 1997). That rapid evolution makes nuclear introns attractive candidates for comparison with mtDNA phylogenies. However, relatively few phylogenies using nuclear intron data have been published in birds (but see Prychitko and Moore 1997, 2000; Johnson and Clayton 2000; all of which use β-fibrinogen intron 7).

Ovomucoid is a single-copy gene that acts as a storage protein and protease inhibitor (Laskowski et al. 1987a), and it was one of the earliest genes for which intron and exon structure was determined (Stein et al. 1980). A large set of partial amino acid sequences exists for avian ovomucoids (Laskowski et al. 1987b, 1990; Apostol et al. 1993), providing data that can be used to develop primers for phylogenetic studies of birds. Here we compare the molecular evolution of intron G in domain III of ovomucoid (OVOG) with that of the mitochondrial cytochrome-*b* (cytb) gene in Galliformes. These comparisons indicate that OVOG is useful for avian phylogenetics.

Methods.—OVOG primers were designed by locating conserved amino acid sequences surrounding the intron (using Laskowski et al. 1987b, 1990; Apostol et al. 1993) and back translating from those sequences. Nucleotide data from the chicken ovomucoid (Stein et al. 1980) was used to establish the nucleotides at 3rd codon positions; one site near the 3′ end of each primer was left degenerate to ensure template matching (OVOG Forward: 5′-CAAGACATACGGCAACAARTG-3′; OVOG Reverse: 5′-GGCTTAAAGTGAGAGTCCCRTT-3′).

DNA was extracted as described elsewhere (Kimball et al. 1997), and most samples were identical to those used in our previous research (Kimball et al. 1997, 1999). OVOG was amplified by PCR using standard protocols, and the products cleaned using QIAquick™ PCR Purification columns (Qiagen, Inc., Valencia, California). Sequencing reactions (one-quarter or one-half volume) were performed using Thermosequenase dye, terminator (Amersham Life Science, Piscataway, New Jersey), or BigDye™ Terminator (Perkin-Elmer Applied Biosystems, Forest City, California) chemistry and the primers used for PCR amplification. Sequence reactions were analyzed using an ABI 310 or 377 automated DNA sequencer. Sequence chromatographs were edited manually and assembled into double-stranded contigs. A few ambiguous sites, probably reflecting heterozygosity, were evident, and those sites were coded using the proper IUPAC symbols. Cytb sequences were published or sequenced for this study as described previously (Kimball et al. 1999). Species and sequence accession numbers are in Table 1.

OVOG sequences were aligned using ClustalW (Thompson et al. 1994), and optimized by eye. The aligned data have been submitted to GenBank. Gaps were treated as missing data, recoded as described by Bruns et al. (1992), or alignment positions with gaps were deleted. Unless otherwise stated, all results reported correspond to the gap positions deleted alignment. On the basis of Sibley and Ahlquist (1990) and Kornegay et al. (1993), phylogenetic trees

TABLE 2. Base composition of complete sequences and of the variable sites of OVOG and cytb.

	A	C	G	T
cytb all sites	27.8	34.7	12.3	25.2
cytb variable	29.0	47.8	5.7	17.5
OVOG all sites	22.2	25.4	22.2	30.2
OVOG variable	25.9	26.2	22.5	25.4

were rooted to the Cracidae (*Ortalis vetula* and *Crax pauxi*).

Analyses were performed using PAUP* 4.0b4a (Swofford 1999), unless otherwise noted. The most parsimonious trees were identified using equally weighted parsimony with 100 random addition sequence replicates. The incongruence length difference test was conducted using 1,000 replicates and heuristic searches with 10 random addition sequence replicates.

Neighbor joining of maximum likelihood distances were also conducted. Parameters (the transition-transversion [s/v] ratio or substitution model rate matrix, the proportion of invariant sites [inv], and the shape parameter [α] of a Γ-distribution) were estimated using MODELTEST 3.04 (Posada and Crandall 1998). The best fitting models, based upon the hierarchical likelihood ratio test implemented in MODELTEST, were used. Synonymous and nonsynonymous p-distances were calculated using MEGA 1.02 (Kumar et al. 1993).

Reliability of specific groupings in parsimony and distance analyses was assessed using 1,000 bootstrap replicates. For parsimony analyses, 10 random addition sequence replicates were used per bootstrap replicate. Clades with <50% bootstrap support were collapsed.

Results and discussion.—The OVOG alignment was quite variable, containing 505 sites, of which 182 (36%) were variable and 112 (22%) were parsimony informative. After removal of gaps, 388 sites remained, with 151 (39%) variable and 97 (25%) parsimony informative. A total of 126 equally parsimonious trees requiring 209 steps were found.

For OVOG, the best fitting model was HKY+Γ, with a slight transition bias (s/v ratio = 2.33) and relatively weak among-site rate heterogeneity (α = 1.18). The biological significance of the observed among-site rate variation is unclear, because few sites in intron sequences are expected to be constrained. Although absolute conservation of the 5′ and 3′ splice sites for OVOG (see Li 1997 for description) was observed, exclusion of the conserved nucleotides near the 5′ and 3′ splice sites did not alter the model selected (it remained HKY+Γ) and had little affect upon the shape parameter (α = 1.23). Thus, OVOG exhibits currently unappreciated sources of among-site rate variation.

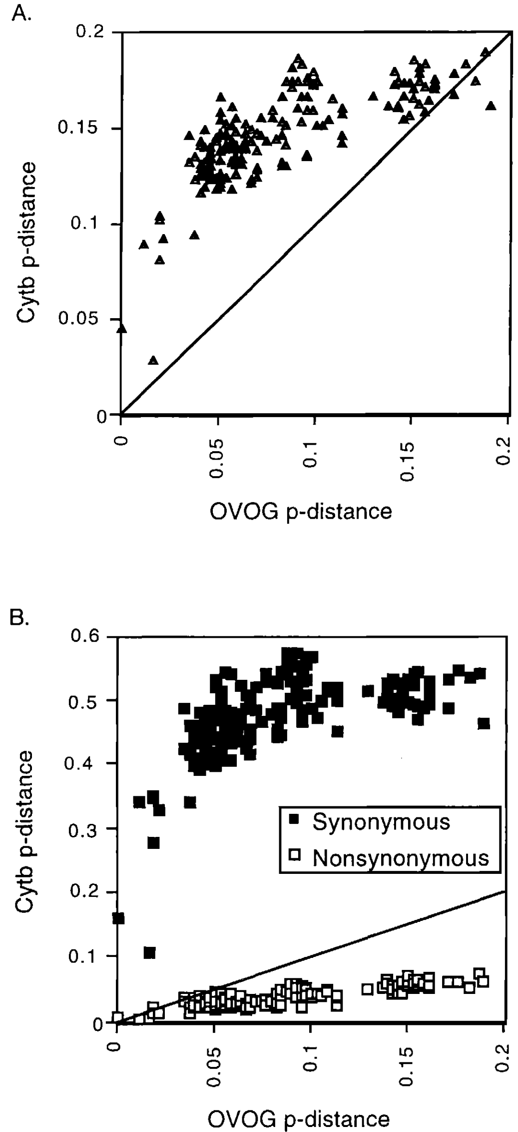

FIG. 1. Sequence divergence between OVOG and cytb. (A) OVOG p-distance compared to total cytb p-distance. (B) OVOG p-distance compared to cytb synonymous (filled squares) and nonsynonymous (open squares) p-distances. The diagonal line represents the expectation if the accumulation of substitutions were identical in OVOG and cytb.

The cytb alignment contained 1,143 sites with no insertions or deletions (see Kimball et al. 1999). As reported previously (Kornegay et al. 1993, Randi 1996, Kimball et al. 1997, 1999), cytb is highly variable, with 473 variable (41%) and 375 parsimony informative sites (33%). A single most parsimonious tree requiring 1,536 steps was identified.

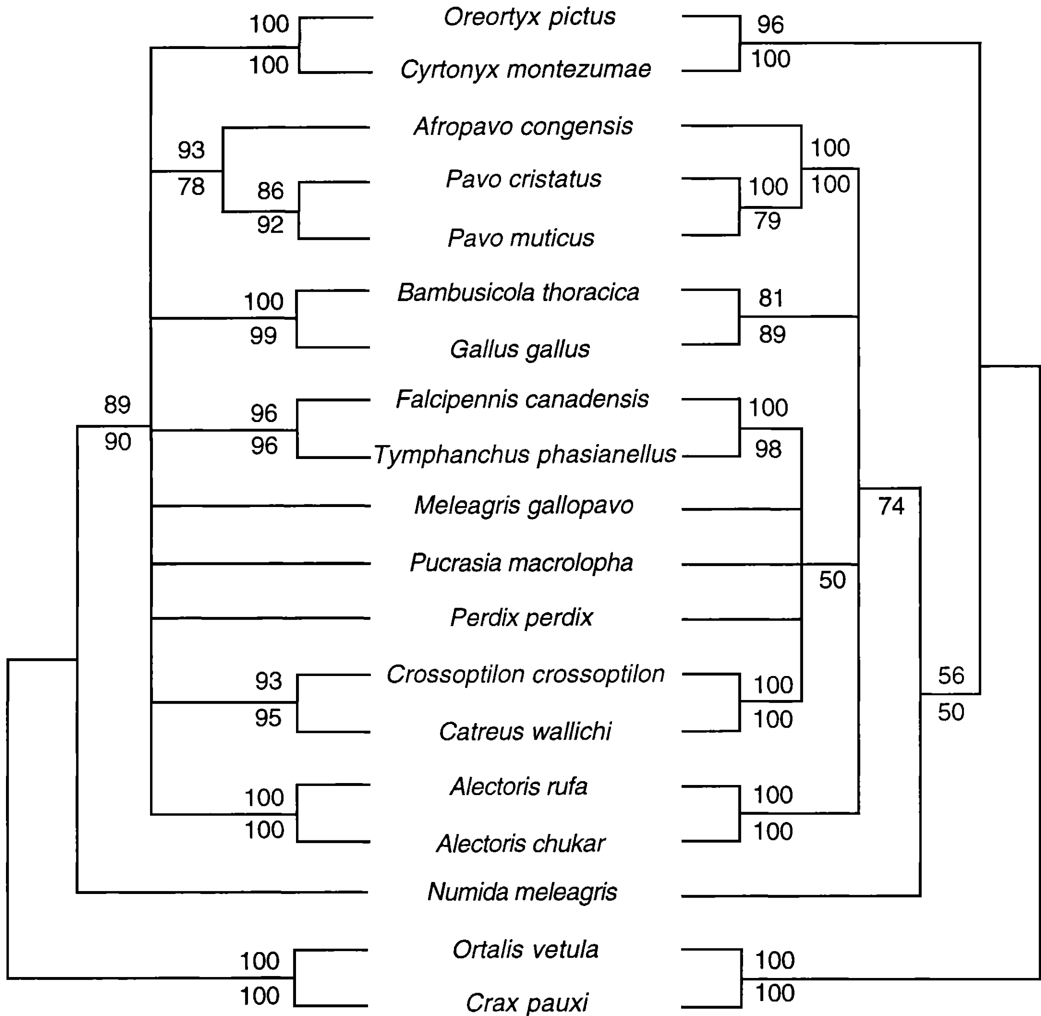

FIG. 2. Bootstrap consensus trees of OVOG (left) and cytb (right). Values represent percentage of 1,000 bootstrap replicates for equally-weighted parsimony (above) and neighbor-joining of maximum likelihood distances (below). Branches were collapsed when both bootstrap values were <50%.

In contrast to OVOG, cytb is characterized by a more complex model of evolution (also see Kimball et al. 1999), exhibiting the best fit to the GTR+Γ+inv model. More than half of cytb sites appear to be invariant (inv = 0.51), and the remaining sites exhibit moderate among-site rate variation (α = 0.73). As expected, there is a bias toward transition substitutions, with the rate parameter for the most slowly accumulating (A–G) transitions being 3.8× greater than that of the most rapidly accumulating (A–T) transversions. There was also more than two-fold variation in rates among different classes of transitions and transversions (data not shown).

The base composition of cytb is highly biased (Table 2), which can lower the amount of detectable variation in an alignment (e.g. Meyer 1994). More interestingly, the base composition of cytb variable sites differed from that of all cytb sites (Table 2). In contrast, the base composition of the OVOG alignment, after removing sites aligned with gap positions, was similar to an equal base composition. Likewise, there was little difference between the variable sites and all sites in OVOG (Table 2).

Genetic distances in OVOG comparisons were about 50% that of cytb, but that relationship is not linear (Fig. 1a). Between closely related taxa, the degree of sequence divergence in cytb increases more rapidly than OVOG as cytb accumulates synonymous mutations; between more distant taxa, sequence divergence continues to increase in OVOG,

but cytb sequence divergence slows as synonymous mutations appear to reach saturation and only nonsynonymous substitutions continue to accumulate (Fig. 1b). Thus, the evolution of OVOG is characterized by accumulation of mutations in a manner substantially more linear relative to time than cytb. A comparison of the β-fibrinogen intron 7 (Prychitko and Moore 1997, 2000; Johnson and Clayton 2000) suggests that, relative to cytb, rate of sequence divergence in OVOG is at least twice that of the β-fibrinogen intron, assuming similar rates of cytb evolution in galliforms, woodpeckers (Picidae), and doves (Columbiformes).

Trees constructed using OVOG were compared to those constructed using cytb (Fig. 2). OVOG and cytb represented congruent data partitions on the basis of the incongruence length difference test ($P = 0.945$), suggesting both partitions support similar estimates of galliform phylogeny. However, the consistency indices (CI) excluding uninformative characters were substantially higher for OVOG (CI = 0.780) than for cytb (CI = 0.408).

Gaps (insertions or deletions) in OVOG provided modest phylogenetic information, with most parsimony informative gaps uniting groups that were clearly monophyletic on the basis of other analyses. We found that gap treatment had little effect on topology or levels of bootstrap support (data not shown).

All nodes well supported in cytb analyses (those with >70% bootstrap; see Hillis and Bull 1993) were also well supported in OVOG analyses (Fig. 2). OVOG strongly supported a close relationship between *Afropavo* and *Pavo* (Kimball et al. 1997) and between *Gallus* and *Bambusicola* (Kimball et al. 1999). However, our analysis could not support the relationship between *Meleagris* and grouse (*Falcipennis* and *Tympanchus*) suggested by Sibley and Ahlquist (1990) but not yet confirmed using sequence data (e.g. Mindell et al. 1997, Kimball et al. 1999).

Relative to cytb, one additional well-supported node was evident in analyses using OVOG (Fig. 2). That node involved the New World quail (*Cyrtonyx* and *Oreortyx*), which have been difficult to place within the galliforms (Crowe 1988, Kornegay et al. 1993). Analyses of morphological data (reviewed by Crowe 1988) and OVOG (Fig. 2) place the New World quail in a derived position relative to the guineafowl, such as *Numida*. However, DNA–DNA hybridization place the New World quail in a basal position (Sibley and Ahlquist 1990), and the New World quail are currently placed in their own family (Odontophoridae) distinct from the other phasianids (which include the guineafowl; American Ornithologists' Union 1997).

Deep coalescence at the OVOG locus is unlikely to explain the differences between estimates of phylogeny using this marker and those obtained using cytb or DNA–DNA hybridization. Inspection of phylograms from Sibley and Ahlquist (1990), Kornegay et al. (1993), Kimball et al. (1999), and OVOG (data not shown) coupled with consideration of the galliform fossil record (reviewed by Kornegay et al. 1993, Kimball et al. 1997) suggest that the internodes between the relevant groups are too long to be attributed to intraspecific coalescence times. A complete understanding of the relationship between the New World quail and other galliforms may require data from additional nuclear loci.

Overall, OVOG appears to be a reliable marker for phylogenetic studies. It provided novel information that may change previous conclusions regarding placement of the New World quail, and further emphasizes the value of examining multiple markers, including both nuclear and mitochondrial, in avian phylogenetics.

Acknowledgments.—We thank L. Bean and J. Parkhani for laboratory assistance. Additional samples were generously provided by J. Hagelin and H. B. Tordoff. Much of this work was conducted in the National Science Foundation-supported (RIMI program grant HRD-9550649) Molecular Biology Facility at the University of New Mexico.

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Received 17 April 2000, accepted 23 January 2001.
Associate Editor: F. Sheldon