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### Malate Dehydrogenase Isozymes Provide a Phylogenetic Marker for the Piciformes (Woodpeckers and Allies)

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Nuclear genes encode two major forms of malate dehydrogenase (MDH; E.C. 1.1.1.37) in birds: S-MDH (or MDH-1), which is found in the soluble fraction of the cell cytoplasm; and M-MDH (or MDH-2), which is housed in mitochondria (Karig and Wilson 1971). Under standard starch-gel electrophoretic conditions, S-MDH typically migrates toward the anode while M-MDH migrates cathodally. In terms of general zymogram appearance and electromorph frequencies across avian taxa, both S-MDH and M-MDH are very conservative evolutionarily (Kitto and Wilson 1966, Aquadro and Avise 1982, Kuroda et al. 1982). In particular, S-MDH assayed in pH 7.0 gels exhibited the same common electromorph in representatives of 22 avian orders (Kitto and Wilson 1966). Although additional S-MDH variation was detected under the multiple electrophoretic conditions employed by Aquadro and Avise (1982), S-MDH remains extremely conservative in comparison with most other enzyme systems.

Kitto and Wilson (1966) first noticed an unusual S-MDH zymogram pattern in three species of Piciformes. Unlike the typical avian S-MDH pattern, which consists of an intense major band with one or two much lighter anodic subbands, the Piciformes were "exceptional in having two S-MDH subbands

of approximately equal intensity" (Kitto and Wilson 1966). This distinctive S-MDH pattern was subsequently observed in a Japanese woodpecker (Kuroda et al. 1982: fig. 3) and in three species of North American woodpeckers (Aquadro and Avise 1982).

Phylogenetic relationships of the woodpeckers and allies have been the subject of intense debate (Simpson and Cracraft 1981, Swierczewski and Raikow 1981, Olson 1983, Raikow and Cracraft 1983, Sibley and Ahlquist 1986). In most traditional taxonomic lists, six families are included in the Piciformes: Galbulidae (jacamars), Bucconidae (puffbirds), Capitonidae (barbets), Indicatoridae (honeyguides), Ramphastidae (toucans), and Picidae (woodpeckers). Recently, this classification has been challenged on the grounds that the assemblage may be polyphyletic (Olson 1983, Sibley and Ahlquist 1986). Raikow and Cracraft (1983), however, defended the validity of the synapomorphs (hypothesized shared-derived traits) used to infer monophyly of the Piciformes. Additional questions concern the relationships of true piciform families to one another. In an effort to help resolve some of these phylogenetic issues, we surveyed members of all the taxa listed above, plus other possible piciform allies, for occurrence of the distinctive and obviously derived S-MDH woodpecker pattern.

Frozen tissue samples (usually heart, liver, or muscle) were homogenized separately in an equivalent volume of grinding solution (0.01 M Tris, 0.001 M EDTA; pH 6.8) and centrifuged at 49,000 g. Electro-

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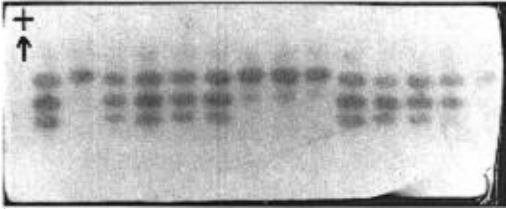


Fig. 1. Representative S-MDH zymogram patterns in various species of "piciform" birds. From left to right: (1) *Melanerpes carolinus* (Picidae); (2) *Galbula ruficauda* (Galbulidae); (3) *Veniliornis nigriceps* (Picidae); (4) *Melanerpes carolinus* (Picidae); (5) *Melanerpes cruentatus* (Picidae); (6) *Picus rivolii* (Picidae); (7) and (8) *Chelidoptera tenebrosa* (Bucconidae); (9) *Monasa nigrifrons* (Bucconidae); (10) *Melanerpes carolinus* (Picidae); (11) *Andigena cucullata* (Ramphastidae); (12) *Pteroglossus castanotis* (Ramphastidae); (13) *Ramphastos culminatus* (Ramphastidae); (14) *Malacoptila semicincta* (Bucconidae). The 3-band S-MDH pattern is evident in lanes 1, 3-6, and 10-13. The typical S-MDH pattern in nonpiciform birds is pictured in fig. 1 of Aquadro and Avise (1982) and is like that in lanes 2, 7-9, and 14 above.

phoresis of supernatant extracts involved horizontal starch gels (12.5%), run at 75 mA for 5-7 h. The following amine-citrate (A-C) buffer system was employed routinely: electrode buffer, 0.04 M citric acid, pH adjusted to 6.1 with N-(3-aminopropyl) morpholine (purchased from Aldrich Chemicals); gel buffer, 1 to 19 dilution of electrode buffer. This system was chosen because, among 12 buffers utilized previously, it provided the best resolution for S-MDH (Aquadro and Avise 1982). Nonetheless, other buffer conditions yielded comparable results. MDH was stained using the recipe of Selander et al. (1971).

The avian material consisted of 29 species that represented 22 of the 74 piciform genera (30%) recognized in most current checklists. In addition, members of Coliiformes and several families of Coraciiformes (possible relatives of the Piciformes) were surveyed (Table 1). The samples originated from three continents: Africa, North America, and South America (Table 1).

*S-MDH zymograms.*—The distinctive "woodpecker-type" S-MDH electrophoresed with the amine-citrate buffer exhibits three major bands (Fig. 1), the two most anodal of which apparently correspond to the "two subband" zymogram pattern originally noticed by Kitto and Wilson (1966). This pattern is readily distinguishable from the usual S-MDH pattern in other birds, which on A-C gels consists of a single intense band, with occasional, much lighter surrounding bands observable upon heavy staining (Fig. 1).

In both the Downy Woodpecker (*Picoides pubescens*) and Northern Flicker (*Colaptes auratus*), we assayed

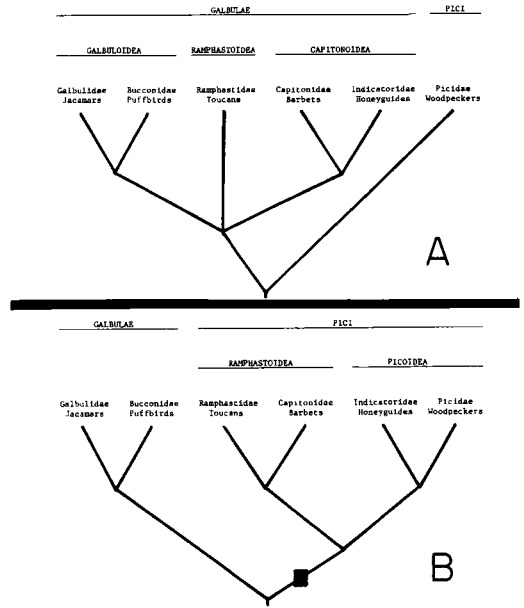


Fig. 2. Two (among several) possible phylogenies for "piciform" birds. (A) Inferred from a traditional classification proposed by Peters (1948). (B) Proposed by Simpson and Cracraft (1981) and independently by Swierczewski and Raikow (1981). The postulated origin of the derived 3-band S-MDH zymogram is indicated by the black rectangle.

pectoral muscle, leg muscle, heart, liver, gizzard wall, intestine wall, eye, and brain. There were no obvious, differential tissue specificities for any S-MDH isozymes (although all bands were darkest in the muscle and heart samples).

In the woodpecker-type S-MDH zymogram, all three bands were invariably present, and the middle band stained most intensely. Thus, the gel pattern gives the impression of "fixed heterozygosity" for S-MDH, which is known to be a dimeric molecule in birds. One plausible explanation is that woodpeckers possess a gene duplication for S-MDH, such that the middle band in the zymogram represents an interlocus heterodimer, flanked by the two respective intralocus homodimeric products. Similar zymogram patterns and assembly of interlocus hybrid molecules are known in some fishes that carry an S-MDH gene duplication (Bailey et al. 1970, Wheat et al. 1971). If the S-MDH gene is also duplicated in woodpeckers, individuals detectably heterozygous at either locus should exhibit a 6-band zymogram (three homo- and three heterodimers). Unfortunately, despite assay of more than 100 piciform specimens (Table 1), we did not detect any heterozygotes. This may not be surprising, however, because S-MDH is essentially monomorphic in most avian species (Aquadro and Avise 1982).

TABLE 1. Woodpeckers and possible nonpasseriform allies assayed for presence vs. absence of the unique S-MDH isozyme pattern.

Taxon	<i>n</i>	Continent of collection	Unique "woodpecker" S-MDH zymogram?
<b>Piciformes</b>			
<b>Picidae (woodpeckers)</b>			
<i>Colaptes auratus</i> <sup>a</sup>	3	North America	Yes
<i>Dryocopus pileatus</i>	1	North America	Yes
<i>Melanerpes carolinus</i>	34	North America	Yes
<i>Melanerpes erythrocephalus</i>	7	North America	Yes
<i>Melanerpes formicivorus</i>	11	North America	Yes
<i>Picoides pubescens</i>	21	North America	Yes
<i>Picoides scalaris</i>	5	North America	Yes
<i>Picoides villosus</i>	5	North America	Yes
<i>Sphyrapicus varius</i>	2	North America	Yes
<i>Melanerpes cruentatus</i>	1	South America	Yes
<i>Piculus rivolii</i>	1	South America	Yes
<i>Veniliornis nigriceps</i>	1	South America	Yes
<i>Veniliornis passerinus</i>	1	South America	Yes
<i>Campeothera abingoni</i>	1	Africa	Yes
<i>Dendropicos fuscescens</i>	1	Africa	Yes
<b>Indicatoridae (honeyguides)</b>			
<i>Indicator minor</i>	2	Africa	Yes
<b>Capitonidae (barbets)</b>			
<i>Lybius torquatus</i> <sup>b</sup>	1	Africa	Yes
<i>Trachyphonus vaillantii</i>	3	Africa	Yes
<i>Tricholaema leucomelas</i>	1	Africa	Yes
<b>Rhamphastidae (toucans)</b>			
<i>Andigena cucullata</i>	1	South America	Yes
<i>Pteroglossus castanotis</i>	1	South America	Yes
<i>Ramphastos culminatus</i>	2	South America	Yes
<i>Ramphastos toco</i> <sup>b</sup>	1	South America	Yes
<b>Bucconidae (puffbirds)</b>			
<i>Chelidoptera tenebrosa</i>	2	South America	No
<i>Malacoptila semicincta</i>	1	South America	No
<i>Monasa nigrifrons</i>	1	South America	No
<i>Nonnula ruficapilla</i>	1	South America	No
<i>Nystalus striolatus</i>	1	South America	No
<b>Galbulidae (jacamars)</b>			
<i>Galbula ruficauda</i>	1	South America	No
<b>Coraciiformes</b>			
<b>Alcedinidae (kingfishers)</b>			
<i>Ceryle alcyon</i>	1	North America	No
<b>Bucerotidae (hornbills)</b>			
<i>Tockus erythrorhynchus</i>	2	Africa	No
<i>Tockus flavirostris</i>	1	Africa	No
<b>Coraciidae (rollers)</b>			
<i>Coracias caudata</i>	1	Africa	No
<b>Momotidae (motmots)</b>			
<i>Momotus momota</i> <sup>a</sup>	1	South America	No
<b>Phoeniculidae (wood-hoopoes)</b>			
<i>Phoeniculus purpureus</i>	2	Africa	No

TABLE 1. Continued.

Taxon	n	Continent of collection	Unique "woodpecker" S-MDH zymogram?
Upupidae (hoopoe)			
<i>Upupa epops</i> <sup>a</sup>	1	Africa	No
Coliiformes			
Coliidae (colies)			
<i>Colius striatus</i>	3	Africa	No
<i>Urocolius indicus</i>	1	Africa	No

<sup>a</sup> One specimen also assayed by Kitto and Wilson (1966).

<sup>b</sup> Assayed by Kitto and Wilson (1966) but not in the present study.

On the other hand, from available data we cannot exclude the possibility that some "epigenetic" phenomenon, such as posttranslational protein modification, might account for the multiplicity of S-MDH bands in woodpeckers. For example, subbands of alcohol dehydrogenase in *Drosophila* result from the binding of a NAD-carbonyl complex to the protein (Everse et al. 1971). Regardless of its particular molecular basis, the unique S-MDH zymogram pattern should provide an informative phylogenetic marker.

*S-MDH and piciform phylogeny.*—The distinctive, 3-band S-MDH zymogram was present in all species of Picidae, Indicatoridae, Capitonidae, and Ramphastidae assayed, but not in the Bucconidae or Galbulidae (Table 1; see examples in Fig. 1). The "woodpecker-like" S-MDH pattern was absent from all six families of Coraciiformes, and from the two representatives of the Coliiformes (Table 1). Together with taxa previously assayed (Table 2), S-MDH has now been surveyed in 26 of the approximately 27 living avian orders, and in 16 families of Passeriformes. Only in a subset of the Piciformes—woodpeckers, honeyguides, barbets, and toucans—has the distinctive 3-band S-MDH pattern been observed.

One traditional and widely recognized classification of Piciformes (by Peters 1948; see also Wetmore 1960) implies the phylogeny shown in Fig. 2A. It is incompatible with the observed taxonomic distribution of the 3-band S-MDH pattern, provided this MDH pattern has not been lost secondarily by the common ancestor of jacamars and puffbirds. The likelihood of such secondary loss is difficult to assess critically. Note that the 3-band S-MDH pattern must be very old, however, because it is present in four families and in both Old and New World Piciformes. Sibley and Ahlquist (1985, 1986) estimated that some of these groups diverged nearly 80 million years ago. The earliest known fossils referable to the true Pici (as defined below) are about 50 million years old (S. L. Olson pers. comm.). In any event, the retention of the unusual S-MDH genotype over such time spans suggests that it may have some adaptive significance.

If true, the probability of secondary loss of the pattern presumably would be low.

Cladistic analyses of skeletal morphology (Simpson and Cracraft 1981) and limb myology (Swierczewski and Raikow 1981) of the Piciformes produced the phylogeny shown in Fig. 2B. The observed distribution of the 3-band S-MDH pattern is supportive of the Pici clade composed of Ramphastidae, Capitonidae, Indicatoridae, and Picidae. Together with results from DNA-DNA hybridizations (Sibley

TABLE 2. Avian taxa (orders and passeriform families) previously assayed for S-MDH isozyme patterns. No assayed representatives of these groups exhibited the S-MDH pattern characteristic of woodpeckers.

Tinamiformes <sup>a</sup>	Passeriformes <sup>d</sup>
Rheiformes <sup>a</sup>	Eurylaimidae <sup>a</sup>
Apterygiiformes <sup>a</sup>	Pipridae <sup>a</sup>
Struthioniformes <sup>a</sup>	Tyrannidae <sup>a</sup>
Gaviiformes <sup>a,b</sup>	Pittidae <sup>a</sup>
Podicipediformes <sup>a</sup>	Hirundinidae <sup>a,b</sup>
Sphenisciformes <sup>a</sup>	Corvidae <sup>a,b</sup>
Procellariiformes <sup>a</sup>	Paridae <sup>b</sup>
Pelicaniformes <sup>a</sup>	Troglodytidae <sup>b</sup>
Ciconiiformes <sup>a</sup>	Muscicapidae <sup>a,b</sup>
Anseriformes <sup>a,b</sup>	Mimidae <sup>a,b</sup>
Falconiformes <sup>a,b</sup>	Bombycillidae <sup>b</sup>
Galliformes <sup>a,b</sup>	Sturnidae <sup>a,b</sup>
Gruiformes <sup>a,b</sup>	Picathartidae <sup>a</sup>
Charadriiformes <sup>a</sup>	Nectariniidae <sup>a</sup>
Columbiformes <sup>a,b</sup>	Emberizidae <sup>a,b</sup>
Psittaciformes <sup>a</sup>	Passeridae <sup>a,b</sup>
Cuculiformes <sup>a,b</sup>	
Strigiformes <sup>a,b</sup>	
Caprimulgiformes <sup>a</sup>	
Apodiformes <sup>a</sup>	
Trogoniformes <sup>a</sup>	

<sup>a</sup> From Kitto and Wilson (1966).

<sup>b</sup> From Aquadro and Avise (1982).

<sup>c</sup> From Kuroda et al. (1982: fig. 3).

<sup>d</sup> Olson (1983) suggested that the closest living relatives of the Pici (as in Fig. 2B) may be found among the Passeriformes.

and Ahlquist 1986), and further analyses of morphology (Olson 1983), the monophyletic status of the Pici now seems well established. Most of the further debate about piciform phylogeny concerns whether the Galbulae and Pici are sister groups (as argued by Raikow and Cracraft 1983), or whether each has closest avian relatives elsewhere (as argued by Olson 1983, and Sibley and Ahlquist 1986).

Wiley (1981) noted that "one true synapomorphy is enough to define a unique genealogical relationship. The problem is that the synapomorphies we hypothesize may or may not be true synapomorphies." Much of the debate about piciform phylogeny has centered on whether a few particular morphological features reflect shared ancestry or convergence. Sibley and Ahlquist (1983) argued that quantitative DNA-DNA hybridization values indicate true cladistic relationships because they are proportional to the relative times of divergence between taxa. They also predicted that qualitative morphological or genetic traits, properly interpreted, "will prove to be congruent with the DNA evidence in all cases" (Sibley and Ahlquist 1986). In this instance at least, the derived S-MDH genotype (as well as certain previously studied morphological traits) are indeed congruent with the DNA-hybridization data in predicting monophyletic status for a Pici assemblage composed of the toucans, barbets, honeyguides, and woodpeckers.

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