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SYSTEMATIC RELATIONSHIPS AMONG SOME ANATINI AS DERIVED FROM RESTRICTION-ENDONUCLEASE ANALYSIS OF A REPEATED DNA COMPONENT

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ABSTRACT.—We recently isolated and cloned a highly repeated retropseudogenelike DNA sequence (*RBMI*) from the genome of the Red-breasted Merganser (*Mergus serrator*). This sequence was used to probe Southern blots of restriction-enzyme-digested DNA from members of the waterfowl tribe Anatini in order to infer phylogenetic relationships. The digested DNA of all the species studied contained major and minor *RBMI*-like components. Some *RBMI*-like components were common to all the species examined, others were only found in groups of species, and still others were specific to individual species. The Mallard (*Anas platyrhynchos*) and the American Black Duck (*A. rubripes*) could be differentiated on the basis of *RBMI*-probed Southern blots. Thus, the *RBMI* sequence can be used to distinguish very closely related species. We suggest that the Anatini consist of three major groups: the mallard-pintail group; the gadwall-wigeon group; and the blue-winged ducks. The Versicolor Teal (*A. versicolor*) had major elements in common with both the blue-winged ducks and the mallard-pintail group. The Baikal Teal (*A. formosa*) and the Marbled Teal (*Marmaronetta angustirostris*) do not appear to be members of any of these three groups. *Received 19 February 1990, accepted 13 January 1992.*

MOLECULAR DATA are used increasingly to study avian phylogenetic relationships. Many of these studies have focused on proteins (Sibley and Ahlquist 1972, Brush 1976, Wilson et al. 1977, Prager and Wilson 1980, Aquadro and Avise 1982, Barrowclough 1983, Evans 1987). Two distinct types of analyses have been done using avian DNA. One compares total unique-copy DNA sequences by heteroduplex melting analysis (Sibley and Ahlquist 1983, 1990). The other approach focuses on restriction-nuclease analysis of mitochondrial DNA (mtDNA; Glaus et al. 1980, Kessler and Avise 1984, Ovenden et al. 1987, Shields and Wilson 1987) and of nuclear-DNA sequences coding for proteins (Helm-Bychowski and Wilson 1986) or minisatellites (Quinn and White 1987). Minisatellite-DNA analysis allows only for the identification of individuals within a species and cannot be used to determine the relationships among species (Jeffreys et al. 1985). Mitochondrial DNA, on the other hand, only provides limited phylogenetic information because the data are confined to a small segment of DNA that is maternally inherited. The low rate of sequence evolution of avian mtDNA may not allow study of closely related species or different populations of one species (Kessler and Avise 1984, Ovenden et al. 1987). Furthermore, although avian blood contains small amounts of mtDNA (Kocher et al. 1989), analytical techniques usually involve isolation and purification from heart or kidney tissue (Lansman et al. 1981) involving sacrifice of the animal. We prefer avian blood as a source of nuclear DNA, as erythrocytes provide a source of genomic DNA without harm to the specimen.

We (McHugh et al. 1990) have isolated, cloned and characterized a highly repeated 2.6-kilobase (Kb) DNA-sequence element (*RBMI*) from the Red-breasted Merganser (*Mergus serrator*). This sequence resembles that of a rearranged retropseudogene. The *RBMI*-like sequences occur in high, but variable, copy number, accounting for up to 10% of the genome in virtually all members of the Anatidae (McHugh, Tuohy, and de Kloet, in prep.) Only the Oxyurini and a number of swans (e.g. *Cygnus buccinator*) contain very little (<0.05%) of this material. In the Anatini, *RBMI*-like material amounts to approximately 1% of individual genomes. *RBMI*-like sequences were found in both clustered and dispersed patterns.

We used restriction-pattern analysis, with respect to *RBMI*, to determine phylogenetic relationships among waterfowl species. We focused on the Anatini (Johnsgard 1978) because information on mitochondrial restriction pat-

terns already exists for this tribe (Kessler and Avise 1984). The rate of nuclear pseudogene evolution has been reported to be intermediate between that of mtDNA and the average rate of nuclear-DNA evolution (Nei 1987). Therefore, phylogenetic conclusions drawn from the use of the *RBMI* sequence should complement those derived from the study of mitochondrial restriction patterns.

METHODS

All DNA samples were obtained from birds maintained at Mergus Waterfowl Farms, Tallahassee, Florida. DNA was isolated from blood as described previously (McHugh et al. 1990). Blood samples of at least three different individuals were mixed before DNA extraction.

The restriction endonucleases *EcoRI, HincII, HindIII, HaeIII, PstI, PvuII,* and *SstI* were obtained commercially (Bethesda Research Laboratories [BRL], Life Technologies, Inc., Gaithersburg, Maryland; International Biotechnology Industries, Inc. [IBI], New Haven, Connecticut) and used according to the manufacturers' directions. Agarose (BRL) gel electrophoresis was carried out in 1% agarose gels in TPE buffer (0.09 M Tris base, 0.02 M phosphoric acid, 0.002 M EDTA; pH 7.8). Southern blotting on modified-charge nylon membranes (Dupont, Wilmington, Delaware) was carried out as described by Maniatis et al. (1982).

The reiterated DNA-component RBMI was cloned as a 2.6-Kb *EcoRI* fragment from *M. serrator* as described previously (McHugh et al. 1990). The excised insert was labelled with α-P^{32}dCTP (3,000 Ci/mmol; Dupont NEN, Boston, Massachusetts) by random-primer extension using a kit (BRL). Unincorporated dCTP was removed by spun-column chromatography (Maniatis et al. 1982). Hybridization of the filters with the labelled probe was carried out for 15 h at 65°C using the heparin procedure as described by Singh and Jones (1984). Following hybridization, filters were washed in 1 × SSC (0.15 M NaCl, 0.015 M Na_3-citrate; pH 7.6) five times at room temperature, followed by a single 15-min wash at 65°C. Autoradiographs were made on Kodak XAR-5 film with an intensifying screen (Dupont Cronex Lightning-plus AD) at −70°C over a range of exposure times and, subsequently, were scanned on a Beckman DU8 densitometer (Beckman Instruments, Carlsbad, California).

RESULTS

RBMI-like fragment patterns can show a great degree of species specificity. This is illustrated in Figure 1, which shows a Southern blot of *SstI* digests from samples representing different tribes of waterfowl (Johnsgard 1978). Major and minor fragments with similar electrophoretic mobility were found in a number of different species. For instance, 1.5-Kb fragments were found in *A. platyrhynchos* (lane 10), *A. clypeata* (lane 11) and *A. formosa* (lane 12) of the Anatini; 1.8-Kb fragments (also see Fig. 2A) were found in these species, as well as in *Dendrocygna guttata* (lane 3), *Neochen jubata* (lane 5), *Cairina moschata* (lane 6), *Aix sponsa* (lane 7), *Pteronetta hartlaubi* (lane 9), *Anas formosa* (lane 12) and *Bucephala islandica* (lane 13).

Figures 2 and 3 show the *RBMI*-like fragment patterns of DNA from 19 different Anatini species (Johnsgard 1978) obtained with the restriction nucleases *SstI, PvuII, EcoRI, HincII* and *PstI*. The data demonstrate that such patterns can be relatively simple with only a few fragments of identical size in virtually all species (e.g. *PvuII*; Fig. 2B), or quite complex and very species specific as defined by the number of fragments of variable size and copy number (e.g. *HincII*; Fig. 3B). All enzymes produced identical patterns among individuals of a given species. Furthermore, no differences in pattern were evident between individuals of two different breeds of the Mallard (*A. platyrhynchos*; Khaki Campbell and Rouen, data not shown).

Of the Anatini sampled, only *Lophonetta specularoides* yielded patterns of largely unique fragment size. Moreover, both *Marmaronetta angustirostris* and *A. formosa* had relatively few fragments in common with the other species. All other species shared major *SstI* fragments of 1.5 and 1.8 Kb, *PvuII* fragments of 1.1 and 1.8 Kb, *EcoRI* fragments of 3.6 and 5.0 Kb, and a *HincII* fragment of 2.0 Kb (Figs. 2, 3A, and 3B); *HaeIII* fragments of 1.2 Kb and *HindIII* fragments of 3.2 and 3.6 Kb also were shared (Fig. 4).

Several fragments had a more limited distribution in the digests. *EcoRI* fragments of 1.7 and 2.6 Kb (Fig. 3A) and a *PstI* component of 1.8 Kb (Fig. 3C) that were present in most Anatini were absent in *A. platalea, A. clypeata, A. discors* and *A. cyanoptera*. However, these species as well as *A. versicolor* contained a *PstI* fragment of 2.1 Kb (Fig. 3C), a *HincII* fragment of 5.5 Kb (Fig. 3B), and high-molecular-weight (HMW) *SstI* fragments of approximately 10 Kb (Fig. 2A), all of which were absent in other species. *Anas strepera, A. falcata, A. americana* and *A. penelope* contained distinct 3.2- and 3.8-Kb *EcoRI* fragments (Fig. 3A), and HMW material in *SstI, HincII* and

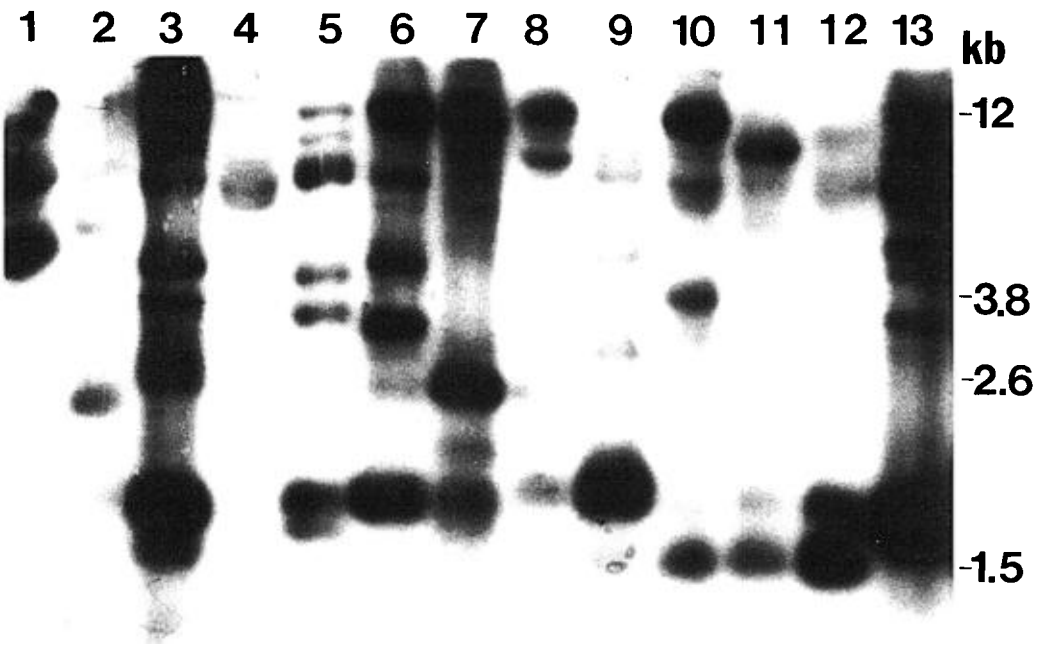

Fig. 1. Comparison of *RBMI*-like *SstI* restriction-digest patterns of 13 waterfowl representing 7 anatid tribes. Specimens are: (1) *Anseranas semipalmata* (Magpie Goose); (2) *Dendrocygna viduata* (White-faced Whistling-Duck); (3) *D. guttata* (Spotted Whistling-Duck); (4) *Cygnus atratus* (Black Swan); (5) *Neochen jubatus* (Orinoco Goose); (6) *Cairina moschata* (Muscovy Duck); (7) *Aix sponsa* (Wood Duck); (8) *Sarkidiornis melanotos* (Comb Duck); (9) *Pteronetta hartlaub* (Hartlaub's Duck); (10) *A. platyrhynchos* (Mallard); (11) *A. clypeata* (Northern Shoveler); (12) *A. formosa* (Baikal Teal); (13) *Bucephala islandica* (Barrow's Goldeneye). Tribes represented as follows (with specimen numbers in parentheses): Anseranatini (1); Dendrocygnini (2–3); Anserini (4); Tadornini (5); Cairinini (6–9); Anatini (10–12); Mergini (13).

PstI digests (Figs. 2A, 3B, and 3C). *Anas platyrhynchos, A. rubripes, A. crecca, A. acuta, A. bahamensis, A. hottentota* and *A. castanea* all shared a major 5-Kb *HincII* fragment (Fig. 3B) and major HMW *SstI* components of approximately 11 Kb (Fig. 2A).

Other fragments were found as major components in only a few species. A 4.6-Kb *EcoRI* (Fig. 3A) and a 3.4-Kb *HincII* (Fig. 3B) fragment were only found in *A. strepera* and *A. falcata,* whereas a 4.2-Kb *EcoRI* component (Fig. 3A) occurred only in *A. americana* and *A. penelope.* A major 3.0-Kb *HincII* fragment (Fig. 3B) was found in *A. acuta* and *A. bahamensis.* Both *A. clypeata* and *A. discors* contained distinguishing, minor 1.0-Kb *SstI* fragments (Fig. 2A).

In addition to the above qualitative relationships between the restriction patterns of anatid *RBMI*-like sequences, quantitative differences also were evident. A 3.6-Kb *EcoRI* component (Fig. 3A) found in high concentration in many of the species examined occurred in much lower concentration in others, such as *A. strepera, A. falcata, A. americana, A. penelope* and *A. hottentota.*

In digests made with certain enzymes, the minor components can show more species specificity than the major fragments (Figs. 2 and 3). This is most evident in the *SstI* digests of *A. strepera, A. falcata, A. americana* and *A. penelope,* which contained similar major components, but also minor fragments of 3 to 8 Kb that can be specific to one or two species (Fig. 2A). A similar observation is applicable to *A. platalea, A. clypeata, A. discors, A. cyanoptera* and *A. versicolor,* as well as *A. platyrhynchos* and *A. rubripes,* two very closely related species (Johnsgard 1978). Figure 5 shows that *A. rubripes* contained a distinguishing 3.8-Kb *SstI* fragment, which was absent in *A. platyrhynchos.*

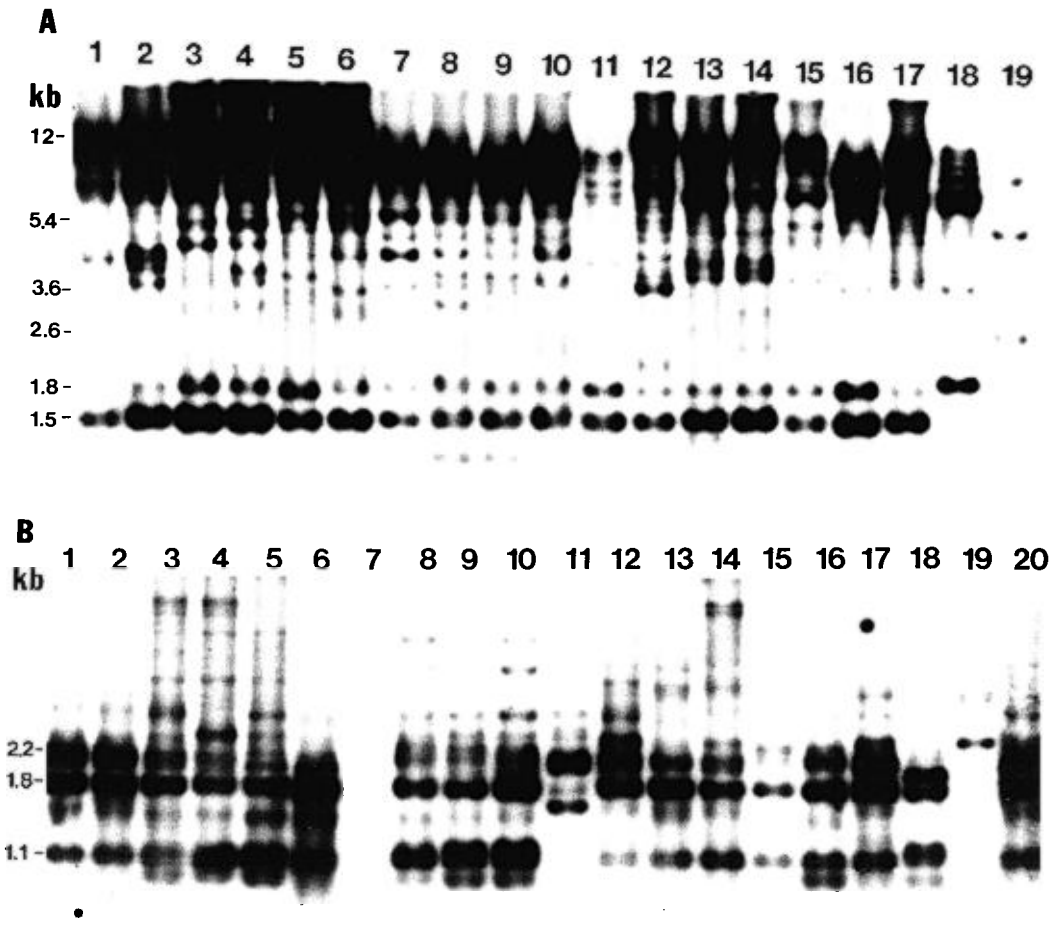

Fig. 2. Comparison of *RBMI*-like restriction-fragment patterns of 19 Anatini species obtained with restriction nucleases (A) *SstI* and (B) *PvuII*. Specimens are: (1) *A. platyrhynchos* (Mallard); (2) *A. rubripes* (American Black Duck); (3) *A. strepera* (Gadwall); (4) *A. falcata* (Falcated Duck); (5) *A. americana* (American Wigeon); (6) *A. penelope* (Eurasian Wigeon); (7) *A. platalea* (Argentine Shoveler); (8) *A. clypeata* (Northern Shoveler); (9) *A. discors* (Blue-winged Teal); (10) *A. cyanoptera* (Cinnamon Teal); (11) *A. formosa* (Baikal Teal); (12) *A. crecca* (Green-winged Teal); (13) *A. acuta* (Northern Pintail); (14) *A. bahamensis* (White-cheeked Pintail); (15) *A. hottentota* (Hottentot Teal); (16) *A. versicolor* (Versicolor Teal); (17) *A. castanea* (Chestnut-breasted Teal); (18) *Marmaronetta angustirostris* (Marbled Teal); (19) *Lophonetta specularoides* (Crested Duck); and (20) *A. platyrhynchos* (Mallard).

DISCUSSION

RBMI-like sequences can form specific patterns of major and minor fragments in Southern blots of waterfowl DNA. Major fragments of similar electrophoretic mobility are found in a number of species belonging to different tribes (e.g. a 1.8-Kb *SstI* fragment), while other fragments are more species specific (Fig. 1). Among the Anatini, we found *A. formosa*, *M. angustirostris* and *L. specularoides* to yield quite different *RBMI* restriction patterns from other Anatini. On this basis, we suggest that these three species are quite distinct from each other, as well as from the other sampled Anatini. With respect to *M. angustirostris* and *L. specularoides*, our conclusion supports the earlier morphological (Delacour 1954–1964, Livezey 1986) and behavioral

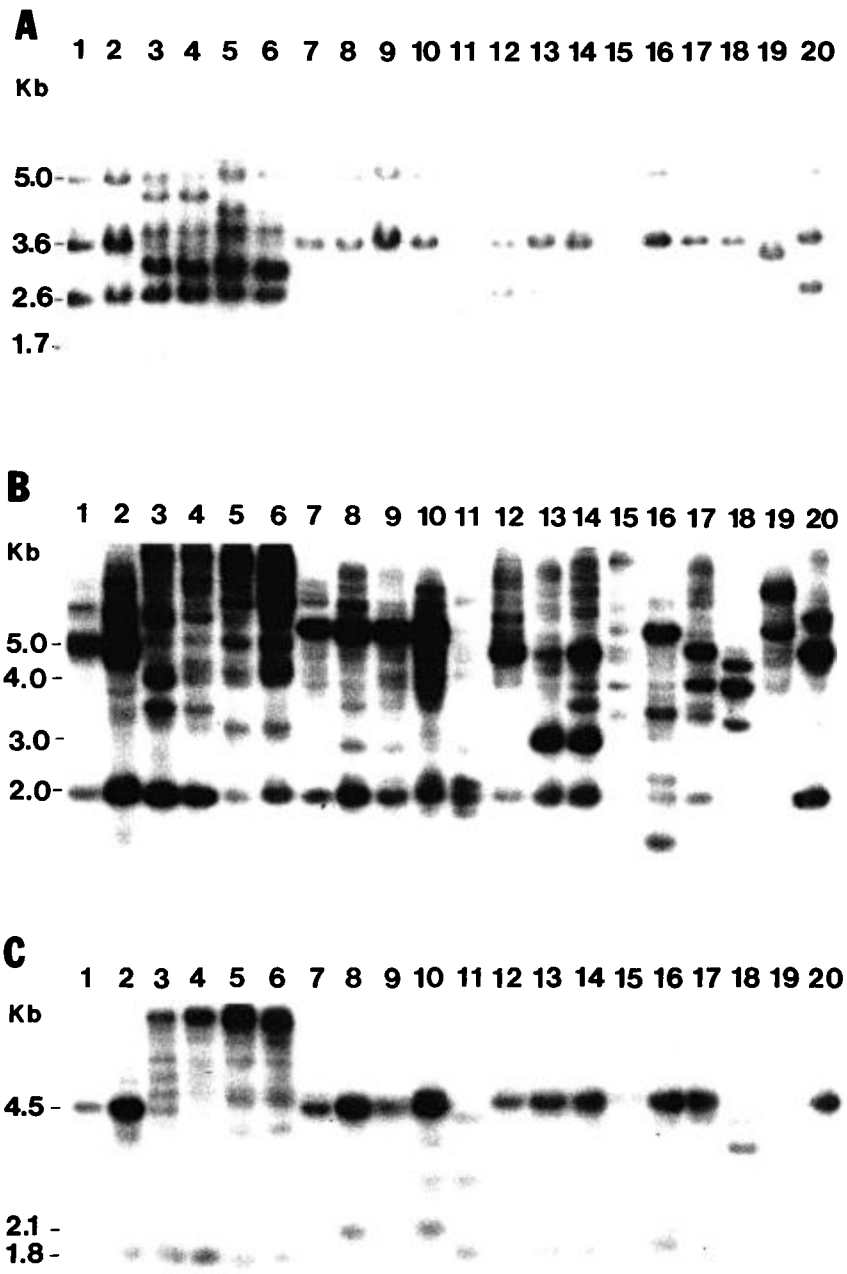

Fig. 3. Comparison of *RBMI*-like restriction-fragment patterns of 19 Anatini species obtained with restriction nucleases (A) *EcoRI*, (B) *HincII*, and (C) *PstI*. Specimens as indicated in Figure 2.

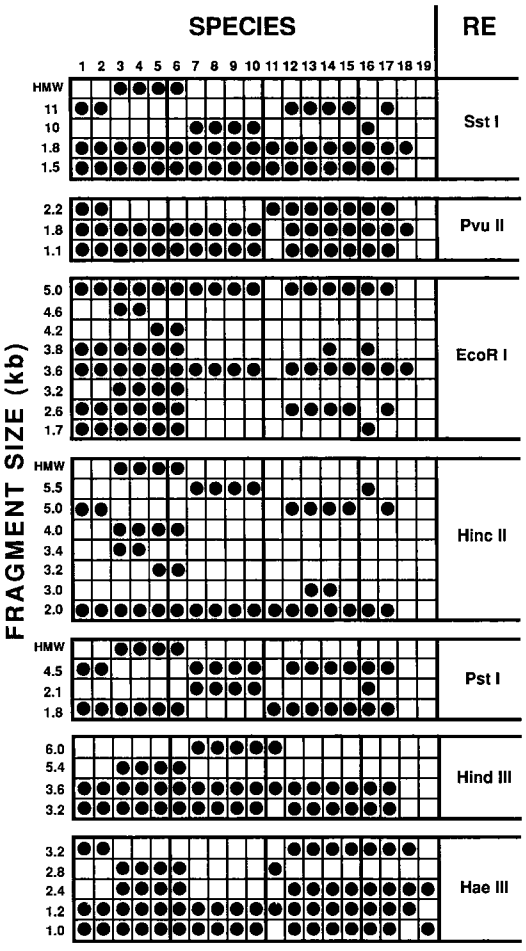

Fig. 4. Restriction-endonuclease fragments and their occurrence in 19 Anatini species. Specimens as indicated in Figure 2.

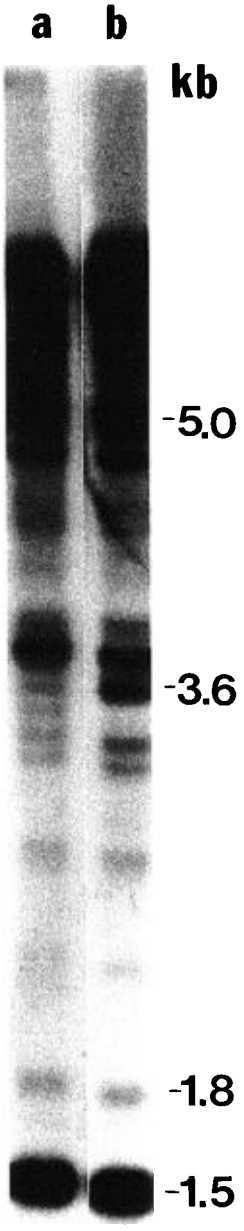

Fig. 5. Comparison of the *RBMI*-like *SstI* restriction patterns of (A) *A. platyrhynchos* and (B) *A. rubripes*.

(Johnsgard 1978) data, which also suggest that these two species are quite distinct from the Anatini. The isolated position of *A. formosa* is less expected, as it is often considered a close relative of *A. crecca* (Johnsgard 1978). However, our conclusion is endorsed by anatomical evidence (Delacour 1954–1964).

All of the other investigated Anatini can be grouped together according to the degree of similarity of the *RBMI* patterns. The enzyme digests of *A. strepera, A. falcata, A. americana,* and *A. penelope* contain similar components. The close relationship among these species also has been proposed on anatomical and behavioral grounds (Delacour 1954–1964, Johnsgard 1978) and is, with particular regard to *A. strepera* and *A. americana,* also supported by restriction patterns of mtDNA (Kessler and Avise 1984). Similarly, the close relationship between the members of the blue-winged duck group (i.e. *A. platalea, A. clypeata, A. discors* and *A. cyanoptera*)

TABLE 1. Matrix of shared *RBMI*-like fragments in selected Anatini. Fragments listed in Figure 4 were combined to generate matrix of shared fragments for selected species. Shared fragments are described in terms of $F = 2N_{xy}/(N_x + N_y)$, where N_x and N_y are total number of restriction fragments considered in species X and Y, respectively, and N_{xy} is total number of fragments shared by X and Y. Numbers below diagonal represent $2N_{xy}/(N_x + N_y)$, corresponding to raw data; those above diagonal represent calculated F-value.

	1	2	3	4	5	6	7
1 *A. platyrhynchos*		0.67	0.67	0.92	0.48	0.84	0.38
2 *A. falcata*	30/45		0.54	0.64	0.41	0.71	0.32
3 *A. clypeata*	24/36	22/41		0.69	0.48	0.77	0.36
4 *A. acuta*	36/39	28/44	24/35		0.44	0.81	0.32
5 *A. formosa*	14/29	14/34	12/25	14/32		0.50	0.13
6 *A. versicolor*	36/43	34/48	30/39	34/42	14/28		0.24
7 *M. angustirostris*	10/26	10/31	8/22	8/25	2/15	6/25	

has been indicated previously (Delacour 1954–1964, Johnsgard 1978). The latter three species present similar mtDNA restriction patterns (Kessler and Avise 1984). Note that *A. versicolor* contains some major *RBMI*-like restriction fragments, such as the HMW 10-Kb *SstI* fragment, a 2.1-Kb *PstI* fragment, and a 5.5-Kb *HincII* fragment, which are typical of the blue-winged ducks. On the other hand, the 1.7-Kb *EcoRI* and 3.2- and 2.4-Kb *HaeIII* fragments do not occur in the digests of the blue-winged ducks, but are more typical of the other Anatini. In addition, a major 1.5-Kb *HincII* fragment in *A. versicolor* occurs as a minor fragment in the blue-winged ducks. A major 3.4-Kb *HincII* fragment is found in *A. versicolor* and *A. falcata*. Johnsgard (1978) has suggested that, on the basis of behavioral and anatomical studies, *A. versicolor* is intermediate between the blue-winged ducks and the mallard-pintail group. Our data support this conclusion, although the data also are compatible with a hybrid origin of this species.

In order to further illustrate the usefulness of the *RBMI*-like fragments for phylogenetic analysis, we constructed a data matrix based on fragment patterns (Table 1). For simplification, some species were grouped together on the basis of similarity of patterns (>95% similarity). The following groups were assembled: (1) *A. platyrhynchos, A. rubripes, A. crecca, A. hottentota,* and *A. castanea* (mallard group); (2) *A. strepera, A. falcata, A. americana,* and *A. penelope* (gadwall-wigeon group); (3) *A. platalea, A. clypeata, A. discors,* and *A. cyanoptera* (blue-winged duck group); and (4) *A. acuta* and *A. bahamensis* (pintail group). The patterns of *A. platyrhynchos, A. falcata, A. clypeata,* and *A. acuta* were used to represent these groups in the data matrix. In addition (5) *A. formosa,* (6) *M. angustirostris* and (7) *A. versicolor* were used as individual species in the analysis because of the uniqueness of their patterns.

The relationship between *RBMI*-like elements in different species involves qualitative and quantitative components, both of which have value as phylogenetic parameters contributing to the complexity of the calculation of relationships (Sneath and Sokal 1973). In this analysis we have focused only on qualitative parameters, with particular reference to major fragments. We define major fragments as those fragments that amount to at least 10% of the total amount of *RBMI*-like material as determined by densitometric analysis of autoradiograms (Fig. 6). Equal significance has been assigned to all such fragments. The selected fragments and their occurrence in the different species are shown in Figure 4 and were combined in a distance matrix of shared fragments between individual species (Table 1). The data were processed using the PHYLIP computer package (version 3.3; J. Felsenstein, University of Washington, Seattle), which is based on the phylogenetic-tree construction method of Fitch and Margoliash (1967).

The results of this analysis are presented in Figure 7 as a dendrogram generated by PHYLIP after examination of 97 alternative trees. The dendrogram indicates that, with respect to the *RBMI* component, lineages leading to *A. formosa* and *M. angustirostris* have demonstrated the greatest sequence divergence relative to the other species examined. On the same criterion, the mallard and pintail groups are closely related to each other.

From the dendrogram, *A. versicolor* appears to

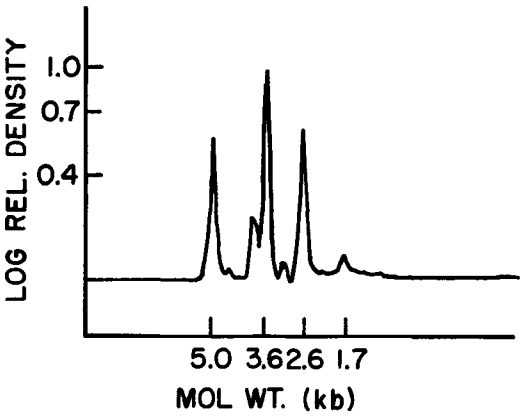

Fig. 6. Densitometric analysis of an autoradiograph of *EcoRI*-digested *A. platyrhynchos* DNA probed with *RBMI*.

be more closely related to the mallard-pintail grouping than any other. However, Table 1 shows that the relative fragment similarities between this species and *A. clypeata*, *A. platyrhynchos*, and *A. acuta* are relatively high with an average *F*-value ($\bar{F}$) of 0.81. The patterns of *A. clypeata* and those of *A. platyrhynchos* (and *A. acuta*) are much less similar ($\bar{F}$ = 0.68). Moreover, if quantity of the fragments is considered of primary interest, the prominent 10-Kb *SstI* and 5.5-Kb *HincII RBMI*-like components (Figs. 2A and 3B) tend to indicate a close relationship between *A. versicolor* and the blue-winged ducks. Thus, one possible reason for the discrepancy between aspects of the restriction-fragment data and the dendrogram may lie with our failure to take these quantitative differences into consideration in our analysis. A weighted analysis with copy-number variables factored into the equation might give a different resolution of this issue. Another possibility may involve the fact that fragments of equal electrophoretic mobility may not be identical. It is clear from recent experiments (McHugh, Tuohy, and de Kloet, in prep.) that different *RBMI*-like elements contain both common and element-specific sequences. Thus, the multiple forms in which *RBMI*-like elements occur may lead to erroneous estimates of *F* and of the phylogenetic relationships of *A. versicolor*.

The patterns of *L. specularoides* show so little similarity with the other species that we conclude that it is relatively phylogenetically remote from these species. On the other hand, *RBMI*-like fragment patterns also can be used to distinguish very closely related species as determined by the *SstI* digests of *A. platyrhynchos* and *A. rubripes* (Fig. 5). This result suggests that such patterns can be of use in the understanding of the evolution of the Mallard-like waterfowl.

The constructed dendrogram is in agreement with the findings of Kessler and Avise (1984) with regard to the phylogenetic placement of the gadwall-wigeon group, the mallard-pintail group and the blue-winged duck group (Kessler and Avise 1984:figs. 2 and 3). Therefore, the use

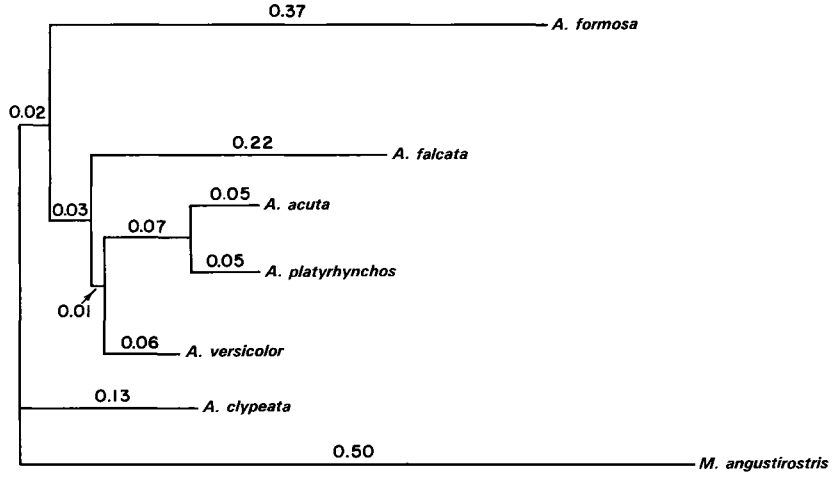

Fig. 7. Data in Table 1 analyzed to generate an unrooted dendrogram of seven species using PHYLIP program (version 3.3). Numbers shown are indices of relative sequence divergence (average percent standard deviation = 7.3).

of the *RBMI* sequence for the determination of phylogenetic relationships can complement such estimates based on mitochondrial data. Furthermore, we believe that the former approach will prove to be the more powerful, after the addition of the quantitative data to the analysis, by virtue of the heterogeneity of the sequence. In conclusion, the data indicate that highly repeated nuclear-DNA sequence elements such as *RBMI* can be used to investigate the relationships among a relatively wide range of waterfowl species.

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