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Slow Evolutionary Loss of the Potential for Interspecific Hybridization in Birds: A Manifestation of Slow Regulatory Evolution

(anatomical evolution/protein evolution/chromosomal evolution/frogs/mammals/immunology)

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ABSTRACT **Birds have lost the potential for interspecific hybridization slowly. This inference emerges from protein comparisons made on 36 pairs of bird species capable of hybridization. Micro-complement fixation tests show that hybridizable pairs of bird species differ by an average of 12 units of albumin immunological distance and 25 units of transferrin immunological distance. As these proteins evolve at a known and rather steady rate, it is inferred that the average hybridizable species pair diverged from a common ancestor about 22 million years ago. The corresponding period for frog species pairs capable of hybridization is about 21 million years, while for hybridizable placental mammals it is only 2 to 3 million years. Thus birds resemble frogs in having lost the potential for interspecific hybridization about 10 times as slowly as have mammals.**

Birds have also been evolving very slowly at the anatomical level, particularly within the last 25 million years, according to Simpson, Romer, and many other vertebrate zoologists. In this respect they resemble frogs and differ from placental mammals, which have been undergoing unusually rapid anatomical evolution. Chromosomal evolution is also thought to have proceeded very slowly in both birds and frogs, relative to mammals.

The above observations are consistent with the hypothesis that evolutionary changes in regulatory systems, that is, changes in the patterns of gene expression, provide the basis for both anatomical evolution and the evolutionary loss of hybridization potential.

Several authors have suggested that in order to understand organismal evolution one needs to focus attention on the control of gene expression rather than on the amino acid sequences of proteins coded for by structural genes (1–5). This article and the preceding ones in this series (6–8) present evidence consistent with this suggestion. This evidence comes from studies of the relative rates of evolution of anatomy, chromosomes, hybridization potential, and proteins in three major groups of vertebrates. The data on frogs and mammals have already been published (6, 7). We show here that similar conclusions may be drawn from analogous studies on birds.

The data presented in this article are based on studies of species capable of interspecific hybridization. Studies of interspecific hybridization have often provided valuable new insights into the mechanism of evolution (9). There are, of course, many natural barriers to interspecific hybridization. Geographical, ecological, behavioral, and anatomical barriers normally prevent contact between an egg of one species and sperm of another. If these barriers are circumvented and fertilization occurs, the resulting interspecific zygote may develop into a viable hybrid organism. We have examined the problem of what relationship, if any, exists between hybridization potential and degree of protein sequence difference among species. At first thought it might seem likely that degree of protein similarity between parental species should be the major factor affecting the probability of successful development of an interspecific zygote. The more similar the proteins of two species, the more likely it is, one might suppose, that their genomes would be compatible enough to permit development of viable hybrids. However, our first study of this produced a result inconsistent with this expectation. Mammals that can hybridize differ only slightly at the protein level, whereas frogs that differ substantially at the protein level hybridize readily (6).

To explain this result, it has been proposed that the principal molecular barriers to interspecific hybridization are regulatory differences between the parental genomes and that placental mammals appear to have been undergoing more rapid regulatory evolution than frogs have (6). We consider regulatory differences to be differences in the patterns of gene expression. Since mammals have also undergone more rapid anatomical evolution than frogs have, it was suggested that rapid regulatory evolution in mammals may account for both their rapid anatomical evolution and their rapid evolutionary loss of the potential for interspecific hybridization. In view of the observation that mammals have undergone rapid evolutionary changes in gene arrangement compared to frogs (7), it was further suggested that the phenomenon of gene rearrangement may be an important means of achieving new systems of regulation.

To explain the observation that protein evolution has gone on at the same rate in frogs as in mammals, despite the occurrence of far more rapid organismal evolution in mammals than in frogs, it has been proposed that protein evolution may not be at the basis of organismal evolution (6, 7). Thus there may be two types of molecular evolution: (*1*) protein evolution, which proceeds relentlessly in a predominantly time-dependent manner, and (*2*) regulatory evolution, which parallels organismal evolution.

We now present the results of a study of protein resemblance within pairs of bird species capable of hybridization. Birds, like frogs, seem to have lost the potential for interspecific hybridization slowly. We also review evidence that birds have evolved slowly at both the anatomical and the chromosomal levels.

MATERIALS AND METHODS

Avian Samples. Bird egg whites, sera, and tissue extracts were obtained, prepared, and stored as described (10). Thirty-three different avian species capable of hybridizing with one or more other species were studied. They were

Abbreviation: MY, million years.

TABLE 1. *Immunological distances within avian species pairs capable of hybridization*

Pair*†	Immunological distance: Albumin	Transferrin
Rheiformes		
Rhea americana		
× *Pterocnemia pennata*	0	ND‡
Anseriformes		
Anas platyrhynchos		
× *Anas laysanensis*	0	1
× *Anas poecilorhyncha*	0	2
× *Anas castanea*	0	3
× *Anas rubripes*	0	4
× *Anas bahamensis*	2	3
× *Anas acuta*	0	11
× *Aix galericulata*	8	16
× *Aix sponsa*	8	29
× *Cairina moschata*	9	21
× *Netta rufina*	8	21
× *Tadorna tadorna*	13	34
× *Anser cygnoides*	10	65
× *Anser anser*	13	69
× *Branta canadensis*	12	61
Galliformes		
Gallus gallus		
× *Gallus sonnerati*	ND§	0
× *Gallus varius*	11	6
× *Lophophorus impeyanus*	18	33
× *Lophura nycthemera*	ND§	50
× *Phasianus colchicus*	29¶	35¶
× *Chrysolophus pictus*	26	32
× *Pavo cristatus*	24¶	34¶
× *Coturnix coturnix*	23	35
× *Numida meleagris*	34	53
× *Meleagris gallopavo*	18¶	32¶
Phasianus colchicus		
× *Lagopus mutus*	ND§	16
× *Lophura nycthemera*	ND§	26
× *Syrmaticus reevesi*	18	25
× *Chrysolophus amherstiae*	14	13
× *Chrysolophus pictus*	12	12
× *Meleagris gallopavo*	19¶	15¶
Pavo cristatus		
× *Pavo muticus*	0	0
× *Numida meleagris*	25	39
× *Meleagris gallopavo*	16¶	20¶
Meleagris gallopavo		
× *Meleagris ocellata*	4	0
× *Numida meleagris*	20	44
Average	12	25
Range	0–34	0–69

* The species pairs are listed according to order. Within each order they are listed consistent with standard taxonomic practice. The two rheiform birds are the only existing members of the Rheiformes. All species given within the order Anseriformes are members of the family Anatidae; species in the genera *Anser* and *Branta* are geese and members of the subfamily Anserinae, while the remaining anseriform species in the table are ducks and members of the subfamily Anatinae. Within the order Galliformes, all species given belong to the superfamily Phasianoidea. Within this superfamily, the following categories exist for the species tabulated: genus *Numida* (guinea fowls), family Numididae; genus *Meleagris* (turkeys), family Meleagrididae; genus *Lagopus* (ptarmigans), family Tetraonidae; all other genera, family Phasianidae. Within the Phasianidae, *Coturnix coturnix* (Japanese quail) belongs to the subfamily Perdicinae, while all the others are members of the subfamily Phasianinae and consist of chickens and jungle fowls (genus *Gallus*), peafowls (genus *Pavo*), and pheasants (genera *Lophophorus*, *Lophura*, *Phasianus*, *Chrysolophus*, and *Syrmaticus*).

† For each pair the species to which antisera were made is given first. Cases in which antisera to both members of the pair were available are indicated (footnote ¶).

‡ ND, not done. Antisera to rhea transferrin not available.

§ ND, not done. Only egg white, no serum or tissue, available in our collection.

¶ The average of reciprocal measurements is given.

chosen from three of the 27 bird orders: Rheiformes, containing the large, flightless rheas of South America; Anseriformes, made up of waterfowl; and Galliformes, consisting of game birds. The first footnote to Table 1 gives further details regarding the classification of the species examined. This constituted a representative sample of hybridizable species pairs, since more than half of all reported cases of avian interspecific hybrids occur within the orders Anseriformes and Galliformes (11–13). We prepared antisera (see below) to albumin from six species and to transferrin from five species and made immunological comparisons of the proteins of 36 different avian species pairs capable of interspecific hybridization.

Protein Purification. Eleven avian proteins—six serum albumins, two serum transferrins, and three ovotransferrins—were purified and used as immunogens. The purification of common rhea (*Rhea americana*) albumin and chicken (*Gallus gallus*) ovotransferrin has been described (10).

Serum albumin from Peking duck (*Anas platyrhynchos*), chicken, ring-necked pheasant (*Phasianus colchicus*), peafowl (*Pavo cristatus*), and turkey (*Meleagris gallopavo*) was purified by Rivanol precipitation and subsequent regeneration of the albumin (14) followed by preparative polyacrylamide gel electrophoresis (15). Eight percent polyacrylamide gels and a continuous buffer system of 0.1 M Tris plus 0.05 M glycine were generally used. Occasionally 6.4% gels and a discontinuous buffer system (16) were employed. 8-anilino-1-naphthalene sulfonate (17) or simply the difference in refractive index was used to detect the albumin band on the gels. Elution of the albumin from the polyacrylamide discs (15) into isotris buffer (18) containing merthiolate (2.5 μg per ml) was done at room temperature for 1 day and then at 4° for two or more days with occasional shaking. The albumins (except that of the chicken) were then concentrated by vacuum dialysis, further purified by a repetition of the electrophoresis in polyacrylamide, and again eluted. In certain cases three polyacrylamide gel purification steps were utilized.

Duck and peafowl serum transferrin were purified essentially as described (19). A small amount of ferrous ammonium sulfate was added prior to the Rivanol precipitation and polyacrylamide gel electrophoresis steps to saturate the transferrin with iron. Two successive runs using the 8% polyacrylamide gel system were employed. The transferrin was located by the pink color of the iron–transferrin complex. Elution and concentration were performed as described above. To purify pheasant and turkey ovotransferrin, a small amount of ferrous ammonium sulfate was first added to

TABLE 2. *Estimation of the average time elapsed since the common ancestor of a hybridizable species pair lived*

Vertebrate group	Protein	Rate of protein evolution*	Number of hybridizable species pairs tested†	Mean immunological distance within pairs†	Time elapsed since common ancestor lived (MY)
Mammals	Albumin	1.7	31	3.2	2
	Transferrin	2.6	21	8	3
Frogs	Albumin	1.7	50	36	21
Birds	Albumin	0.6	32	12	20
	Transferrin	1.1	35	25	23

* Measured in immunological distance units per MY. The values given are from ref. 10.

† The data for mammals and frogs are from ref. 6.

whole egg white and then two polyacrylamide gel purification steps were carried out as described for serum transferrin.

Immunoelectrophoresis and Ouchterlony double diffusion (10) with antisera elicited to whole serum or whole egg white from *Anas platyrhynchos* and a number of gallinaceous birds were used to demonstrate the purity of the proteins prior to immunization.

Antisera. The antisera to rhea albumin and chicken ovotransferrin were those described by Prager *et al.* (10). Each of the remaining purified avian antigens was injected into four Dutch Belted rabbits according to a published schedule (20) with the following alterations: (*1*) Freund's supplemented complete adjuvant (21) was used for the initial immunization; (*2*) the final intravenous injections were at 10 and 11 weeks; (*3*) bleeding, by cardiac puncture, was at 12 weeks. The importance of using a 3-month immunization period has been explained elsewhere (18, 21); (*4*) very small amounts of immunogen were used, each rabbit receiving as little as 50 μg of transferrin or 100 μg of albumin per injection, with a maximum of about 250 μg of immunogen per injection; (*5*) the same amount of protein was given at each injection for any particular immunogen.

Antisera to each of the 11 immunogens were heated, stored, and pooled as described (18, 21). The purity of the antisera was evaluated as previously (10). Any antibodies produced to proteins other than the immunogen specified were found to be so weak as not to interfere with micro-complement fixation tests. The average micro-complement fixation titers (18) of the pools of antisera elicited toward albumin and transferrin were 4000 and 11,000, respectively.

Micro-Complement Fixation. Quantitative micro-complement fixation was carried out as described (18). The degree of antigenic difference in this test is given in immunological distance units, which appear to be proportional to the degree of amino acid sequence difference between two homologous proteins. The relationship between immunological distance (y) and percent sequence difference (x) is generally found to be $y \simeq 5x$ (for example, refs. 10, 18, 21, and 22). Experiments involving antisera to transferrin were done with bird egg whites, sera, or tissue extracts as antigen sources; ovotransferrin and serum transferrin can be used interchangeably in micro-complement fixation tests (10). Studies involving antisera to albumin were conducted with bird sera or tissue extracts as antigen sources (10).

Criteria of Hybridization Potential. Only those avian species pairs capable of producing viable, full-term interspecific hybrids were considered (11–13, 23, 24). Cases of questionable, alleged, or presumed hybridization were excluded. As is the case with the interspecific frog and mammal hybrids previously studied (6), many of these avian hybrids are sterile.

RESULTS

Table 1 presents the albumin and transferrin immunological distances between members of 36 avian species pairs capable of hybridization. These species are members of three different orders: Rheiformes (1 pair), Anseriformes (14 pairs), and Galliformes (21 pairs) (compare the first footnote to Table 1).

As Table 1 shows, the average albumin immunological distance for hybridizable bird species pairs is 12 units, with a range of 0–34 units. The average transferrin distance for hybridizable bird species is 25 units, with a range of 0–69 units. The larger values for transferrin are consistent with the greater rate of evolutionary change of vertebrate transferrin relative to albumin (10).

DISCUSSION

Evolutionary age of hybridizable pairs

After a species splits in two, the genomes of the two resulting species slowly diverge to the point where an interspecific zygote fails to develop into a viable adult. It is desirable to know how long such species pairs retain the capacity to hybridize. Our protein studies on hybridizable species pairs allow this problem to be approached.

The principle of the method of determining the time since divergence of species pairs capable of hybridization is illustrated by our studies on placental mammals (6), for which the average immunological distance between the albumins of a hybridizable pair is 3.2 units. This distance can be related to time. There is now strong evidence that albumin evolves rather steadily at the rate of about 1.7 units per million years (MY) in both marsupial (L. R. Maxson, V. M. Sarich, and A. C. Wilson, unpublished data) and placental mammals (compare ref. 25), as well as in a wide variety of other vertebrates, including frogs (refs. 10, and 25–27; Maxson, Sarich, and Wilson, unpublished; Maxson and Wilson, unpublished). Thus, using albumin as a dating device, we conclude that it generally takes about 2 MY for an albumin difference of 3.2 units to arise. Accordingly, the average hybridizable pair of placental mammal species is estimated to have arisen from a common ancestor that lived 2 MY ago (Table 2).

As shown in Table 2, a similar analysis conducted with transferrins of 21 hybridizable pairs of mammal species produces an estimate of 3 MY.

An analogous study of 50 hybridizable frog species pairs yielded an average albumin immunological distance of 36 units, which corresponds to a 21 MY divergence time (Table 2). Thus frogs retain the potential for interspecific hybridization far longer than placental mammals do.

The results of similar calculations for birds are also given in Table 2. Since albumin and transferrin have evolved about three times as slowly in birds as in other vertebrates (10), the rate constants used for the bird calculations are different from those used for mammals and frogs. The average time

since divergence of hybridizable pairs of bird species is 20 MY, based on albumin comparisons, and 23 MY, based on transferrin comparisons. The fragmentary fossil evidence available is consistent with these estimates. For example, at least 20 MY have elapsed since the divergence of lineages leading to the following hybridizable pairs of species: (*1*) ducks and geese (28) and (*2*) turkeys and guinea fowls (29, 30). Thus birds are like frogs in having retained the potential for hybridization much longer than mammals have.

Slow anatomical evolution in birds

We have proposed (6) that slow regulatory evolution in frogs may account for both their slow anatomical evolution and their slow evolutionary loss of hybridization potential. The finding that birds have lost the potential for interspecific hybridization slowly leads us to ask whether birds too have experienced slow anatomical evolution. Since ornithologists unanimously divide living birds into at least 27 orders (31–35), whereas placental mammals are usually divided into 16 orders and frogs (Anura) make up a single order, one is initially tempted to infer that birds are comparable to mammals in being far more diverse in anatomy and way of life than frogs are. However, such an inference is unwarranted if one is to judge from the many comments by those anatomically-trained vertebrate zoologists such as Simpson (36), Romer (37), and Bock (38) who have considered the evolution and classification of birds relative to other vertebrates:

> From the point of view of structural progression, it is hardly an exaggeration to say that birds have not evolved since the Miocene.... Wetmore and others have also emphasized this lack of progression in birds since the early Tertiary, and it is one of the striking generalizations of paleornithology [36].

> The different bird orders have, in general, no more differences between them than exist between families of other classes of vertebrates [37].

> Of all vertebrate classes, the Aves are the easiest to define because of.... their extreme degree of uniformity in phenotypical features [38].

Birds are also remarkably homogeneous in their physiology (39). It therefore seems reasonable to conclude that the slow evolutionary loss of hybridization potential in birds is paralleled by slow anatomical and physiological evolution.

Taxonomic distance between hybridizable species

The idea that birds are oversplit into higher taxonomic categories is supported by an additional line of evidence, summarized in Table 3. This table gives an indication of the taxonomic distance between species capable of hybridizing with each other. First let us consider frogs. About 400 different pairs of frog species are known to hybridize, to the extent of producing hybrids that successfully complete metamorphosis from tadpole to frog. The great majority of these pairs (97%) involve species belonging to the same genus. Intergeneric hybridization is extremely rare in frogs, the best examples being the crosses between *Pseudacris* and *Hyla*. For placental mammals also, most of the 256 known cases of hybridization involve species within a genus; only 11% of the cases are intergeneric. Thus taxonomic distance is a moderately good predictor of hybridization potential: if two frog or two mammal species belong to different genera, they are unlikely to hybridize. For birds, however, the situation differs. Nearly half (44%) of the known cases (about 1000) of hybridization in birds are between genera. Indeed, many of the cases listed in Table 3 as intergeneric are between species belonging to different subfamilies or families; examples include the duck–goose cross and the turkey–chicken cross. The observation that intergeneric hybridization occurs far more often in birds than in frogs or mammals is exactly the result expected from the vertebrate zoologists' hypothesis that birds are victims of an inflated system of organismal classification.

TABLE 3. *Taxonomic distance between species capable of hybridization*

Vertebrate group	Hybridizable pairs of species*	
	Percent interspecific	Percent intergeneric
Frogs	97	3
Placental mammals	89	11
Birds	56	44

* Cases of interspecific hybridization are those reported in refs. 40–44 (frogs), ref. 45 (mammals), and refs. 11–13, 23, and 24 (birds).

Slow chromosomal evolution in birds

Since birds have experienced both slow loss of hybridization potential and slow anatomical evolution, it is pertinent to ask whether birds have also undergone slow evolutionary changes in gene arrangement. Our previous analyses of rate of evolutionary change in chromosome number (7) showed that chromosomal evolution has proceeded roughly 20 times faster in mammals than in frogs. This led to the suggestion that gene rearrangement might be an important mechanism for achieving new systems of gene regulation and thereby provide an important basis for evolutionary changes in anatomy and hybridization potential (7). Unfortunately, this hypothesis cannot be tested quantitatively with birds. Birds have very high chromosome numbers and a rather low DNA content per cell (46–49). Many of the chromosomes are so small ("microchromosomes") that accurate determination of their total number is extremely difficult.

Nevertheless, two qualitative lines of evidence show that bird chromosomes are exceedingly conservative. First, birds exhibit a narrow range of chromosome numbers. About half of the species examined to date have a diploid number of 78 or 80 (46, 47, 50–52). Second, application of the G-banding technique reveals striking homologies among the macrochromosomes of birds belonging to diverse orders (52). No such homologies are detectable among the autosomes of placental mammals belonging to different orders (52). Yet the placental mammals are geologically younger than birds. Both of these qualitative approaches indicate that gene rearrangement has proceeded more slowly in birds than in mammals. In this respect, birds resemble frogs.

Conclusions

Birds resemble frogs in having undergone slow loss of hybridization potential, slow anatomical evolution, and slow chromosomal evolution. These findings are consistent with the proposal that evolution at the organismal level is a manifestation of evolution at the level of gene arrangement and expression.

Note Added in Proof. Coyne (53) has postulated that the evolution of hybrid inviability would be more rapid in those organisms which make a "substantial parental investment in the production and care of progeny" (53) than in those organisms which do not make such an investment. This hypothesis is consistent with our observations (6) that mammals have lost the potential for interspecific hybridization 10 times as fast as have frogs. However, Coyne's hypothesis is inconsistent with the bird data presented here: birds, though making a considerable investment in producing and caring for their offspring, have lost the potential for interspecific hybridization just as slowly as frogs have. Thus it appears that evolutionary changes in regulatory systems may provide the major basis for loss of hybridization potential and that the degree of parental involvement with progeny is a much less significant factor.

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