Restriction Enzyme Cleavage of Nuclear DNA Reveals Differences in Repetitive Sequences in Two Species Pairs (*Ficedula* and *Parus*)

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The haploid nuclear genome of birds contains ca. 1.8 picograms of DNA (Shields 1983), which represent approximately 1×10^9 base pairs (bp). Passerine species have a larger amount of repeated DNA (35% of the genome) than nonpasserine species (10–20%) (Shields 1983), and repetitive sequences are dispersed among single-copy DNA throughout the genome. In birds, 40–50% of the single-copy DNA is organized into blocks of 4.5 kilobases (kb) interspersed with repetitive sequences of 2–4 kb (Arthur and Straus 1978, Eden and Hendrick 1978, Epplen et al. 1978, Shields 1983). The rest of the genome consists of long stretches (>17 kb) of unique single-copy sequences (Eden and Hendrick 1978, Shields 1983).

Some moderately repetitive sequences ($10–10^5$ copies) are transcribed (Bouchard 1982), but most of the repetitive sequences have unknown function and can exhibit large differences in size or copy number between closely related species (Dover et al. 1982, Flavell 1982). In birds, interspecific variation in DNA content is low relative to that of other vertebrates (Shields 1983). Nontranscribed highly repetitive DNA sequences ($>10^5$ copies) consist of very short (5–12 bp) tandemly repeated sequences (Singer 1982), and their function is unknown. It has been suggested that they function in chromosome recognition events and chromosome recombination (Jeffreys et al. 1985), or DNA sequence organization and reorganization (Gillespie et al. 1982). Amplification of different repeated DNA sequences in different populations might cause misalignment and failure of synapsis during meiosis in hybrids, causing hybrid sterility (Flavell 1982, Rose and Doolittle 1983). Differentiation in repetitive sequences therefore may be a factor that contributes to postzygotic isolation between species and promotes speciation (Dover et al. 1982, Flavell 1982).

Differentiation of highly repetitive sequences between species can be investigated by digestion of total DNA with restriction enzymes and comparison of fragment patterns by electrophoresis. Highly repeated sequences appear as distinct bands in the continuous smear of DNA fragments after electrophoresis. These bands contain sequences that represent at least 0.5% of the genome (Singer 1982). To investigate the extent to which highly repeated sequences reflect interspecific differentiation, we compared the digestion patterns of repetitive sequences from two species pairs. The two Old World flycatchers *Ficedula hypoleuca* and *F. albicollis* are thought to have a recent phylogenetic origin, whereas the two titmice, *Parus major* and *P. caeruleus*, are more distantly related.

We extracted genomic DNA from blood and muscle tissue of two individuals each of *Ficedula albicollis*, *F. hypoleuca*, *Parus caeruleus*, and *P. major* according to Gelter et al. (MS). Approximately 1 μg of DNA was digested with 3 units of restriction endonuclease in 400 μl for 5 h at +37°C with the appropriate buffer specified by the supplier, extracted once with phenol/chloroform, once with chloroform, precipitated with 99% ethanol at −70°C, pelleted at 12,000 g for 20 min, washed with 70% ethanol, and vacuum dried. Five restriction enzymes (*Hae*III, *Alu*I, *Hin*fI, *Eco*RI, and *Taq*I; obtained from Boehringer Mannheim or Pharmacia P-L Biochemicals) were used to analyze repetitive sequences. The digested DNA was redissolved overnight in 25 μl of TE (10 mM Tris, pH 7.0). Electrophoresis of DNA fragments was performed in 0.8% agarose gels for 5–12 h at 2–5 V/cm with phage lambda DNA digested with *Hin*dIII as fragment size standard. Gels were stained with ethidium bromide for 10 min, DNA was visualized on a short-wave UV transilluminator, and photographed.

All the five restriction enzymes used gave distinct

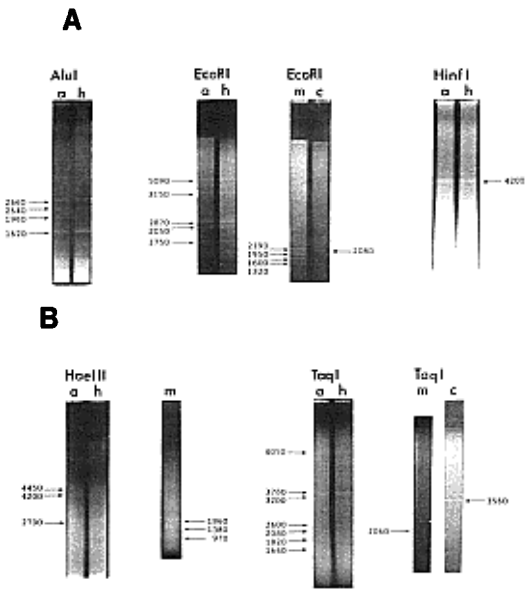

Fig. 1. Representative electropherograms of high-molecular-weight nuclear DNA after digestion with restriction endonucleases and electrophoresis in 0.8% agarose gels. Arrows indicate fragments of repetitive sequences, and the numbers denote sizes (base pairs) of DNA fragments. (A) *Alu*I, *Eco*RI, *Hin*fI; (B) *Hae*III, *Taq*I. (a) = *Ficedula albicollis*. (h) = *F. hypoleuca*, (c) = *Parus caeruleus* and (m) = *P. major*.

TABLE 1. Fragment sizes of repetitive sequences after digestion with five restriction enzymes. *Ficedula albicollis* (*F.a.*), *F. hypoleuca* (*F.h.*), *Parus caeruleus* (*P.c.*), and *P. major* (*P.m.*).

Enzyme	Species	Fragment no.						
		1	2	3	4	5	6	7
*Alu*I	*F.a.*	3,660	2,640	1,960	1,620			
	F.h.	3,660	2,640	1,960	1,620			
	P.c.	—						
	P.m.	5,160						
*Eco*RI	*F.a.*	5,900	3,150	2,870	2,050	1,750		
	F.h.	5,900	3,150	2,870	2,050	1,750		
	P.c.	2,080						
	P.m.	2,190	1,950	1,600	1,320			
*Hae*III	*F.a.*	4,450	4,200	2,790				
	F.h.	4,450	4,200	2,790				
	P.c.	—						
	P.m.	1,860	1,380	970				
*Hin*fI	*F.a.*	4,200						
	F.h.	4,200						
	P.c.	—						
	P.m.	—						
*Taq*I	*F.a.*	4,050	3,760	3,200	2,600	2,080	1,820	1,660
	F.h.	4,050	3,760	3,200	2,600	2,080	1,820	1,660
	P.c.	3,560						
	P.m.	2,060						

bands of repetitive sequences in the two *Ficedula* species (Fig. 1). In contrast, *Alu*I, *Hae*III, and *Hin*fI failed to reveal any repetitive fragments in *Parus caeruleus*, and *P. major* lacked repetitive fragments after digestion with *Hin*fI. The two *Ficedula* species showed a higher number of repetitive fragments than the two *Parus* species (Table 1). No fragments were identical between the two genera.

The two *Ficedula* species had identical repetitive sequence fragments for all five restriction enzymes (Table 1). Digestion with *Hin*fI gave one fragment. The other restriction enzymes produced three to seven repetitive fragments, which indicates either multiple restriction sites within the repetitive sequence or the presence of the same restriction site in different repetitive sequences.

In contrast, *P. caeruleus* and *P. major* showed many differences in repetitive sequences. After digestion with *Taq*I, both *P. caeruleus* and *P. major* showed only one fragment, but it had a different size in each species (Table 1). After digestion with *Eco*RI, *P. caeruleus* showed one fragment while *P. major* showed four fragments. *Parus major* showed one fragment after digestion with *Alu*I and three fragments after digestion with *Hae*III, but none of these fragments were present in *P. caeruleus*. *Parus caeruleus* has fewer repetitive fragments overall than *P. major*.

All five restriction enzymes produced ladderlike fragment patterns after incomplete digestion. This validated the repetitive nature of the fragments. When a recognition site for a particular restriction endonuclease occurs within a repeat unit, incomplete digestion converts the tandem array of repeats to a set of DNA fragments of multiples of the length between the repeated units, which appears as a "ladder" of fragments in the smear after electrophoresis (Hörz and Zachau 1977). The technique cannot distinguish between multiple cuts within a certain repetitive sequence and cuts in different repetitive sequences. Despite these limitations, the technique may be valuable for investigating differences in highly repetitive sequences between congeneric species.

We found identical fragment patterns of highly repetitive sequences in the two *Ficedula* species. The more distantly related *Parus* species have differentiated in their repetitive sequences. Lack of differentiation of highly repetitive sequences between the two *Ficedula* species indicates that sequence divergence was not involved in speciation, and is in accordance with a close allozymic similarity (Nei's genetic distance $D = 0.0006$, Gelter et al. 1989), similarity in morphology, behavior, and ecology. Hybrids between the two *Ficedula* species occur frequently in areas of sympatry (Löhrl 1950, Alatalo et al. 1982, Gelter 1987). Female hybrids are sterile, the male hybrids are fertile (Gelter et al. MS). Unless hybrid infertility evolves as a consequence of base-pair substitutions in single repeats of repetitive sequences, the infertility of hybrid *Ficedula* females cannot be attributed to divergence in highly repetitive sequences. Observed female hybrid sterility may rather be a consequence of lack of interactions between loci

on the Z and W chromosome (Gelter et al. MS). No hybrids between the two *Parus* species have been reported.

The similarity of the highly repetitive sequences in *Ficedula* and the observed differentiation of repetitive sequences in *Parus* suggest divergence of highly repetitive sequences by differentiation after speciation and correlation to overall differentiation between each species pair.

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Literature Cited

ALATALO, R. V., L. GUSTAFSSON, & A. LUNDBERG. 1982. Hybridization and breeding success of Collared and Pied flycatchers on the island of Gotland. Auk 99: 285–291.

ARTHUR, R. R., & N. A. STRAUS. 1978. DNA-sequence organization in the genome of the domestic chicken (*Gallus domesticus*). Can. J. Biochem. 56: 257–263.

BOUCHARD, R. A. 1982. Moderately repetitive DNA in evolution. Int. Rev. Cytol. 26: 113–193.

DOVER, G. A., S. BROWN, E. COEN, J. DALLAS, T. STRACHAN, & M. TRICK. 1982. The dynamics of genome evolution and species differentiation. Pp. 343–372 *in* Genome evolution (G. A. Dover and R. B. Flavell, Eds.). New York, Academic Press.

EDEN, F. C., & J. P. HENDRICK. 1978. Unusual organization of DNA sequences in the chicken. Biochemistry 17: 5838–5844.

EPPLEN, J. T., M. LEIPOLDT, W. ENGEL, & J. SCHMIDTKE. 1978. DNA sequence organization in avian genomes. Chromosoma 69: 307–321.

FLAVELL, R. B. 1982. Sequence amplification, deletion and rearrangement: major sources of variation during species divergence. Pp. 301–323 *in* Genome evolution (G. A. Dover and R. B. Flavell, Eds.). New York, Academic Press.

GELTER, H. P. 1987. Song differences between the Pied Flycatcher, *Ficedula hypoleuca*, the Collared Flycatcher, *F. albicollis*, and their hybrids. Ornis Scandinavica 18: 205–215.

———, H. TEGELSTRÖM, & G. STÅHL. 1989. Allozyme similarity and speciation in the Pied and Collared flycatcher (Aves: *Ficedula hypoleuca* and *F. albicollis*). Hereditas 111: 65–72.

GILLESPIE, D., L. DONEHOWER, & D. STRHYER. 1982. Evolution of primate DNA organization. Pp. 113–133 *in* Genome evolution (G. A. Dover and R. B. Flavell, Eds.). New York, Academic Press.

HÖRZ, W., & H. G. ZACHAU. 1977. Characterization of distinct segments in mouse satellite DNA by restriction nucleases. European J. Biochem. 73: 382–392.

JEFFREYS, A. J., V. WILSON, & S. L. TEIN. 1985. Hypervariable 'minisatellite' regions in human DNA. Nature 314: 67–73.

LÖHRL, H. 1950. Ein Bastard Halsbandschnäpper-Trauenschnäpper. Ornithol. Ber. 3: 126–130.

ROSE, M. R., & W. F. DOOLITTLE. 1983. Molecular biological mechanisms of speciation. Science 220: 157–162.

SHIELDS, G. F. 1983. Organization of the avian genome. Pp. 271–290 *in* Perspectives in ornithology (A. H. Brush and G. A. Clark Jr., Eds.). Cambridge, Cambridge Univ. Press.

SINGER, M. F. 1982. Highly repeated sequences in mammalian genomes. Int. Rev. Cytol. 76: 67–112.

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Terminal-Egg Chilling and Hatching Intervals in the American White Pelican

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With the onset of pipping, incubation behavior of many bird species alters rapidly as parents adjust their behavior to the changing nest contents. Typically, parents settle less tightly over pipped eggs and apply their brood patch(es) less closely (Drent 1973). In species where the young hatch asynchronously, further disruptions to incubation arise when parents begin to tend their first hatched young (Beer 1962, Nelson 1978). In extreme cases, parents may permanently neglect late-hatching eggs, which die (Gullion 1954, Beer 1962, Spellerberg 1971).

The adaptive significance of terminal-egg neglect has not been determined, but it is noteworthy that it has been reported mainly for ground-nesting nonpasserine species that commonly exhibit brood reduction after hatching. Graves et al. (1984) have suggested that neglect of the terminal egg would be an easy way for parents in one such species (the Herring Gull, *Larus argentatus*) to achieve brood reduction. Less extreme forms of terminal-egg neglect may also be relevant to brood reduction. It has been suggested, although not as yet directly proven under natural