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Divergence of the Single-Copy DNA Sequences of the Western Grebe (*Aechmophorus occidentalis*) and Clark's Grebe *(A. clarkii)*, as Indicated by DNA-DNA Hybridization

Jon E. Ahlquist, Anthony H. Bledsoe, John T. Ratti, and Charles G. Sibley

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Abstract

Single-copy nuclear DNA sequences of individuals of *Aechmophorus occidentalis* and *A. clarkii* were compared by DNA-DNA hybridization. In each of three experimental sets the average thermal stability of homoduplex and within-species DNA-DNA hybrids did not differ, but the between-species DNA-DNA hybrids dissociated at an average temperature 0.57°C below the median melting temperature of homoduplex and within-species hybrids. The difference was highly significant in all three sets. The median DNA sequence distance between *A. occidentalis* and *A. clarkii* is comparable to such distances between other closely related congeneric species.

Key Words

DNA-DNA hybridization, grebes, molecular evolution, avian systematics.

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Introduction

In 1858 George N. Lawrence described two new species of large grebes from specimens collected in the western United States and Mexico. Lawrence named the species *Podiceps occidentalis,* the Western Grebe, and *P. clarkii,* Clark's Grebe. Lawrence described *clarkii* as "a near ally of '*P. occidentalis,*' but . . . quite distinct. In general appearance and color they somewhat resemble each other, but [*clarkii*] is smaller, has the bill differently colored, and a conspicuous white mark before the eye." The specimens, which were collected between October and April, lacked the colorful "ruffs and crests" characteristic of the breeding plumages of most other large grebes, and Lawrence assumed that the birds were in winter plumage.

In a synopsis of North American loons and grebes, Coues (1862a) described a new genus, *Aechmophorus,* for *occidentalis* and *clarkii.* The breeding plumages were still unknown, and Coues' descriptions, like those of Lawrence, emphasized bill and loral differences. Coues (1862b) subsequently described a female specimen of *A. clarkii* "in full plumage" collected in late April. The bird had "a *decided occipital crest*" but "no decided colorful ruffs." Coues predicted that the breeding plumage of *occidentalis* would prove to be similar to that of *clarkii.*

Coues' prediction was proved correct

when breeding specimens of *occidentalis* became available. Additional specimens showed that the differences in body size and bill shape noted by Lawrence (1858) and Coues (1862a) were not consistent between *occidentalis* and *clarkii,* and the differences between them remained those of bill and loral color. Coues apparently considered these characters insufficient as the basis for specific rank and, by 1873, Coues was treating *clarkii* as a "variety" of *occidentalis.*

It now seemed clear that *clarkii* and *occidentalis* were conspecific, and even their separation as "varieties" was omitted by the committees that prepared the American Ornithologists' Union check-lists of North American birds from the first (1886) through the fifth (1957) editions. Similarly, Peters (1931) and Mayr and Short (1970) did not mention *clarkii.* Deignan (1961) designated specimen number 9930 in the collection of the United States National Museum as the lectotype for *Podiceps clarkii.* Dickerman (1963) restricted *P. clarkii* to this specimen and applied the name *Aechmophorus occidentalis clarkii* to the resident population of Mexico. The sixth edition of the A.O.U. check-list (1983, p. 10) recognized only *A. occidentalis,* but included a note concerning the two "morphs" and the possibility that they may " . . . represent distinct species." In the 35th supplement to the A.O.U. check-list (1985), *Aechmophorus clarkii* was considered to be a separate species from *A. occidentalis.*

In 1965, Storer reported the first evidence of positive assortative mating of sympatric populations of "dark phase" (Lawrence's *occidentalis*) and "light phase" (Lawrence's *clarkii*) grebes.

Between 1975 and 1977 Ratti (1979) studied sympatric populations of the two "morphs" in Utah, California, and Oregon. He observed 1977 mated pairs, of which 67.2% were between dark-phase birds, 32% were between light-phase birds, and 0.8% were mixed pairs. These values were significantly different from those expected if random mating were occurring, and it was apparent that the two-color types were almost completely reproductively isolated. Ratti (1979, p. 582) concluded that the two populations were functioning biologically "as separate species."

Nuechterlein (1981a, 1981b), Nuechterlein and Storer (1982) also observed assortative mating in mixed populations in Oregon and Manitoba. Using playback experiments, he demonstrated that the two morphs discriminate like from unlike on the basis of differences in their "advertising" calls, which provide the initial stimulus for courtship.

Storer and Nuechterlein (1985) analyzed the plumage and morphological characters of "the two color forms of the Western Grebe" and stated their preference for "the conservative treatment, that is, to recognize the two phases as species if and when the color phases in the Mexican population are shown to be reproductively isolated from each other."

Thus, the evidence concerning the taxonomic status of the Western and Clark's Grebes is mixed and a consensus has not yet been reached.

DNA-DNA Hybridization Comparisons

We have used DNA-DNA hybridization to measure the divergence between the single-copy nuclear DNA sequences of *A. occidentalis* and *A. clarkii.* The goals of the comparisons were 1) to determine if the DNA hybridization technique employed in studies of higher avian systematics can be used to assess genetic divergence between closely related species, such as the *Aechmophorus* grebes, and 2) to compare the level of genetic divergence between *A. occidentalis* and *A. clarkii* with the range of genetic distances between other congeneric and intergeneric avian species.

Methods

Our procedures are based on those of Britten and Kohne (1968), Kohne and Britten (1971), and Britten et al. (1974). The methods used in this study were described by Sibley and

Ahlquist (1981, 1982, 1983, 1984). Erythrocytes from 20 individuals each of *A. occidentalis* and *A. clarkii* were collected by Ratti at Bear River, Utah, in 1977, and each erythrocyte sample was prepared and analyzed separately. At three different times since collection, we have radio-labeled DNAs of these grebes and have treated them separately. In each of the three experimental sets the mean thermal stability of a homoduplex DNA-DNA hybrid (an individual's DNA hybridized with itself) was compared with the mean thermal stabilities of DNA-DNA hybrids between different individuals of the same species (=within-species hybrids), and between members of the two different species (=between-species hybrids). Three *occidentalis* and two *clarkii* radio-labeled single-copy (>Cot 500) DNA tracers were hybridized with eleven driver DNAs of each species, and each comparison was replicated five times. For each set, the mean $T_{50}H$ value was calculated for homoduplex, within-species, and between-species DNA-DNA hybrids. The $T_{50}H$ statistic provides an index to the median thermal stability of each DNA-DNA hybrid (see Sibley and Ahlquist 1983, p. 257–60 or Sibley and Ahlquist 1984, p. 234 for details). The equality of variances of mean $T_{50}H$ values was tested, and *t*-tests of the difference between two means were employed to determine the statistical significance of differences in mean thermal stability of homoduplex, within-species, and between-species DNA-DNA hybrids.

Table 1
Mean $T_{50}H$ values (x), variances (s^2), standard errors (se), and sample sizes (*N*) of homoduplex, within-species, and between-species comparisons between *Aechmophorus occidentalis* and *A. clarkii.*

	Homoduplex	Within-species	Between-species
Set 1:			
x	86.1	86.1	85.5
s^2	0.4	0.2	0.2
se	0.2	0.1	0.1
N	10	20	20
Set 2:			
x	85.5	85.6	84.9
s^2	0.2	0.1	0.3
se	0.2	0.1	0.1
N	10	20	20
Set 3:			
x	83.6	83.5	83.1
s^2	0.3	0.2	0.3
se	0.1	0.1	0.1
N	15	28	28

Results

Table 1 lists the mean $T_{50}H$, the variance, and the standard error of the mean, for homoduplex, within-species, and between-species comparisons. Within each analysis, the variances of the means did not differ significantly ($P \gg 0.05$, two-tailed tests of equality of variances), and the mean $T_{50}H$ values of homologous and within-species comparisons were not significantly different (*t*-tests of the difference between two means; analysis 1: $P \gg 0.05$, df = 28; analysis 2: $P \gg 0.05$, df = 28; analysis 3: $P \gg 0.05$, df = 41). However, the between-species duplexes dissociated at a mean temperature 0.6°, 0.6°, and 0.5°C below the melting temperature of the within-species duplexes. These differences were highly significant in all three analyses (analysis 1: $P < 0.001$, df = 38; analysis 2: $P < 0.001$, df = 38; analysis 3: $0.001 < P < 0.01$, df = 54), regardless of which species provided the radio-labeled tracer. The slight variation in mean thermal stability of the three homologous comparisons may have resulted from small differences in the average sizes of the tracer DNAs used in the three analyses (see Crothers et al. 1965 and Hayes et al. 1970).

Discussion

The results of the three analyses indicate that the DNA hybridization technique is sensitive to differences in sequence complementarity between closely related species. The average delta $T_{50}H$ between *A. occidentalis* and *A. clarkii* = 0.57 and corresponds to ca. 4.3 ×

Table 2

Average delta $T_{50}H$ values between congeneric taxa. The sample sizes of the averages are in parentheses next to the delta $T_{50}H$ for each comparison. We have not included comparisons for which only one measurement was available. Some of the pairs of species have been considered to be conspecific (e.g., *Passer,* Wolters 1982; *Promerops,* White 1963; *Sphecotheres,* Ford 1975), while others are so distant that they have been placed in separate genera (e.g., *Cormobates* for *Climacteris leucophaea,* Sibley et al. 1984; *Urocolius* for *Colius indicus,* Sibley and Ahlquist 1985).

Radio-labeled taxon (tracer)	Unlabeled taxon (driver)	Delta $T_{50}H$
Burhinus bistriatus	*B. superciliaris*	0.2 (2)
Passer domesticus	*P. hispaniolensis*	0.2 (2)
Fregata magnificens	*F. ariel*	0.4 (2)
Fregata magnificens	*F. minor*	0.4 (2)
Promerops cafer	*P. gurneyi*	0.5 (3)
Sphecotheres flaviventris	*S. vieilloti*	0.6 (2)
Apteryx australis mantelli	*A. australis australis*	0.7 (5)
Phalacrocorax auritus	*P. olivaceus*	0.8 (2)
Indicator maculatus	*I. variegatus*	0.9 (3)
Myiarchus tyrannulus	*M. ferox*	0.9 (3)
Apteryx australis	*A. haastii*	1.0 (16)
Coracias caudata	*C. benghalensis*	1.2 (2)
Cisticola chiniana	*C. tinniens*	1.2 (2)
Toxostoma longirostre	*T. dorsale*	1.3 (2)
Apteryx australis	*A. owenii*	1.4 (27)
Phalacrocorax auritus	*P. pelagicus*	1.5 (3)
Phalacrocorax auritus	*P. carbo*	1.6 (3)
Pelecanus erythrorhynchos	*P. onocrotalus*	1.8 (6)
Pelecanys erythrorhynchos	*P. rufescens*	1.8 (6)
Scytalopus femoralis	*S. latebricola*	2.0 (2)
Regulus calendula	*R. satrapa*	2.1 (3)
Telophorus olivaceus	*T. sulphureopectus*	2.3 (2)
Calidris minutilla	*C. alpina*	2.4 (2)
Parus atricapillus	*P. bicolor*	2.5 (3)
Formicarius rufipectus	*F. nigricapillus*	2.6 (2)
Toxorhamphus iliolophus	*T. novaeguineae*	2.8 (3)
Pelecanus erythrorhynchos	*P. occidentalis*	2.8 (3)
Pelecanus erythrorhynchos	*P. conspicillatus*	3.0 (2)
Sula dactylatra	*S. nebouxii*	3.3 (2)
Parus atricapilllus	*P. major*	3.6 (3)
Cuculus pallidus	*C. canorus*	4.1 (2)
Pterocles bicinctus	*P. decoratus*	4.6 (2)
Climacteris rufa	*C. leucophaea*	5.4 (2)
Pitta versicolor	*P. gujana*	7.3 (3)
Colius indicus	*C. striatus*	8.9 (3)

10^6 nucleotide substitutions in each lineage, because the single-copy fraction of the avian genome contains ca. 1.7×10^9 nucleotide pairs (Bachmann et al. 1972) and a delta value of 1.0 indicates that ca. 1% of the base pairs are mismatched (Bonner et al. 1973). This level of genetic differentiation between *A. occidentalis* and *A. clarkii* does not bear upon the question of their status as biological species. However, coupled with their widespread sympatry, positive assortative mating (Storer 1965, Ratti 1979, Nuechterlein 1981b, Lindvall and Low 1982), and weak morphological differentiation (Ratti et al. 1983), it supports the hypothesis that they are separate species.

The average delta $T_{50}H$ between *A. occidentalis* and *A. clarkii* is compared to

Table 3

Average delta $T_{50}H$ values between closely related genera. The sample size of the average is in parentheses next to the delta $T_{50}H$ for each comparison. We have included only comparisons for which more than one measurement was available.

Radio-labeled species (tracer)	Unlabeled species (driver)	Delta $T_{50}H$
Paradisaea minor	*Ptiloris paradiseus*	0.6 (3)
Grus canadensis	*Anthropoides paradisea*	0.7 (3)
Gymnogyps californianus	*Vultur gryphus*	1.1 (2)
Phoeniconaias minor	*Phoenicopterus chilensis*	1.5 (3)
Vultur gryphus	*Gymnogyps californianus*	1.5 (5)
Sarcoramphus papa	*Vultur gryphus*	1.7 (2)
Psarocolius angustifrons	*Cacicus cela*	1.9 (4)
Toxostoma longirostre	*Oreoscoptes montanus*	2.0 (2)
Cladorhynchus leucocephalus	*Himantopus himantopus*	2.1 (4)
Toxostoma longirostre	*Mimus polyglottos*	2.1 (3)
Cladorhynchus leucocephalus	*Recurvirostra americana*	2.3 (2)
Daptrius americanus	*Phalcobaenus megalopterus*	2.4 (2)
Sarcoramphus papa	*Cathartes aura*	2.4 (2)
Vultur gryphus	*Cathartes aura*	2.4 (7)
Gymnogyps californianus	*Cathartes aura*	2.5 (2)
Myiarchus tyrannulus	*Rhytipterna holerythra*	2.5 (2)
Monarcha guttula	*Chasiempis sandwicensis*	2.6 (2)
Mycteria ibis	*Ciconia ciconia*	2.8 (3)
Ptilonorhynchus violaceus	*Chlamydera nuchalis*	2.8 (4)
Dendroica striata	*Helmitheros vermivorus*	2.9 (5)
Sarcoramphus papa	*Coragyps atratus*	2.9 (3)
Vultur gryphus	*Coragyps atratus*	3.0 (5)
Mycteria ibis	*Ephippiorhynchus asiaticus*	3.0 (4)
Monarcha guttula	*Trochocercus cyanomelas*	3.1 (2)
Grus canadensis	*Balearica pavonina*	3.3 (3)
Psarocolius angustifrons	*Gymnomystax mexicanus*	3.3 (5)
Pooecetes gramineus	*Junco hyemalis*	3.3 (4)
Myiarchus tyrannulus	*Pitangus sulphuratus*	3.5 (3)
Sicalis luteola	*Nephelornis oneilli*	3.7 (2)
Pityriasis gymnocephala	*Cracticus quoyi*	3.9 (2)

those between other avian congeners in Table 2. There is a wide range of delta values between morphologically similar pairs. It is not surprising to find low delta $T_{50}H$ values between morphologically similar species such as *Passer domesticus* and *P. hispaniolensis,* but other species such as *Colius indicus* and *C. striatus* are distant enough genetically to warrant separate generic status. The delta $T_{50}H$ between the grebes falls near the low end of the range of delta values between congeners, but several taxa considered to be "good" species have lower delta values than the *Aechmophorus* grebes. Conversely, the delta $T_{50}H$ between two subspecies of Brown Kiwi (*Apteryx australis*) is slightly larger than the delta value between the grebes (Sibley and Ahlquist, personal communication). Thus, although delta $T_{50}H$ values cannot be used to establish species boundaries they do reflect the time of divergence of lineages and the extent of the median DNA sequence divergence between taxa.

The congeneric comparisons provide few examples of low delta values between morphologically dissimilar species because avian systematists traditionally have placed such species in different genera. Table 3 presents a sample of delta $T_{50}H$ values between species in different genera. Some of the species (e.g., *Paradisea minor* and *Ptiloris paradiseus*) differ markedly in appearance but are no more divergent genetically than the *Aechmophorus* grebes. It may be argued that some of these genera are not valid, and the comparisons of DNA sequence divergence support that argument. However, taxonomic rearrangement will not alter the fact that morphological and genetic differentiation do not "track" one another in many instances.

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The Authors

Jon E. Ahlquist, Anthony H. Bledsoe and **Charles G. Sibley.** Department of Biology and Peabody Museum of Natural History, Yale University, New Haven, CT 06511
John T. Ratti. Department of Zoology, Washington State University, Pullman, WA 99164

Present Addresses

JEA: Department of Zoological and Biomedical Sciences, Ohio University, Athens, OH 45701
AHB: Section of Birds, Carnegie Museum of Natural History, Pittsburgh, PA 15213
JTR: College of Forestry, University of Idaho, Moscow, ID 83843
CGS: Tiburon Center, San Francisco State University, Tiburon, CA 94920