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PHYLOGEOGRAPHIC PATTERNS IN MITOCHONDRIAL DNA OF THE OSTRICH (*STRUTHIO CAMELUS*)

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ABSTRACT.—We assayed restriction-site differences in mitochondrial DNA (mtDNA) within and among populations of the Ostrich (*Struthio camelus*) throughout much of its African distribution. Little genetic diversity was evident among samples drawn from localities throughout southern Africa (*S. c. australis*), while deep divisions in the mtDNA gene tree exist between representatives of the eastern (*S. c. molybdophanes* and *S. c. massaicus*) and northern African subspecies (*S. c. camelus*). The low mtDNA variability within *australis* and the presence of widespread mtDNA genotypes in this subspecies suggest considerable historical interconnectedness among populations, either through gene flow and/or recent colonization from smaller source populations. The strong phylogeographic structuring evident in eastern and northern Africa aligns with the currently accepted subspecies designations. Data indicate that the Ethiopian system of the Great Rift Valley has been effective in disrupting east-west gene flow between *molybdophanes* and *camelus*, while ecological differences and behavioral/reproductive cues have contributed to maintaining the genetic and phenotypic discreteness of *molybdophanes* and *massaicus* in east Africa. Although contemporary Ostrich populations are effectively divided into southern and northern populations by a belt of *Brachystegia* woodland, arid-corridor links in the recent evolutionary past appear to have allowed for periodic contact between *australis* and *massaicus* populations. Consequently, the development of subspecific differences between these two taxa has occurred within the context of shallow evolutionary separation. *Received 28 July 1992, accepted 25 November 1992.*

THE OSTRICH (*Struthio camelus*) is currently regarded as comprising four extant subspecies separated by fairly marked phenotypic differences; a fifth, *S. c. syriacus*, whose range once reportedly extended into Arabia, is now considered extinct (Brown et al. 1982). As presently understood, the species' natural range is restricted to the African continent, generally south of the Sahara. This distribution is disrupted by a belt of *Brachystegia* ("miombo") woodland in south-central Africa (Hamilton 1982) that effectively divides the Ostrich into northern and southern populations with the former incorporating *S. c. camelus*, *S. c. molybdophanes* and *S. c. massaicus*, while *S. c. australis* is confined to southern Africa (Brown et al. 1982).

Restriction-enzyme analysis of mitochondrial DNA (mtDNA) has been useful in the study of evolutionary relationships over microevolutionary time scales in a variety of avian species (e.g. Mack et al. 1986, Ovenden et al. 1987, Shields and Wilson 1987a, Avise and Zink 1988, Avise et al. 1990), often providing resolution beyond that yielded by more conventional approaches such as protein electrophoresis (Barrowclough 1983). Part of the utility of mtDNA analyses as a means for determining the magnitude of intraspecific polymorphism in organisms stems from its high rate of nucleotide-sequence evolution by base substitution, which can give rise to extensive sequence heterogeneity among conspecific individuals. Population genetic variation in mtDNA depends both on the maternal effective population size and life-history processes, with accumulating evidence clearly pointing to the dominant role that historic demographic and zoogeographic factors play in shaping intraspecific phylogenies (Avise 1989).

Geographic variation in size, plumage, eggshell porosity and assorted phenotypic characteristics, underpinnings of the intraspecific classification of the Ostrich (Brown et al. 1982), may be indicative of relatively low interpopulation gene flow across obvious geographic barriers, possibly promoted to some extent by the species' inability to fly. Given the tendency for mtDNA discontinuities to align with geographic boundaries in other species (Avise et al. 1987), we sought to determine whether geographic structuring in mtDNA haplotypes among the Ostrich subspecies and/or the northern and southern Ostrich populations may exist. We present a geographic survey of mtDNA variation in the Ostrich that includes representatives of the four extant subspecies drawn from lo-

TABLE 1. Ten mtDNA haplotypes in Ostriches from 26 African localities. Each haplotype composed of morph designations for restriction enzymes *Hinc* II, *Pvu* II, *Hind* III, *Xba* I, *Ava* I, *Dra* I, *Sac* I, *Ksp* I, *Bcl* I, *Ssp* I, *Stu* I, *Bgl* I, *Pst* I, *Sca* I, *Xho* I. Locality numbers correspond to those in Figure 1.

Lineage	Composite haplotype	*n*	Localities
A	AAAAAAAAAAAAAAA	41	1-3, 5-7, 10-14, 16, 18, 20-22
B	BBAAAAAAAAAAAAA	26	1, 4-12, 14, 15, 17, 18
C	AABAAAAAAAAAAAA	6	2, 6, 14, 15
D	BAAAAAAAAAAAAAA	11	1, 4, 22, 23, 24
E	BAABAAAAAAAAAAA	1	18
F	CAAAAAAAAAAAAAA	1	19
G	BACAAAAAAAAAAAA	2	24
H	AACBBBBBBAAAAAA	1	26
I	DCDCCCCBCBBBBA	7	25
J	ECDCCCCBCBBBBA	1	25

calities originating in southern, eastern and northern Africa. Our data show that, although strong geographic structure is evident from the genotypes distinguishing the eastern and north African subspecies, the *australis* clones are closely related and widespread suggesting considerable gene flow between populations. The low levels of sequence divergence distinguishing *australis* and *massaicus* and the presence of a shared lineage spanning the *Brachystegia* woodland indicate the transient nature of this contemporary barrier.

MATERIALS AND METHODS

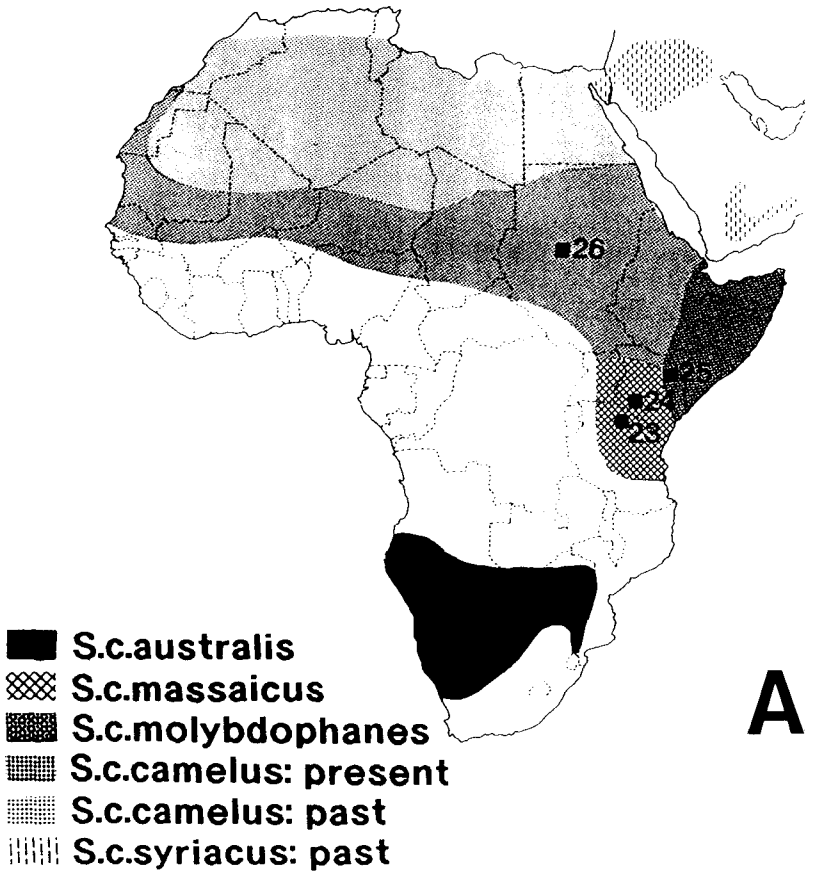
Since domestication has led to large-scale movement of birds to farms across national and geographic boundaries, the utmost care was taken to ensure the provenance of specimens used in this investigation. As a result, where possible, collection preference was given to material obtained from national parks and wildlife reserves, since extant populations in these facilities were almost invariably determined to have descended from birds resident in these areas prior to fencing. We also evaluated these populations on the grounds that there was no evidence of subsequent augmentation of these wild populations from elsewhere.

Ostrich samples ($n = 97$) were collected in the form of soft tissue or blood from wild and domestic representatives of the four extant ostrich subspecies from 26 African localities: *australis*, $n = 78$; *massaicus*, 10; *molybdophanes*, 8; *camelus*, 1 (Fig. 1). The single *camelus* specimen used in this study was obtained from Saudi Arabia, which falls outside of the species' natural range, but was initially derived from a population in the Sudan (Greth, Taif, Saudi Arabia). In instances of multiple sampling of subspecies, attempts were made to collect randomly from within different populations and, in the one instance where young birds were sampled, from single specimens taken from different areas within the Kleinsee Nature Reserve. Samples were assigned to a particular subspecies on the basis of morphological examination as well as collection locality.

Where soft tissue was available, mtDNA was extracted from heart and liver using conventional techniques (Lansman et al. 1981). In instances where birds could be restrained and blood samples drawn, high-molecular-weight total genomic DNA was obtained from venous blood (Wetton et al. 1987). Ample quantities of DNA were obtained from blood samples as small as 25 μL. Furthermore, as reported by other workers (Quinn and White 1987), we found minimal degradation in the DNA extracted from nucleated erythrocytes maintained at room temperature over extended periods.

Mitochondrial DNA (20-30 ng) and genomic DNA (800-900 ng) samples were digested with a suite of 15 type II restriction endonucleases (Table 1) following the conditions recommended by the manufacturers. The selection of restriction enzymes was based on consistent digestion of the Ostrich mtDNA. Restriction-fragment analyses were performed using two different experimental approaches. First, purified mtDNA fragments were end-labelled (Brown 1980) in the presence of (α-^{32})PdCTP and DNA polymerase (Klenow fragment). Subsequently, the end-labelled fragments were separated electrophoretically by molecular weight in 1.0% agarose gels, dried on 3MM Whatman paper on a slab gel drier and revealed by autoradiography. In cases where total genomic DNA was used, DNA fragments were separated through 0.8% agarose gels and transferred to nylon Hybond N membranes (Amersham) by Southern blotting (Southern 1975). Membrane-bound fragments were probed with purified (α-^{32})PdCTP-labelled mtDNA and the mtDNA-specific hybridizing fragments detected by autoradiography. Fragments were sized according to the molecular weight standards run on each gel.

Samples that produced unique fragment patterns with a particular enzyme were assigned a letter designation beginning with *A* and then proceeding alphabetically. The relative loss or gain of restriction


26
25
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23
S.c.australis
S.c.massaicus
S.c.molybdophanes
S.c.camelus: present
S.c.camelus: past
S.c.syriacus: past
A

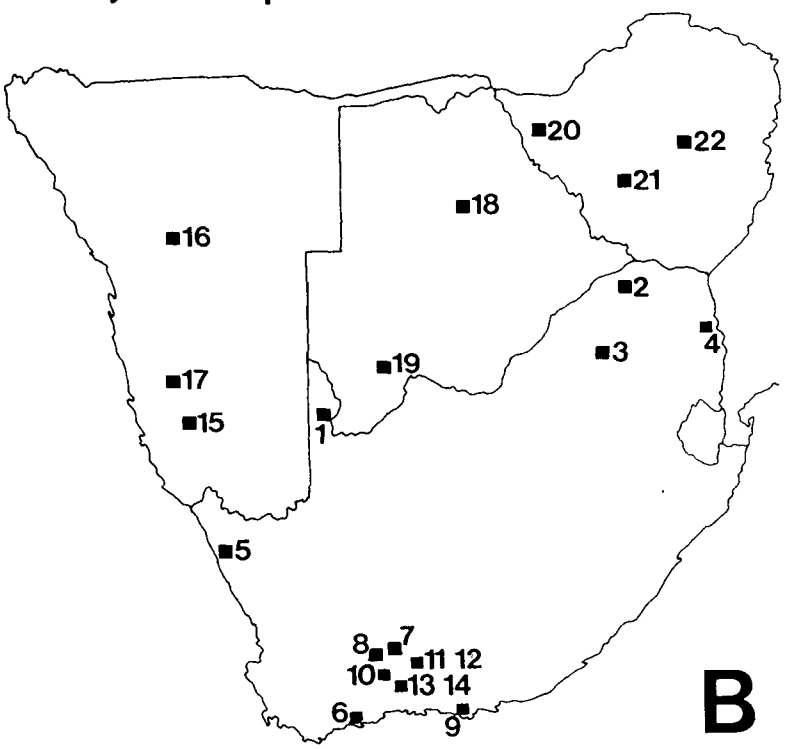
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TABLE 2. Percentage sequence divergence (nucleotide substitutions/site) between 10 mtDNA clones in the Ostrich (above diagonal). Values below diagonal are standard errors obtained via bootstrapping. Jukes-Cantor correction applied to all values. Comparisons along lineages A to H based on 15 enzymes, while those involving I and J based on 14 enzymes.

	A	B	C	D	E	F	G	H	I	J
A	—	0.26	0.13	0.13	0.26	0.47	0.26	1.15	7.59	7.66
B	0.16	—	0.40	0.13	0.26	0.52	0.26	1.40	6.68	6.74
C	0.13	0.21	—	0.25	0.40	0.60	0.39	1.33	7.54	7.61
D	0.12	0.12	0.17	—	0.13	0.40	0.12	1.32	7.30	7.36
E	0.20	0.18	0.22	0.14	—	0.53	0.26	1.17	7.22	7.29
F	0.59	0.47	0.68	0.45	0.50	—	0.53	1.84	8.69	8.80
G	0.17	0.19	0.26	0.11	0.20	0.45	—	1.15	7.33	7.39
H	0.47	0.19	0.48	0.41	0.39	0.65	0.45	—	8.24	8.31
I	1.75	1.48	1.73	1.76	1.60	2.09	1.62	2.08	—	0.15
J	1.70	1.66	1.44	1.63	1.66	1.89	1.59	1.90	0.13	—

sites between samples was determined by the additive loss or gain of appropriately sized fragments. A composite mtDNA haplotype derived from all digestion patterns was constructed for each individual. Individuals sharing identical composite haplotypes were grouped into maternal lineages or clones. Nucleotide-sequence-divergence estimates (between haplotypes and subspecies) were determined using equations 5.53–5.55 of Nei (1987). In estimating distances between subspecies, nucleotide diversity within each taxon was taken into account (Nei 1987: equation 10.21). All calculations were done by the Restsite computer program vI.I (Nei and Miller 1990). The Jukes-Cantor correction (Nei 1987:equation 5.3) was applied and standard errors were obtained by bootstrapping using 200 replications. The resulting matrix of genetic distance between clones was analyzed phenetically using the unweighted pair-group method with arithmetic averages (UPGMA; Sneath and Sokal 1973).

RESULTS

The 15 restriction endonucleases employed produced 63 to 70 scored fragments in each Ostrich analyzed, revealing an average of 67.1 cleavage sites. This represents approximately 739.2 base pairs (bp) in recognition sequence and 4.03% of the *Struthio* mitochondrial genome. By summing fragment sizes produced by five endonucleases yielding fragments smaller than 9.6 kb, we estimated the size of the Ostrich mitochondrial genome to be 18.348 kb ± SE of 83 bp (Fig. 2). No mtDNA size variants were observed with each specimen appearing homoplasmic for a specific mtDNA genotype.

The *Sca* I restriction digestion profile was identical in all 97 specimens, while *Xho* I fragment patterns were invariant among all *australis*, *massaicus* and *camelus* individuals. Unfortunately, *Xho* I data for *molybdophanes* could not be obtained due to consistent partial digestions (or the complete absence of digestion) of the genomic DNA samples and this enzyme was, thus, omitted from our analyses involving this subspecies. On the basis of shared composite haplotypes the 97 study specimens could be grouped into 10 discrete maternal lineages or clones (Table 1).

Pairwise estimates of nucleotide divergence among the 10 clones are presented in Table 2. While lineages H to J are relatively distinct,

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Fig. 1. **(A)** Historic distribution of Ostriches across the African continent and Arabia (from Brown et al. 1982 and Greth, Taif, Saudi Arabia) and map positions of samples collected in: **Tanzania:** (23) Arusha. **Kenya:** (24) *Kajiado (Three Point Ostrich Farm); (25) *Lewa Downs, northern slopes of Mt. Kenya. **Sudan:** (26) Exact Sudanese locality unknown; specimen provided from an *S. c. camelus* population maintained at Taif, Saudi Arabia. **(B)** Southern African collection localities of Ostrich samples. **South Africa:** (1) Kalahari Gemsbok National Park; (2) Langjan Nature Reserve; (3) Nylsvlei Nature Reserve; (4) Kruger National Park; (5) Kleinsee Nature Reserve; (6) De Hoop Nature Reserve; (7) *Prince Albert; (8) *Ladismith; (9) *Joubertina; (10) *Matjiesrivier; (11) *Oude Muragie, De Rust; (12) *Doornkraal, De Rust; (13) *Proefplaas, Oudtshoorn; (14) *Van Wykskraal, Oudtshoorn. **Namibia:** (15) Gorrasis Game Farm; (16) Ongombeanavita Farm; (17) Escourt Farm. **Botswana:** (18) Makgadikgadi Pans; (19) Mabuasehube Game Reserve. **Zimbabwe:** (20) Hwange National Park; (21) Northern Matabeleland; (22) Centeral Estate. Those with an asterisk (*) represent domestic stocks.

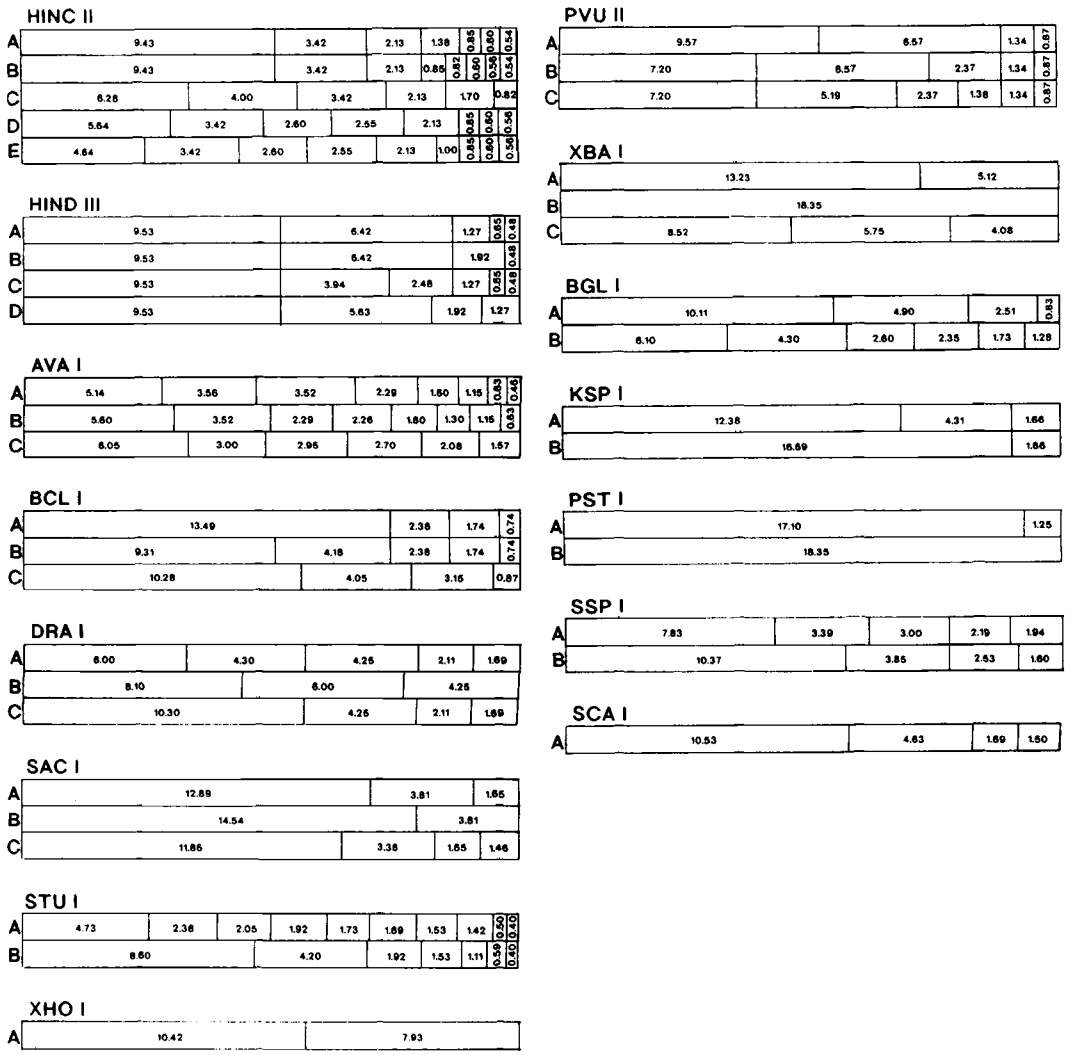

Fig. 2. Graphic representation of the fragment composition of restriction profiles identified from Ostrich mtDNA. Fragment sizes scaled so that their sum equals overall mean mitochondrial genome size.

estimates between clones A to G are low and range from 0.12 to 0.60%. Nucleotide-divergence estimates between subspecies, allowing for within-taxon sequence diversity, indicate (Table 3) that *australis* and *massaicus* are closely allied (0.06%), while *camelus* and *molybdophanes* are the most distantly related (8.23%).

The relationship among the 10 mtDNA clones is summarized in the UPGMA phenogram (Fig. 3). As expected all *australis* and *massaicus* mtDNA haplotypes clustered together at low levels of genetic distance, implying an absence of geographic structuring in the clonal branches, while *camelus* and *molybdophanes* join the major cluster as more distinct genotypes.

Although no meaningful population differentiation was evident from the extensively surveyed southern African range of the Ostrich, a marked degree of phylogeographic structuring was found in northeastern Africa, with mtDNA lineages aligning with subspecific designations (Fig. 4). The two *molybdophanes* lineages (I and J) which differ by a single restriction site are, respectively, separated from the *camelus* genotype (H) to the north, and the *massaicus* genotypes (D and G) to the south by a minimum of

TABLE 3. Percentage sequence divergence (nucleotide substitutions/site) among *S. c. australis*, *S. c. camelus*, *S. c. molybdophanes* and *S. c. massaicus* (above diagonal). Values below diagonal are standard errors obtained via bootstrapping. Jukes-Cantor correction applied to all values. Comparisons involving *S. c. australis*, *S. c. massaicus* and *S. c. camelus* based on 15 enzymes, while those involving *S. c. molybdophanes* based on 14 enzymes. Subspecific nucleotide diversity (presented along diagonal) taken into account in these calculations.

Subspecies	*australis*	*massaicus*	*camelus*	*molybdophanes*
australis	0.15	0.06	1.21	7.16
massaicus	0.04	0.05	1.27	7.25
camelus	0.39	0.38	—	8.23
molybdophanes	1.67	1.62	2.09	0.04

45 mutational steps. Interestingly, the *camelus* lineage H is more closely allied to the northernmost, and most abundant, *australis* lineage (A), differing by nine mutational steps, which is in sharp contrast to the more pronounced differences between *camelus* and the geographically more closely allied *molybdophanes*.

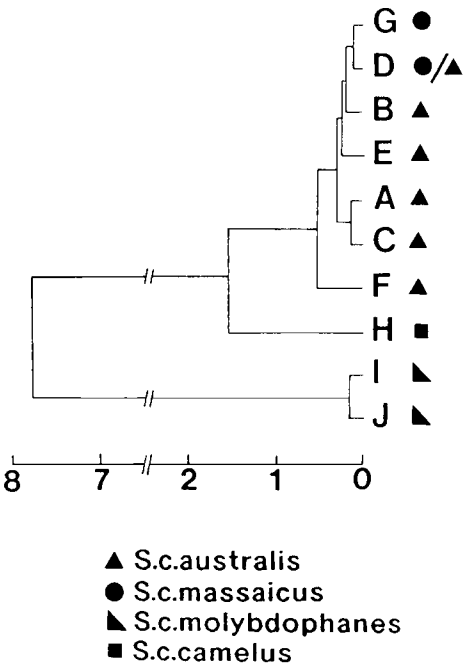

Fig. 3. Phenogram derived from UPGMA cluster analysis of 10 mtDNA haplotypes of the Ostrich. A to J represent individual haplotypes. Scale reflects percentage sequence divergence.

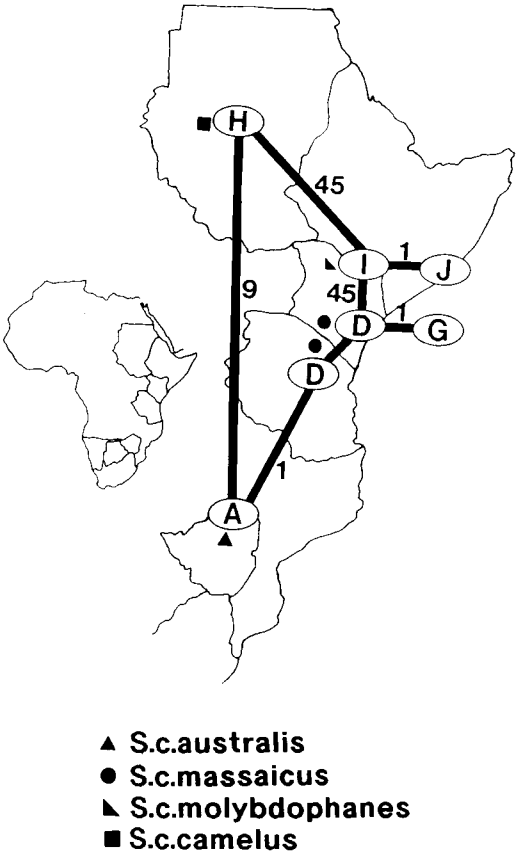

Fig. 4. Parsimony network (Lansman et al. 1983) of minimum number of mutational steps linking the most common and widespread *S. c. australis* haplotype (A) to those detected in *S. c. massaicus*, *S. c. molybdophanes* and *S. c. camelus*. Numbers indicate minimum numbers of restriction-site changes for adjacent lineages.

DISCUSSION

The 78 southern African *australis* specimens drawn from 22 populations were characterized by low levels of mtDNA diversity, both in terms of the number of clones present and the close relationship among them (Tables 1 and 2). In our widespread survey of this subspecies, only six maternal lineages were detected, a low number when compared to that found in most regional surveys of other vertebrate species (e.g. Avise et al. 1979, Brown and Simpson 1981, Lansman et al. 1983). The close affinity between matrilines is clear, with most differing at either one or two restriction sites, and the subspecies being characterized by a mean nucleotide diversity of 0.15 ± 0.08%.

Several reasons could account for these observations. First, the limited mtDNA diversity could result from directional selection (Avise and Ball 1991) favoring particular genotypes, thereby reducing the level of mtDNA polymorphism within this subspecies. A second possibility is that the southern African Ostrich population has gone through a population bottleneck in its recent evolutionary past (similar to that recorded in the period 1886–1890, when severe drought and unknown epidemics caused heavy Ostrich mortality in the region; Smit 1963). Such a reduction in effective population size could have been followed by rapid expansion and subsequent recolonization from small refugial populations. Finally, although there have been relatively few large-scale studies of avian geographic mtDNA structuring to date, examples of limited geographic partitioning of mtDNA genotypes include the Red-winged Blackbird (*Agelaius phoeniceus*; Ball et al. 1988) and the Downy Woodpecker (*Picoides pubescens*; Ball and Avise 1992). In these species, historical interconnectedness and the lack of barriers to migration were invoked to account for the limited mtDNA diversity. Clearly, any of these factors, either individually or in concert, may have influenced mtDNA variability in the Ostrich. However, it seems likely that although flightless, the species' high vagility and the absence of meaningful zoogeographic barriers has undoubtedly promoted high levels of gene flow in southern Africa.

The low mtDNA diversity within the southern African Ostrich population was similarly accompanied by a lack of spatial structuring of the lineages. Populations separated by as much as 2,000 km appeared indistinguishable on the basis of mtDNA analysis, with the most common lineage (A) found at 16 of the 22 collection localities. This lack of genetic geographic structure within *australis* and the associated implication of gene flow within the subspecies' range allows speculation on the possible effects that the historic introduction of extralimital Ostrich may have had on mtDNA diversity in the southern African subregion. These importations, ostensibly to improve feather quality of domesticated Ostrich stocks, involved the 1876 introduction of small numbers of "Barbary birds" (presumably *camelus*; the Barbary States were Tripoli, Algiers, Morocco and Tunisia) and the now extinct *syriacus*. These early introductions appear to have been followed by the importation of 132 *camelus* from Nigeria in 1912 (Thornton et al. 1961 *in* Smit 1963). Subsequently, the uncontrolled relocation of fertile hybrid Ostriches to numerous localities throughout South Africa has raised fears about widespread introgression to the point where conservation agencies are concerned for the genetic integrity of *australis*.

Clearly, no lineage differences were found in *australis* that approximate the magnitude of change characterizing the *camelus* genotype analyzed in our material (see below). Furthermore, the domesticated populations in the Little Karoo (collection localities 7 to 14, Fig. 1), the presumed foci of the introductions, are not noteworthy for increased levels of mtDNA heterogeneity. Given the distinctness of the *camelus* genotype, and assuming that this is representative of lineages in this subspecies, it could be concluded, albeit indirectly, that little evidence for the continued survival of extralimital lineages within the *australis* gene pool exists. However, while all southern African Ostrich populations are genetically very similar as measured by mtDNA analysis, this should not be construed (in isolation) as unequivocal support for the abolition of regional controls on the movement of Ostriches as part of agricultural practices in these regions. Obviously, these data reflect only mtDNA lineage survivorship in extant *australis* populations and not on the possible extent of nuclear introgression.

Our data also show that the contemporary *Brachystegia* or "miombo" woodlands in Tanzania, Zambia, northern Angola and Zaire (Hamilton 1982) separating Ostriches into northern and southern populations has probably not been an effective zoogeographic barrier over evolutionary time. The differentiation of the eastern African *massaicus* from the southern African *australis* is weak (0.06% nucleotide divergence), and is probably due to their relatively recent isolation and probable gene flow in times of a receding *Brachystegia* woodland. During such periods the arid southwestern and Saharo-Sindic regions of Africa were reportedly connected by an arid corridor (Moreau 1966, Verdcourt 1969, Hamilton 1976, 1982, Kingdon 1971, 1990) the most recent of which probably occurred approximately 20,000 to 12,000 y.b.p. (Hamilton 1982). Consequently, the development of subspecific differences between *australis* and *massaicus* has occurred within the context of shallow evolutionary separation. Also,

it is feasible that some of these morphological differences are ecophenotypic (not based entirely on genetic differences) as has been suggested for the Red-winged Blackbird by the nestling transplantation experiments of James (1983).

In contrast, however, to the close mtDNA relationship evident between the *australis* and *massaicus* lineages detected in this study, *camelus* and *molybdophanes* are clearly divergent from other sample genotypes. The solitary *camelus* specimen had a maternal lineage that was distinct from all other sampled genotypes being more divergent from *massaicus* (1.27% sequence divergence) and *australis* (1.21% divergence; Table 3) than representatives of either of these two subspecies were from each other. The eight *molybdophanes* samples comprised two genotypes which were closely related to one another, but were extremely divergent from any of the clones in the other three subspecies. As suggested by Figure 3, *molybdophanes* appears to have diverged from the common ancestor to the other three subspecies approximately 3.6 to 4.1 million years ago (assuming a 2% per million year rate of sequence evolution; see Helm-Bychowski 1984 *in* Avise and Ball 1991, Shields and Wilson 1987b). We speculate that the genetic distinctness of *molybdophanes* arose as a result of isolation probably caused by the Ethiopian system of the Great Rift Valley, which may have served as a major geographic barrier disrupting gene flow. This pattern of geographically replacing species/subspecies pairs on either side of African Rift Valleys has been documented for a variety of birds (Benson et al. 1962). It has been suggested that the floors of these valleys are ecologically different from the surrounding higher elevation areas and, as such, provide an unfavorable biome to species living in the environments at higher levels (Benson et al. 1962).

Attempts to explain the large phylogenetic separation between *molybdophanes* and *massaicus* (7.25% nucleotide divergence; Table 3) are more problematic, given the lack of any readily identifiable zoogeographic barrier separating their ranges and their intrinsic dispersal capabilities. Nonetheless, the mtDNA divergence between these taxa appears to be reflected in their different ecological requirements, as well as behavioral/reproductive cues. Jackson (1938) and Lewis and Pomeroy (1989) reported that *molybdophanes* readily enters more bushed regions (and is a browser) than the strictly open savanna *massaicus* (a grazer). Furthermore, in addition to these adaptational differences, there are reports indicating interbreeding difficulties between them (Brown et al. 1982, Lewis and Pomeroy 1989). This supports published suggestions that *molybdophanes* is phenotypically the most distinct of the Ostrich subspecies, and that separate species status may be warranted (Brown et al. 1982, Lewis and Pomeroy 1989), an observation which is strengthened by the magnitude of sequence divergence between *molybdophanes* and other Ostrich lineages detected in our study.

Acknowledgments

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