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Identification of the subspecies of *Oreochromis niloticus* (Pisces: Cichlidae) using restriction endonuclease analysis of mitochondrial DNA

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ABSTRACT

Seyoum, S. and Kornfield, I., 1992. Identification of the subspecies of *Oreochromis niloticus* (Pisces: Cichlidae) using restriction endonuclease analysis of mitochondrial DNA. *Aquaculture*, 102: 29–42.

Restriction endonuclease analysis of mitochondrial DNA (mtDNA) was used to characterize differentiation among the seven described subspecies of *Oreochromis niloticus* from East Africa and Egypt. Mitochondrial DNAs of 14 populations were examined with 42 restriction endonucleases. Two subspecies of *Oreochromis spilurus* were also examined. Approximately 8% of the mitochondrial genome of *O. niloticus* (17 070±40 base pairs) was assayed; 29 (70%) of the 42 restriction enzymes that were examined displayed restriction phenotypes that varied among the samples analyzed.

Several endonucleases produced restriction phenotypes which were diagnostic for the described subspecies of *O. niloticus*, though a few samples did not agree with the conventional taxonomy of the group. Extensive differentiation of some populations suggests that additional taxonomic recognition is warranted. Because all subspecies of *Oreochromis niloticus* could be distinguished by their unique restriction enzyme profiles, analysis of mtDNA can be used to identify the origin of cultured stocks. This study provides the first molecular key for the objective identification of this taxon.

INTRODUCTION

The cichlid fish *Oreochromis niloticus* (Linnaeus) is one of the most important taxa in global aquaculture today (FAO, 1980). The species occurs naturally in East and West Africa, though its distribution is not continuous. It is probably the most widespread and the most abundant of all the tilapiine species having been extensively introduced into many areas of the world (Welcomme, 1981). These introduced populations of *O. niloticus* have been founded from a variety of both natural and cultured sources (Trewavas,

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1983). The identification of taxa and of cultured stocks is central to aquaculture and effective fishery management of tilapias.

Oreochromis niloticus has been subjected to detailed morphological study and analysis. The most recent systematic treatment (Trewavas, 1983) divided the taxon into seven subspecies: *O. niloticus baringoensis* Trewavas, *O. n. cancellatus* (Nichols), *O. n. eduardianus* (Boulenger), *O. n. filoa* Trewavas, *O. n. niloticus* (Linnaeus), *O. n. sugutae* Trewavas, and *O. n. vulcani* (Trewavas). This classification is based on osteological features and composite differences in meristic and morphometric characters. However, none of these characters, either singly or in combination, can be used to unambiguously identify individual fish: all characters overlap among subspecies. Additionally, this situation can be exacerbated by potential environmental influences on meristic traits, morphometric characters and population parameters (Dentry and Lindsey, 1978; Ryman et al., 1984; Beacham and Murry, 1986). If some attributes on which these taxa have been characterized are indeed plastic, their usefulness for stock identification in aquaculture may be limited.

Protein electrophoresis has been used extensively to delineate species and differentiation among populations of fishes (e.g. Shaklee, 1983; Utter, 1987). In tilapias, some studies using allozymes have very successfully discriminated among species (Kornfield et al., 1979; McAndrew and Majumdar, 1983, 1984). However, many species of cichlids are exceedingly similar when assayed by this technique, possessing identical alleles at most loci (Sage and Selander, 1975; Kornfield, 1984; McKaye et al., 1984; Sage et al., 1984; Kornfield 1991). This is particularly true for taxa of very recent origin. In such cases, protein electrophoresis is not sufficiently sensitive to reveal discriminatory genetic characteristics. Extensive protein electrophoresis was conducted by Seyoum (1989, 1990) for eleven population samples representing five subspecies of *O. niloticus* (*O. n. baringoensis, O. n. cancellatus, O. n. filoa, O. n. sugutae,* and *O. n. vulcani*). Though four of the 32 resolved loci were polymorphic, no diagnostic alleles were present and allele frequencies could not be used to discriminate among subspecies. Thus, an alternative method for objective identification of tilapias was pursued.

The technique of restriction endonuclease analysis of mitochondrial DNA (mtDNA) has been successfully exploited for stock identification and population analysis in fishes (Ferris and Berg, 1987; Gyllensten and Wilson, 1987; Kornfield and Bogdanowicz, 1987). Because of its small size, taxonomic homology, absence of recombination and ease of isolation, the mtDNA genome is an excellent system for population analysis (Wilson et al., 1985; Avise et al., 1987; Moritz et al., 1987). Strict maternal inheritance (Lansman et al., 1981) insures that each lineage accumulates its own mutations in the mtDNA genome. Further, the apparent rapid rate of mtDNA evolution relative to nuclear DNA (Brown, 1983) has provided sensitivity sufficient to resolve acute taxonomic problems in ichthyology (Avise et al., 1986). Restriction enzyme

analysis of mtDNA permits quantification of variation at the nucleotide level and provides a large number of characters for comparative purposes. Here we exploit restriction enzyme analysis of mtDNA to define the subspecies of *O. niloticus*.

MATERIALS AND METHODS

Twelve populations of *Oreochromis niloticus* and two populations of the related congener *O. spilurus* were examined (Table 1). A few of the samples were taken from waters that are interconnected to some degree. Three samples from the Gallo Lakes in the Ethiopian Rift (Lakes Abijata, Langano and Zewai) are interconnected by intermittent streams; these three localities are lumped together in analyses. There are potential interconnections between the Blue Nile and the White Nile, but distances between populations (e.g. in Lake Albert and Lake Tana) are very large and intervening habitats are very diverse.

Specimens were dissected in the field and tissues were placed in cryotubes and transported to the laboratory on dry ice. Tissue samples were stored up to 7 months at $-70°C$ prior to analysis.

Mitochondrial DNA was extracted from ovarian tissue using cesium chloride/ethidium bromide gradient ultracentrifugation (Lansman et al., 1981). Aliquots of purified mtDNA were digested (Maniatias et al., 1982) for 5 h

TABLE 1

Source and samples of *Oreochromis niloticus* and *Oreochromis spilurus*. Nomenclature according to Trewavas (1983); acronym according to the results in this study. *N* indicates sample size

Taxon	Source[a]	Latitude	Longitude	*N*	Acronym
O. n. baringoensis	L. Baringo, Kenya	0° 38′N	36° 05′E	3	BB
O. n. cancellatus	L. Abijata,[b] Ethiopia	7° 38′N	38° 27′E	9	CC
O. n. cancellatus	L. Awassa, Ethiopia	7° 03′N	38° 27′E	4	CC
O. n. cancellatus	L. Akaki, Ethiopia	8° 40′N	38° 35′E	3	FF
O. n. cancellatus	L. Chamo, Ethiopia	5° 50′N	37° 40′E	3	CC
O. n. cancellatus	L. Tana, Ethiopia	12° 00′N	37° 20′E	3	CT
O. n. eduardianus	L. Victoria, Kenya	1° 00′S	33° 00′E	3	EV
O. n. filoa	R. Awash (Metehara), Ethiopia[c]	8° 53′N	39° 53′E	3	FF
O. n. filoa	L. Beseka, Ethiopia	8° 52′N	39° 52′E	3	CC
O. n. niloticus	L. Manzilah, Egypt	31° 08′N	32° 00′E	3	NM
O. n. sugutae	R. Suguta (Lopetakinyanga), Kenya	1° 00′N	36° 15′E	3	SS
O. n. vulcani	L. Turkana (Loyengalini), Kenya	3° 30′N	36° 05′E	3	VT
O. s. spilurus	R. Tana, Kenya	1° 03′N	37° 05′E	5	ST
O. s. niger	L. Turkana, Kenya	3° 30′N	36° 05′E	3	ST

[a]L = Lake (Amaharic, Hyke); R = River (Wonze).
[b]Includes samples from interconnected Lake Langano (latitude 7° 38′N, longitude 38° 35′E) and Lake Zewai (8° 00′N, 38° 50′E).
[c]Names in parentheses are local names of places where samples were collected.

using 42 restriction endonucleases: 27 six-base enzymes (*Apa*I, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*EII, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hpa*I, *Kpn*I, *Mlu*I, *Nar*I, *Nco*I, *Nde*I, *Nru*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, *Sph*I, *Sst*II, *Xba*I, *Xho*I, *Xmn*I), three 5.3-base enzymes (*Ava*I, *Hinc*II, *Sty*I), one 5-base enzyme (*Mbo*II), three 4.6-base enzymes (*Ava*II, *Eco*RII, *Nci*I) and eight four-base enzymes (*Hae*III, *Hin*fI, *Hha*I, *Mbo*I, *Msp*I, *Rsa*I, *Tha*I, *Taq*I). The resulting cleavage fragments were endlabelled with ^{32}P dNTPs by the method of Drouin (1980). Fragments were separated according to size by standard agarose gel (1–2%) electrophoresis using 1000-base-pair (bp)-size standards (Bethesda Research Laboratories, Bethesda, MD) on each gel. Restriction fragment patterns were visualized by autoradiography (Kodak XO-Mat AR film and Dupont Cronex Lightning Plus screens); exposure was typically 2 h at room temperature. Restriction fragment sizes were determined from autoradiographs using a polynomial regression equation derived from the fragment mobilities of the standard.

For each population, a minimum of three individuals was examined. A single letter was assigned to each cleavage phenotype observed for each enzyme; a 29-letter sequence constitutes the composite phenotype or clonal type of a specimen. Thirteen enzymes that did not cleave the mtDNA, or gave identical multibanded patterns, were not included in the composite phenotype.

RESULTS

Of the 42 restriction enzymes examined, ten did not cleave the mtDNA genome (*Bam*HI, *Cla*I, *Eco*RI, *Kpn*I, *Mlu*I, *Nru*I, *Sal*I, *Sma*I, *Sst*II, and *Xho*I). Three additional enzymes (*Bgl*I, *Eco*RV, and *Nar*I) had identical multibanded phenotypes among all subspecies and *O. spilurus*. The mtDNA genome was estimated to be 17 020 ± 40 base pairs in length; based on the number of fragments visualized, we estimate that approximately 8% of the mitochondrial genome was assayed.

A total of 97 phenotypes was observed for the 29 polymorphic enzymes (Table 2). The fragment sizes of these phenotypes are provided in the Appendix. MtDNA restriction fragment patterns produced with *Apa*I are illustrated in Fig. 1. No intrapopulation polymorphisms were observed for any enzyme; from each population, all individuals examined had identical phenotypes.

Several unique and discriminatory cleavage phenotypes were found for the subspecies, with the highest degree of resolution observed using four-base restriction enzymes. A dichotomous key to the described subspecies of *O. niloticus* using diagnostic mtDNA restriction enzyme phenotypes is given in Table 3. The key was constructed employing six enzymes and can be used to objectively identify single individuals in the absence of locality information.

Of the seven populations morphologically assigned to *O. niloticus cancellatus* (Trewavas, 1983), five (Lakes Abijata, Awassa, Chamo, Langano and

TABLE 2

Mitochondrial DNA cleavage phenotypes of *Oreochromis niloticus* subspecies and *Oreochromis spilurus*. Sample size was three individuals for each population. Abbreviations of samples as in Table 1

Restriction enzyme	Samples								
	BB	CC	CT	EV	FF	NM	SS	VT	ST[a]
*Apa*I	I	G	H	M	G	H	H	H	E
*Ava*I	C	A	B	B	A	B	B	B	G
*Ava*II	M	A	E	D	A	D	D	D	C
*Bcl*I	A	A	A	A	B	A	A	A	A
*Bgl*II	A	B	A	A	B	A	A	A	E
*Bst*EII	D	A	A	B	A	B	B	B	B
*Dra*I	B	A	B	H	A	B	B	B	A
*Eco*RII	C	A	A	C	A	A	B	B	E
*Hae*III	A	H	A	B	H	C	A	A	D
*Hha*I	E	B	A	E	D	A	E	E	G
*Hinc*II	B	A	B	B	A	B	B	B	C
*Hind*III	A	A	D	D	A	D	D	D	A
*Hinf*I	C	B	A	A	B	A	D	A	E
*Hpa*I	A	B	A	A	B	A	A	A	A
*Mbo*I	F	D	F	F	A	F	B	F	E
*Mbo*II	A	A	C	A	E	A	A	B	D
*Msp*I	B	E	B	J	E	B	B	B	D
*Nci*I	B	A	B	B	A	X	B	B	E
*Nco*I	A	A	A	A	A	A	A	A	C
*Nde*I	A	A	A	A	A	A	A	A	C
*Pst*I	B	A	A	B	A	A	A	A	A
*Pvu*II	A	A	E	A	A	A	A	A	A
*Rsa*I	B	D	E	G	D	G	A	F	C
*Sph*I	B	A	A	A	A	A	A	A	E
*Sty*I	D	A	A	A	A	A	A	A	E
*Taq*I	C	B	A	D	B	C	C	C	F
*Tha*I	A	B	A	A	B	A	A	A	B
*Xba*I	A	A	A	A	A	A	A	A	B
*Xmn*I	C	A	A	C	A	C	C	C	A
Number of populations sampled	1	6	1	1	2	1	1	1	2

[a]Includes both subspecies of *O. spilurus* (*O. s. niger* and *O. s. spilurus*).

Zewai) showed identical mtDNA composite phenotypes. However, the sample of *O. n. cancellatus* from Lake Tana exhibited different phenotypes for 19 of the 29 polymorphic enzymes and the sample of *O. n. cancellatus* from Lake Akaki showed mtDNA composite phenotypes different from its assigned subspecies. In addition, the samples from Lake Beseka were not similar to *O. n. filoa*, the taxon to which they had been assigned (see Discussion).

Pairwise comparison of the number of differences in mtDNA composite

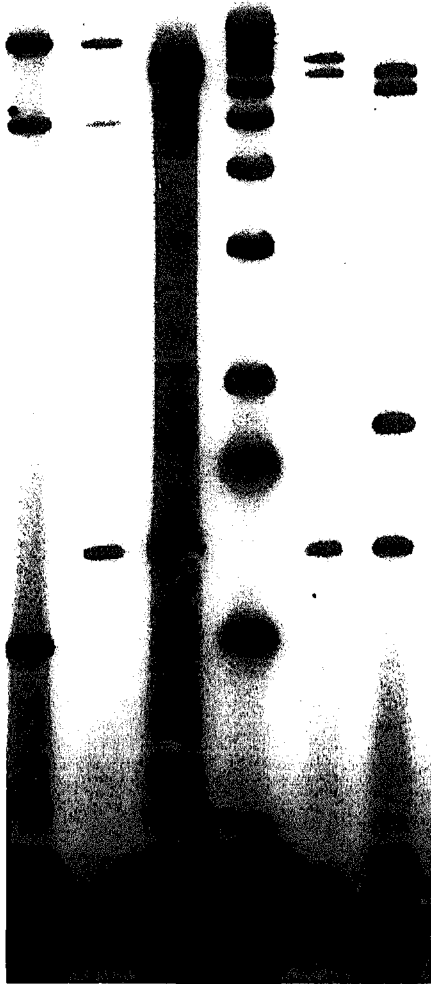

Fig. 1. Mitochondrial DNA restriction fragment patterns produced with *Apa*I. Samples (left to right), *Oreochromis niloticus cancellatus*, *O. spilurus spilurus*, *O. n. vulcani*, 1 kb molecular weight standard, *O. n. sugutae*, and *O. n. baringoensis*.

phenotypes between samples of the subspecies of *O. niloticus* and between *O. spilurus* are provided in Table 4. Each phenotypic difference represents (minimally) one mutation step. Of particular note are differences exhibited by the samples from Lake Tana that had been assigned to *O. n. cancellatus* (Trewavas, 1983). The two subspecies of *O. spilurus* showed identical phenotypes for all 29 polymorphic enzymes. Formal treatment of the evolutionary relationships among the subspecies will be presented elsewhere.

TABLE 3

Dichotomous key to the described subspecies of *Oreochromis niloticus* from East Africa and Egypt, based on mitochondrial DNA restriction enzyme phenotypes. Many other keys can be constructed from the composite phenotypes (see Table 2)

1.	A.	*DraI* "H"	*O. n. eduardianus*
	B.	All other phenotypes	2
2.	A.	*HinfI* "D"	*O. n. sugutae*
	B.	All other phenotypes	3
3.	A.	*RsaI* "F"	*O. n. vulcani*
	B.	All other phenotypes	4
4.	A.	*BclI* "B"	*O. n. filoa*
	B.	All other phenotypes	5
5.	A.	*AvaI* "A" or "E"	6
	B.	All other phenotypes	7
6.	A.	*AvaI* "A"	*O. n. cancellatus*
	B.	*AvaI* "E"	*O. n. cancellatus* (Lake Tana population)
7.	A.	*NciI* "X"	*O. n. niloticus*
	B.	*HinfI* "C"	*O. n. baringoensis*

TABLE 4

Number of mitochondrial DNA cleavage phenotype differences between the subspecies of *Oreochromis niloticus* and *Oreochromis spilurus* (ST). Total number of polymorphic enzymes was 29

		CC	CT	EV	FF	NM	SS	VT	ST
O. n. baringoensis		21	17	13	24	14	12	12	25
O. n. cancellatus	(CC)	-	19	21	4	19	20	21	22
O. n. cancellatus	(CT)	-	-	14	20	9	11	8	23
O. n. eduardianus	(EV)	-	-	-	23	9	10	9	25
O. n. filoa	(FF)	-	-	-	-	21	22	22	23
O. n. niloticus	(NM)	-	-	-	-	-	7	6	24
O. n. sugutae	(SS)	-	-	-	-	-	-	4	24
O. n. vulcani	(VT)	-	-	-	-	-	-	-	24

DISCUSSION

In previous studies, protein electrophoresis could not be used to discriminate among five of the subspecies of *Oreochromis niloticus* (Seyoum, 1989, 1990). Indeed, the tilapiine fishes are morphologically very similar and no earlier work has successfully identified any taxonomic group below the species level with allozyme analysis. However, using restriction endonuclease analysis of mtDNA, we were able to unambiguously distinguish all the subspecies.

Several keys to the subspecies of *O. niloticus* can be drawn from diagnostic restriction enzyme patterns (Table 2, Appendix). One dichotomous key is

illustrated in Table 3. Alternatively, the subspecies can be discriminated by cleavage phenotypes for only three restriction endonucleases: *Apa*I, *Bcl*I and *Rsa*I (Table 2). Thus, for introduced or cultured tilapias, assuming that stocks have remained genetically isolated (i.e., have experienced no hybridization since they were obtained from the wild), mtDNA analysis can be exploited to indicate their origin.

One important factor in the effective exploitation of mtDNA polymorphism for the purpose of stock or subspecies identification is adjustment for intrapopulation variation. Variation of mtDNA within populations has been found in a number of fishes, e.g., rainbow trout, *Oncorhynchus mykiss* (Palva and Palva, 1987), Atlantic herring, *Clupea harengus* (Kornfield and Bogdanowicz, 1987), Great Lakes walleye, *Stizostedion vitreum* (Billington and Hebert, 1988) and southern African hakes, *Merluccius* spp. (Becker et al., 1988). However, no variation was observed within any of the populations we sampled. Indeed, the sample sizes in this study (Table 1) may not be sufficient to expose all clones that may occur within a population. However, the complete absence of differentiation among the lakes inhabited by *O. n. cancellatus* strongly suggests that variation within populations is very limited. On the other hand, a great deal of interspecific polymorphism was observed as differences among the subspecies. Our ability to perceive these differences among populations strongly suggests that we could also have been able to recognize variation within populations had it been present.

Relationships within the *O. niloticus* subspecies complex derived from our analysis of mtDNA basically support the morphological classification of the group; each subspecies can be recognized by four or more unique restriction enzyme patterns (Table 4). However, there are three inconsistencies between the mtDNA findings and expectations from the current taxonomy of the group. The first case involves the position of the specimens from Lake Tana. Fish from Lake Tana, morphologically assigned to *O. n. cancellatus* by Trewavas (1983) differed from other population samples of that subspecies for 19 of 29 informative endonucleases (Table 4). The population in Lake Tana is not *O. n. cancellatus*, but represents an additional, previously unrecognized taxon. This fish has apparently remained static in morphology while its mtDNA diversified; the magnitude of distinctiveness may warrant taxonomic recognition.

A second exception to the conventional taxonomy involves identification of the fish collected from Lake Beseka (Metehara). This small Ethiopian lake possesses a number of hot streams around its periphery. Cichlids from Lake Beseka were thought by Trewavas (1983) to be allied to *O. n. filoa* since she believed that this taxon was adapted to high temperatures. However, analysis of fish from this location showed mtDNA restriction phenotypes identical to those of specimens assigned by Trewavas to *O. n. cancellatus*. Our identification of these fish as *O. n. cancellatus* is consistent with the idea that *O. n. filoa*

is naturally limited in distribution to the Awash system; Lake Beseka has no connection with the River Awash.

The final inconsistency between morphology and mtDNA data concerns the cichlid of Lake Akaki, an intentional impoundment in the River Awash system associated with hydrological development in the region. The lake is cold, shallow and muddy. Trewavas (1983) examined five specimens of *O. niloticus* from Lake Akaki, assigned them to *O. n. cancellatus*, but noted that they had meristics similar to *O. n. filoa*. The low number of dorsal fin elements in some Akaki specimens suggested to her that these fish may have been stocked from an Awash River hot spring since populations in high-temperature areas could display reduced fin elements. However, the fish we collected from Lake Akaki did not show mtDNA phenotypes for *O. n. cancellatus*, but instead had phenotypes characteristic of *O. n. filoa* from hot springs in the Awash near Metehara. Though her taxonomic allocation was incorrect, Trewavas (1973) had anticipated this finding by identifying meristic inconsistencies of these fish.

The use of restriction endonuclease analysis of mitochondrial DNA has allowed us to establish a molecular key to distinguish among the subspecies of *O. niloticus*. With a single individual, identification using morphology is virtually impossible in the absence of locality information. Given the global scale of aquaculture involving *O. niloticus*, we suggest that additional coordinated molecular studies of wild and cultured stocks should be undertaken. Such an effort would be useful for preservation of gene pools and unambiguous identification of cultured or wild stocks. We hope that our findings will assist in stock identification programs and aquaculture research.

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APPENDIX

Restriction enzyme digestion patterns of *Oreochromis niloticus* subspecies. Capital letters denote enzyme-specific phenotypes. Cleavage fragments of identical size are homologous for a given enzyme. Asterisks denote two fragments with equal molecular size which were visualized as extra intense bands on autoradiographs

ApaI					*AvaI*				*AvaII*					*BclI*	
E	G	H	I	M	A	B	C	G	A	C	D	E	M	A	B
9110	9110	7906	6969	9110	6067	4754	4754	6067	5419	4067	4348	4348	3119	16207	17020
5393	5393	6969	6240	5924	4186	4186	4186	5781	2434	2968	3210	3401	2434	813	
1311	980*	1311	1822	1311	2764	2764	3814	2764	1358*	1737	2401	3126	2260		
370*	370	370	1311		1391	1496	1496	1197	1204	1476	1358*	1358	2152		
					1197	1391	1391	937	1164	1358*	1164	1164*	1358		
					937	1197	1197	263	943	1164	943*	943	1164		
					263	937			677*	943*	677	677	943*		
						263			618	677	618	618	677*		
									532*	311	311	311	618		
									444				311		

BglI	*BglII*			*BstEII*			*DraI*			*EcoRII*			
A	A	B	E	A	B	D	A	B	H	A	B	C	E
8146	7900	9162	16983	12023	17020	14577	9940	9940	8145	3472	3472	3472	3472
2044	5252	7900		4997		2443	5730	5119	5119	2121	3058	3058	1888*
1844	3753						657	745	864	1783*	1783*	1783	1783
1771								657	745	1470	1470	1368	1470
1285									657	1292	1292*	1292	1368
751										1136*	962	1136	962*
633										962	817	1128	839
562										817*	560	962*	418
										418	418	817*	
												418	

EcoRV	*HaeIII*					*HhaI*					*HincII*		
A	C	D	A	H	B	A	B	D	E	G	A	B	C
13555	1161*	1161*	1161*	1161*	1161*	2826	2826	2826	2671*	2826	5110	3443	3443
3446	1090*	1090*	1090*	999*	1090*	2671	1994*	2671	1994*	2671	2229*	3079	3079*
	865*	962*	865*	962*	865*	1994	1239*	1994*	1117*	1994*	1598	2229*	2229
	662*	796*	662*	796*	662*	1577	974*	1644	974	1239	1404	1598	1598
	596*	662*	596*	662*	596*	1431	818	1239*	904	974	1118	1118	1118
	562	545*	562*	596*	562*	1239	679*	974	818	904	919*	919*	919*
	516	516	471	562	516	1117	566*	566*	679*	818	769	769	597
	471	471	427	516	471	974	499	499	566	679*	597	597	
	427	427	374*	471	374*	904	358*	358	358*	566			
	374*	359*	359*	374*	459*	818	328*	328	105	499			
	359*	333	325*	359*	325*	679	198*	198		358*			
	325*	325	302*	325*	302*	566	105			198			
	302*	302	288*	302*	288*	358							
	288*	288*	222	288*	232								
	222	222	191	222	191								
	191*	191*		191									

*Hin*dIII		*Hpa*I		*Nco*I		*Nde*I		*Pst*I		*Pvu*II	
A	D	A	B	A	C	A	C	A	B	A	E
12600	12600	8688	8688	17020	16288	17020	15552	7526	11706	11496	13034
4385	4130	3321	6379		732		1468	5296	5296	1232*	1003*
	569	2645	2120					4150		1003*	948*
		2120								970	

*Hin*fI					*Mbo*I					*Msp*I			
A	B	C	D	E	A	B	D	E	F	B	D	E	J
2572	3822	2572	2572	3807	1953*	2177	1953*	1953*	1953*	1961	2838	1430*	3173*
1803*	1446*	1803*	1291	1803	1863	1863	1863	1863	1863	1752	1478*	1138*	1478*
1229*	1229*	1229	1229	1229*	1568*	1324*	1324*	1324*	1324*	1478	1430*	1064*	1430
1047*	1090	1090	1090	1047	1324*	1157	1157	1157*	1157	1430	1064	866*	1138
944*	1047	1047	1047	944*	1019	1019	1019*	1018	1019	1138	866*	797*	660*
798	632*	944	944*	798*	766*	766*	766*	766*	766*	866	660*	753	616*
593	593*	798	798*	477	567	712	567	712*	712	797*	616*	660*	546
477	446*	593*	593*	446	537	666*	537*	567	684	660*	546*	616	318*
446	369*	536*	477*	429*	374	567	400*	537	567	616	454*	546*	309
369*	329*	477	461	369*	324	537	374	374	537	546*	309	454	267*
329*	303	446	446*	329*	302	477	324	302	477	417*	267	309*	224
270*	270	392	369*	270	256*	412*	302	256	412	309*	224*	267	195
187	235	369	329*	235	185	324*	256	185	353	267*	195*	250	125
149	187	345	270	187	154	302*	185	154	256	250*	171	224*	77
77	149	329*	187*	149*	88	256*	154	88	185	224*	125	195*	
	77	270	145	77		185	88		154	195*	77	171	
		235	77			154			88	175		125*	
		187				88				125*		77	
		149								77*			
		77											

*Mbo*II						*Nar*I	*Rsa*I						
A	B	C	D	E	H	A	A	B	C	D	E	F	G
1974*	2250	1974*	1974*	1974*	1974*	14739	1969	1969	1969*	1969*	2313	1969	1969*
1882*	1882*	1882*	1882*	1882*	1882*	2335	1720	1825	1720*	1720	1969	1720	1720
1310*	1310*	1310*	1310*	1535	1310*		1585	1720	1538	1620	1720	1585	1585
1156	1156	1020	1156*	1310*	1156		1492	1538	1128	1585	1533	1492	1492
1020*	1020	775	1020	1020*	1020		1334*	1334*	1068	1334	1128	1334	1334
775*	775*	719*	775*	775*	775		1128	1128*	730*	1128	1068	1128	1128
572*	719	693*	719	385*	719*		1068	1068	674	1068	730*	1068	1068
416*	693	572*	572	332*	693		730*	730	617*	730	674	900*	730
332	572*	492	385		572		601*	601	533	617*	617*	730*	617
	492*	416	324		492		482	482	482	601	482	601*	600
	416	332			416		439	439	439	482	439	439	482
	332*				332		400	400	400	439	400	425	439
							336*	336	336	336	336	336*	425
							308	308		308	308	308	336
							256	256		256	256	256	308
							202	202		159	202	202	256
							159	159			159	159	202
													159

APPENDIX (continued)

*Nci*I A	*Nci*I B	*Nci*I E	*Nci*I X
6503	6503	7504	6503
2584	2584	2584	2584
1793	2030	1793	2466
1528*	1793	1528	1793
798	1528	798	1528
731	731	505	798
437*	658	437	731
339	437	339	437
333	339	333*	291
239	333	325	
	239	239	

*Sph*I A	*Sph*I B	*Sph*I E
16989	9175	10396
	7814	6593

*Sty*I A	*Sty*I D	*Sty*I E
7308	7308	7688
1690	1690	1690
1543	1543*	1543
895*	999	895
825	895	825
768*	825	768
642	768	535
413	642	517*
356	413	472*
288*	356	413
226	288	356
203		226
		203

*Taq*I A	*Taq*I B	*Taq*I C	*Taq*I F
1349*	1349*	1349	1349*
1285	1285	1285	1285*
1176	1176	1176*	1071
1071	1071	1071	961*
991	991	991	814*
961*	961*	961*	780*
814	814	814	594*
780	780	780	546*
621*	621	628	448
546*	594	621*	370
370*	546	546	345
345	525	525	328*
328*	448	448	293
289	370	370	289
261	345*	345*	261*
246*	328	311	209
237*	293*	293	191
209*	261*	261*	166
	246	246	
	191*	209	
	166	191*	
		166	

*Tha*I A	*Tha*I B	*Xba*I A	*Xba*I B	*Xmn*I A	*Xmn*I C
4891	4185	9980	7767	6545	5624
4185	2991	5963	5963	5624	4876
2991	2652	874	2196	2020	2479
2324	2425		874	1084	1459
1165	1381			797*	797*
555	1165				642
516*	766				
	555				
	516*				

For digestion patterns exhibiting one to three restriction fragments, the size of the largest fragment was obtained by subtraction from the estimated molecular size of the mitochondrial DNA.