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Cytochrome-*b* sequence variation among parrots

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The nucleotide sequence of a 307 bp fragment of the mitochondrial cytochrome-*b* gene was determined for 12 species of parrot, using the polymerase chain reaction and direct sequencing. Sequence divergence ranged from 26–54 differences in pairwise comparisons, with the majority of base substitutions occurring at third positions of codons. The transition:transversion ratio was determined to be higher (approximately 24.3:1) in recently divergent parrot lineages than has generally been observed in other groups. Strongly biased base composition, particularly at the third position of codons, is evident among the sequences. Phylogenetic relationships among more divergent taxa were estimated, using only transversion substitutions, while all the substitutions were useful for closely related taxa. The African genera *Psittacus* and *Poicephalus* are closely related, in contrast to the Australian genera *Nymphicus*, *Purpureicephalus* and *Melopsittacus*, which represent more divergent lineages. The cockatoos appear to represent an ancient lineage within the parrots.

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The rapid rate of evolution and lack of recombination endow the vertebrate mitochondrial genome with great utility in studies of population structuring and phylogenetic reconstruction (BROWN et al. 1979; BROWN 1983; AVISE et al. 1987). Until recently, estimates of sequence divergence among mitochondrial genotypes were typically calculated from data generated using restriction enzymes, an approach which estimates the degree of difference over the entire molecule rather than for specific sequences within the genome. However, functional constraints vary considerably over different regions of the mitochondrial genome; hence, rates of evolutionary change also vary (BROWN et al. 1982; THOMAS and BECKENBACH 1989). Until recently, estimation of sequence-specific divergence rates was possible only by employing the time-consuming methods of high resolution restriction site mapping or cloning and sequencing (CANN et al. 1987; BROWN et al. 1982; FERRIS et al. 1983; THOMAS and BECKENBACH 1989). Development of the polymerase chain reaction (PCR) and direct sequencing now permit rapid determination of nucleotide sequences of specific regions of the mitochondrial (and nuclear) genome (SAIKI et al. 1988; KOCHER et al. 1989). This information is of value for the delineation of affinities among evolutionary lineages.

The avian order Psittaciformes (parrots) includes some 332 species, the majority of which are distributed in tropical and subtropical regions of the southern hemisphere (FORSHAW 1978). Previous authors have observed that the parrots are well differentiated from other avian orders (THOMPSON 1899; SMITH 1975; FORSHAW 1978); however, relationships within the order are unclear and several conflicting systematic arrangements based on anatomical and behavioral data have been proposed during the last 150 years. A few studies have addressed this topic at the molecular level (SIBLEY 1960; OVENDEN et al. 1987; RANDI and BERTAGNOLIO 1990; CHRISTIDIS et al. 1991a); yet, no primary DNA sequence information is available. In this regard we report nucleotide sequences for a portion of the mitochondrial cytochrome-*b* gene for 12 species of parrot (representing 9 genera) as determined using PCR amplification and direct sequencing.

Materials and methods

DNA extraction. — Tissues were sampled from captive specimens of the following parrots:

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budgerigar (*Melopsittacus undulatus*), red-cap parakeet (*Purpureicephalus spurius*), double yellow-head amazon (*Amazona ochrocephala oratrix*), yellow-faced amazon (*A. xanthops*), tucuman amazon (*A. tucumana*), red-lored amazon (*A. autumnalis autumnalis*), short-tail parrot (*Graydidascalus brachyurus*), white-cap parrot (*Pionus senilis*), African grey parrot (*Psittacus erithacus timneh*), Senegal parrot (*Poicephalus senegalus*), cockatiel (*Nymphicus hollandicus*), and Goffin's cockatoo (*Cacatua goffini*). This group contains representatives of three of the four subfamilies of parrots recognized by SMITH (1975). Samples from the Loriinae were unavailable. A rock dove (*Columba livia*) was sampled as an outgroup. Crude DNA was prepared from small amounts (approximately 5 mg) of soft tissue (skeletal muscle, pulp from growing feathers, and skin cells associated with the shaft of mature contour feathers) or whole blood (5 µl) sampled from living and dead birds. Tissues were added (without mechanical disruption) to 750 µl of lysis buffer containing 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.1 % SDS and 10 µg/ml proteinase K and incubated overnight at a temperature of 55 °C. Solutions were extracted twice with Tris-saturated phenol and once with chloroform:isoamyl alcohol (24:1).

DNA amplification. — Amplifications were done using primers L14841 and H15149 (KOCHER et al. 1989), which flank a 307 bp region of the mitochondrial cytochrome-*b* gene. Letters refer to light and heavy strands and numbers to positions of the 3′ nucleotides in the human mtDNA sequence (ANDERSON et al. 1981). This primer pair works well for a broad taxonomic group of vertebrate species, and the particular sequence amplified has proven useful for phylogenetic studies in other animal groups (KOCHER et al. 1989). Double-stranded amplifications were performed in 25 µl of a solution consisting of 67 mM Tris pH 8.8, 20 mM $MgCl_2$, 50 mM β-mercaptoethanol, 0.2 mM each dNTP, 0.4 µM each primer, 1 µl crude DNA preparation, and 1 unit *Taq* polymerase (Perkin-Elmer/Cetus). Thirty cycles of the following temperature regime were run: (1) 45 s denaturation at 92 °C; (2) 45 s annealing at 55 °C; and (3) 60 s extension at 72 °C. Aliquots (5 µl) were electrophoresed in 2 % low melting agarose, and small plugs (10 µl) containing the desired product were dissolved in 1 ml water. One microliter of this solution was used as template for asymmetric amplification. Forty cycles of asymmetric amplification were done under the same conditions, except that L primer was diluted 100-fold. The resulting product was desalted, using ultra-filtration (Centricon-30, Amicon) and sequenced, using the chain termination method (SANGER et al. 1977) with Sequenase (US Biochemical Corp.).

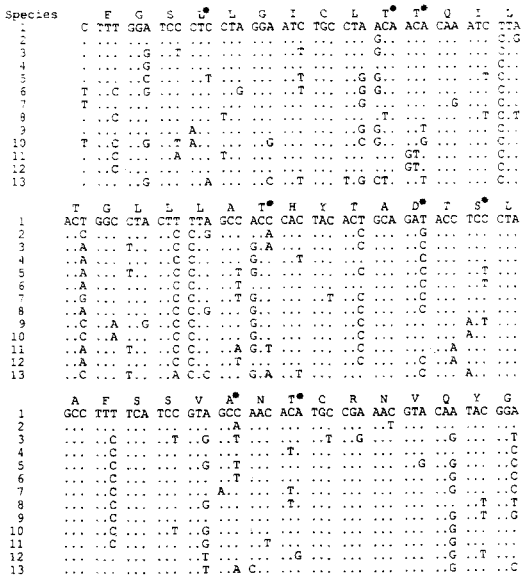
Results and discussion

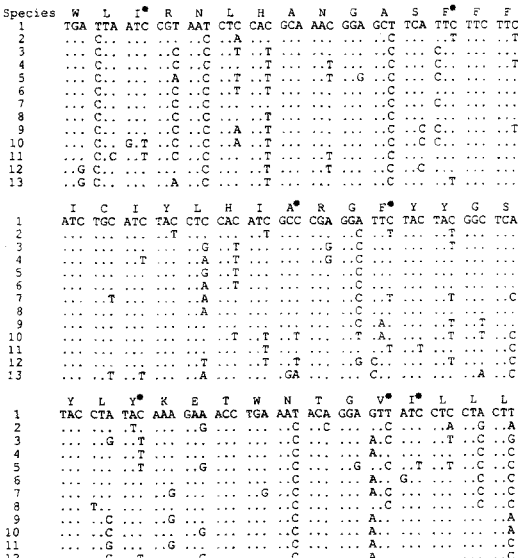
Sequence divergence/base composition

Nucleotide sequences for 12 species of parrot and a rock dove are shown in Fig. 1. The rock dove differed from all parrots at 12 positions. Among all 13 species, 119 (38.8 %) variable nucleotide sites were present. Twenty-one variable sites were at first positions of codons (20.6 % of total), 6 variable sites were at second positions (5.9 %), and 92 were at third positions (89.3 %).

Among the parrots, the most similar sequences differed by 24 base substitutions (*A. xanthops* vs. *Pionus senilis*). The most divergent genotypes differed by 52 substitutions (*A. tucumana* vs. *Poicephalus senegalus*). All pairwise comparisons of parrots vs. the rock dove revealed more than 50 substitutions. Uncorrected sequence divergence values among the 4 *Amazona* species range from 8.1 to 9.8 %, a somewhat more narrow range than among 5 species of Australian babblers (6.2–12 %; EDWARDS and WILSON 1990).

Base composition at first, second and third positions of codons are shown in Table 1. Biased base composition, a common feature of animal mtDNA (BROWN et al. 1982; IRWIN et al. 1991), is apparent in the parrot cytochrome-*b* sequences. The sequences shown in Fig. 1 (light strand) have a mean composition of 41.7 % purine and 58.3% pyrimidine residues. Base composition differs at first, second, and third positions of codons. The four bases occur in nearly equivalent proportions at first positions. Second codon positions contain relatively more thymines and fewer guanines, while third positions are rich in adenine and cytosine. This pattern is similar to that reported by IRWIN et al. (1991) for the entire cytochrome-*b* gene in 20 species of mammals; however, the parrot cytochrome-*b* fragment appears to differ from mammals in having a higher proportion of cytosine and a lower proportion of adenine residues.





Transitions/transversions

Table 2 presents the numbers of transitions and transversions observed between all species pairs. In agreement with previous studies, the ratio of transitions to transversions decreases with taxonomic distance (BROWN et al. 1982; THOMAS and BECKENBACH 1989; SMITH and PATTON 1991). Within the region of the cytochrome-*b* gene considered, the numbers of transition substitutions present between closely related and distantly related taxa are similar. Hence, the number of relatively unconstrained sites is limited, and saturation of these sites occurs rapidly in diverging lineages. To estimate the ratio of transitions to transversions in recently diverged mitochondrial lineages, we considered the sequences of the 3 most similar species pairs, all of which differed by only 1 transversion. The mean ratio for these pairs is 24.3:1. This estimate is consistent with the observations of EDWARDS and WILSON (1990), who reported a ratio of approximately 20:1 among 5 species of *Pomatostomus*. The true ratio is actually somewhat higher since multiple transition hits will have occurred at some sites before transversion substitutions occur (EDWARDS and WILSON 1990). Our estimate does not distinguish between silent and replacement substitutions, although the former greatly outnumber the latter. Transition:transversion ratio estimates at silent sites for other taxonomic groups (mostly mammals) are generally considerably lower than that for the parrots (AQUADRO and GREENBERG 1983; IRWIN et al. 1991; CARR and MARSHALL 1991; BROWN et al. 1982). The apparent high ratio among parrots could result from an unusually rapid rate of transition substitution, and/or a slower than usual rate of transversion substitution. Since there is very little known about the ages of parrot lineages, absolute rates of base substitution cannot be estimated; hence, it is not possible to rule out either possibility.

Fig. 1. Nucleotide sequence of 307 bp segment of cytochrome-*b* gene (light strand) in 12 species of parrot. Nucleotides that do not differ from those of the budgerigar are indicated with dots. Amino acid sequence for the budgerigar is shown above the nucleotide sequence; variable amino acid positions are indicated with asterisks. Species represented are: 1) *Melopsittacus undulatus*, 2) *Purpureicephalus spurius*, 3) *Amazona o. oratrix*, 4) *A. xanthops*, 5) *A. tucumana*, 6) *A. a. autumnalis*, 7) *Graydidascalus brachyurus*, 8) *Pionus senilis*, 9) *Psittacus erithacus*, 10) *Piocephalus senegalus*, 11) *Nymphicus hollandicus*, 12) *Cacatua goffini*, and 13) *Columba livia*.

Table 1. Base composition (%) at first, second and third positions of codons within 307 bp segment of parrot cytochrome-*b* genes

	FIRST				SECOND				THIRD			
	A	C	G	T	A	C	G	T	A	C	G	T
Melopsittacus	24.5	22.5	24.5	28.4	20.6	26.5	16.7	36.3	37.9	48.5	0	13.6
Purpureicephalus	23.5	25.5	25.5	25.5	19.6	26.5	16.7	37.3	35.9	47.6	4.9	11.7
A. o. oratrix	23.5	25.5	25.5	25.5	20.6	26.5	16.7	36.3	31.1	46.6	9.7	12.6
A. xanthops	24.5	26.5	24.5	24.5	20.6	25.5	16.7	37.3	36.0	50.5	1.9	11.7
A. tucumana	21.6	25.5	27.5	25.5	20.6	26.5	16.7	36.3	30.1	47.6	8.7	13.6
A. a. autumnalis	23.5	24.5	25.5	26.5	20.6	26.5	16.7	36.3	34.0	51.5	4.9	9.7
Graydidascalus	25.5	26.5	23.5	24.5	20.6	25.5	16.7	37.3	30.1	55.3	6.8	7.8
Pionus	23.5	23.5	25.5	27.4	20.6	24.5	16.7	38.2	35.0	56.3	1.9	6.8
Psittacus	25.5	25.5	25.5	23.5	21.6	26.5	16.7	35.3	32.0	50.5	5.8	11.7
Poicephalus	24.5	25.5	26.5	23.5	21.6	26.5	16.7	35.3	30.1	50.5	5.8	13.6
Nymphicus	23.5	23.5	25.5	27.5	20.6	25.5	16.7	37.3	36.9	49.5	3.9	9.7
Cacatua	24.5	26.5	24.5	24.5	20.6	25.5	16.7	37.3	30.1	47.6	6.8	15.5
Columba	21.6	26.5	26.5	25.5	20.6	24.5	17.6	37.3	35.0	45.6	4.9	14.6
Mean	23.8	25.2	25.4	25.6	20.7	25.9	16.8	36.8	33.4	49.8	5.1	11.7

Table 2. Numbers of transition (above diagonal) and transversion (below diagonal) substitutions in 307 bp segment of cytochrome-*b* gene of parrots

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Melopsittacus*	–	24	37	27	39	29	31	25	27	35	31	32	28
2	*Purpureicephalus*	12	–	31	28	31	28	26	23	25	31	29	29	28
3	*A. o. oratrix*	11	13	–	27	22	28	35	32	26	24	29	33	33
4	*A. xanthops*	8	14	3	–	31	24	26	23	21	27	29	29	25
5	*A. tucumana*	11	17	4	3	–	26	36	33	28	32	34	36	30
6	*A. a. autumnalis*	9	15	2	1	2	–	28	29	21	24	29	31	26
7	*Graydidascalus*	9	15	4	1	4	2	–	29	20	29	30	35	32
8	*Pionus*	9	15	4	1	4	2	2	–	21	29	24	26	24
9	*Psittacus*	13	15	16	13	18	16	16	16	–	22	24	27	26
10	*Piocephalus*	15	15	18	17	20	18	16	18	4	–	23	28	28
11	*Nymphicus*	9	19	14	10	14	12	9	12	20	20	–	25	32
12	*Cacatua*	7	17	12	9	12	10	8	10	16	16	8	–	28
13	*Columba*	26	28	23	26	25	25	25	27	29	29	29	25	–

Phylogeny

Phylogenetic relationships among the major groups of Psittaciform birds are unclear, and several conflicting taxonomic arrangements have been proposed. SMITH (1975) included all parrots in a single family containing 4 subfamilies based upon morphological and behavioral characters. Relationships among the more distantly related parrots were here estimated from character state data using the PHYLIP package (DNAboot; FELSENSTEIN 1986). For this analysis, only transversion substitutions were considered and all but 1 of the closely related New World species (*A. o. oratrix*) were excluded. Fig. 2 presents the majority-rule consensus tree generated using the bootstrap procedure. This tree is slightly different from the shortest tree, but is identical to that generated using the UPGMA method. The bootstrapping procedure does not support the associations suggested in Fig. 2 particularly strongly, except for that between *Poicephalus* and *Psittacus*. Among the parrots included in the study, the cockatiel and Goffin's cockatoo form a lineage distant from all others. This is consistent with studies based upon protein variation (ADAMS et al. 1984; CHRISTIDIS et al. 1991a) and cytogenetic evidence (CHRISTIDIS et al. 1991b), and indicates that the cockatoos represent an ancient lineage. The phylogenetic hypothesis suggested by the cytochrome-*b* sequences is therefore not consistent with SMITH's (1975) subfamily Platycercinae, in which he included most species from Australia and some other species from surrounding islands.

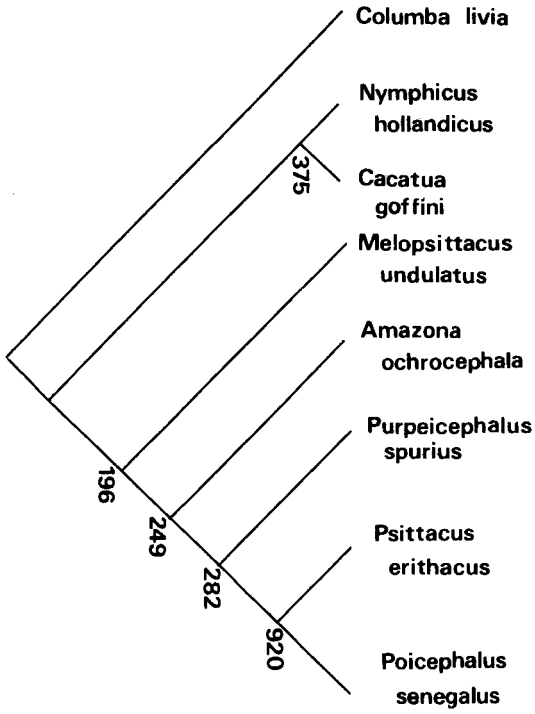


Fig. 2. Consensus tree showing inferred relationships among some parrots and rock dove based upon transversion substitutions. Numbers shown at nodes indicate the number of times particular phylogenetic associations were found in 1000 bootstrap replicates.

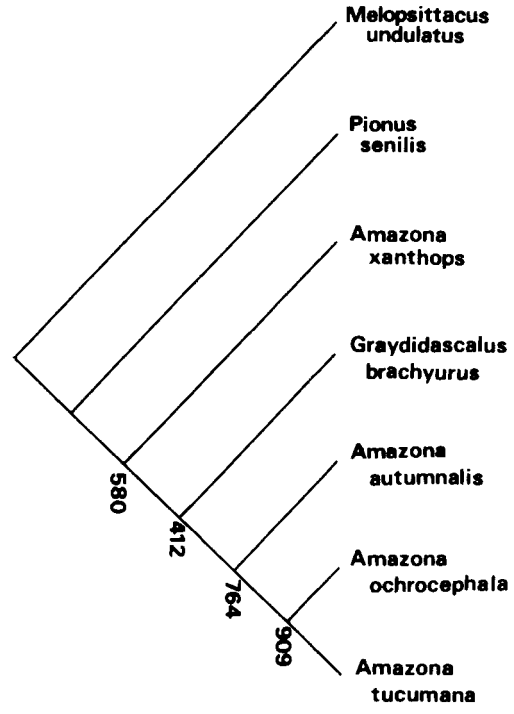


Fig. 3. Consensus tree showing inferred relationships among 6 species of New World parrots and the budgerigar based upon transition and transversion substitutions. The number of times particular phylogenetic associations were found in 1000 bootstrap replicates are shown at nodes.

Transition and transversion substitutions were used to estimate relationships among the recently diverged New World species (EDWARDS et al. 1991). The majority-rule consensus tree (*Melopsittacus* as the outgroup) is not consistent with the current taxonomic status of some of these species (Fig. 3). The only associations well supported by the bootstrapping procedure are between 3 *Amazona* species. The position of *A. xanthops* is of particular interest in light of its cytogenetic similarity to *Pionus* (VALENTINE 1990). Similarly, the position of *Graydidascalus* relative to *Amazona* and *Pionus* remains uncertain. The data suggest that the genus *Amazona* contains more species than currently recognized, or that *xanthops* should be excluded. Clearly, however, phylogenetic inferences based upon the current data set are not well supported statistically. A further caveat relating to DNA sequence-based phylogenies in general and to mtDNA-based phylogenies in particular, is the need to differentiate between gene trees and species trees (AVISE 1989). Since the two types of tree are not always the same, information from several unlinked genes is required before definitive phylogenetic statements are warranted. This underlines the need for additional sequence information before relationships among the Psittaciformes can be established.

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References

ADAMS, M., BAVERSTOCK, P. R., SAUNDERS, D. A., SCHODDE, R. and SMITH, G. T. 1984. Biochemical systematics of the Australian cockatoos (Psittaciformes: Cacatuinae). — *Aust. J. Zool. 32*: 363–377

ANDERSON S., BANKIER, A. T., BARRELL, B. G., DEBRUIJN, M. H. L., COULSON, A. R., DROUIN, J., EPERON, I. C., NIERLICH,

D. P., ROE, B. A., SANGER, F., SCHREIER, P. H., SMITH, A. J. H., STADEN, R. and YOUNG, I. G. 1981. Sequence and organization of the human mitochondrial genome. — *Nature 290*: 457–465

AQUADRO, C. F. and GREENBERG, B. D. 1983. Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. — *Genetics 103*: 287–312

AVISE, J. C. 1989. Gene trees and organismal histories: a phylogenetic approach to population biology. — *Evolution 43*: 1192–1208

AVISE, J. C., ARNOLD, J., BALL, R. M., BERMINGHAM, E., LAMB, T., NEIGEL, J. E., REEB, C. A. and SAUNDERS, N. C. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. — *Annu. Rev. Ecol. Syst. 18*: 489–522

BROWN, W. M. 1983. Evolution of animal mitochondrial DNA. — In: *Evolution of Genes and Proteins* (*eds* M. NEI *and* R. K. KOEHN), *Sinauer Associates Inc., Sunderland, Mass.*, p. 62–88

BROWN, W. M., GEORGE, M. and WILSON, A. C. 1979. Rapid evolution of animal mitochondrial DNA. — *Proc. Natl. Acad. Sci. USA 76*: 1967–1971

BROWN, W. M., PRAGER, E. M., WANG, A. and WILSON, A. C. 1982. Mitochondrial DNA sequences of primates: tempo and mode of evolution. — *J. Mol. Evol. 18*: 225–239

CANN, R. L., STONEKING, M. and WILSON, A. C. 1987. Mitochondrial DNA and human evolution. — *Nature 325*: 31–36

CARR, S. M. and MARSHALL, H. D. 1991. Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome *b* gene of Atlantic cod (*Gadus morhua*) by the polymerase chain reaction. — *Can. J. Fish. Aquat. Sci. 48*: 48–52

CHRISTIDIS, L., SCHODDE, R., SHAW, D. D. and MAYNES, S. F. 1991a. Relationships among the Australo-Papuan parrots, lorikeets, and cockatoos (Aves: Psittaciformes): protein evidence. — *Condor 93*: 302–317

CHRISTIDIS, L., SHAW, D. D. and SCHODDE, R. 1991b. Chromosomal evolution in parrots, lorikeets and cockatoos (Aves: Psittaciformes). — *Hereditas 114*: 47–56

EDWARDS, S. V. and WILSON, A. C. 1990. Phylogenetically informative length polymorphism and sequence variability in mitochondrial DNA of Australian songbirds (*Pomatostomus*). — *Genetics 126*: 695–711

EDWARDS, S. V., ARCTANDER, P. and WILSON, A. C. 1991. Mitochondrial resolution of a deep branch in the genealogical tree for perching birds. — *Proc. R. Soc. Lond. B 243*: 99–107

FELSENSTEIN, J. 1986. PHYLIP; Phylogeny Inference Package, Version 2.9. — *University of Washington, Seattle, Washington*

FERRIS, S. D., SAGE, R. D., PRAGER, E. M., RITTE, U. and WILSON, A. C. 1983. Mitochondrial DNA evolution in mice. — *Genetics 105*: 681–721

FORSHAW, J. M. 1978. Parrots of the World. — *Lansdowne Press, Melbourne*

IRWIN, D. M., KOCHER, T. D. and WILSON, A. C. 1991. Evolution of the Cytochrome *b* gene of mammals. — *J. Mol. Evol. 32*: 128–144

KOCHER, T. D., THOMAS, W. K., MEYER, A., EDWARDS, S. V., PAABO, S., VILLABLANCA, F. X. and WILSON, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. — *Proc. Natl Acad. Sci. USA 86*: 6196–6200

OVENDEN, J. R., MACKINLAY, A. G. and CROZIER, R. H. 1987. Systematics and mitochondrial genome evolution of Australian rosellas (Aves: Platycercidae). — *Mol. Biol. Evol. 4*: 526–543

RANDI, E. and BERTAGNOLIO, P. 1990. Biochemical relationships in the Psittaciformes. — *Abstract 686, International Ornithological Congress, Christchurch, New Zealand*

SAIKI, R. K. GELFAND, D. H., STOFFEL, S., SCHARF, S. J., HIGUCHI, R., HORN, G. T., MULLIS, K. B. and ERLICH, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. — *Science 239*: 487–491

SANGER, F., NICKLEN, S. and COULSON, A. R. 1977. DNA sequencing with chain-terminating inhibitors. — *Proc. Natl. Acad. Sci. USA 74*: 5463–5467

SIBLEY, C. G. 1960. The electrophoretic patterns of avian egg-white proteins as taxonomic characters. — *Ibis 102*: 215–284

SMITH, G. A. 1975. Systematics of Parrots. — *Ibis 117*: 18–68

SMITH, M. F. and PATTON, J. L. 1991. Variation in mitochondrial cytochrome *b* sequence in natural populations of South American Akadontine rodents (Muridae: Sigmodontinae). — *Mol. Biol. Evol. 8*: 85–103

THOMAS, W. K. and BECKENBACH, A. T. 1989. Variation in salmonid mitochondrial DNA: evolutionary constraints and mechanisms of substitution. — *J. Mol. Evol. 29*: 233–245

THOMPSON, D'A. W. 1899. On characteristic points in the cranial osteology of the parrots. — *Proc. Zool. Soc. London 1899*: 9–46

VALENTINE, M. 1990. Chromosome analysis. — In: *Genus Amazona*, (*eds* J. STOODLEY *and* P. STOODLEY), *Bezels Publications, Portsmouth, England*, p. 127–131