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Original article

Location of genes in *Apis mellifera scutellata*-derived mitochondrial DNA of Africanized honey bees

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Summary — The mitochondrial DNA of Africanized honey bees having a mtDNA haplotype known from the African subspecies *Apis mellifera scutellata,* was analysed for the location of 6 mitochondrial genes by hybridization to mitochondrial gene probes from *Saccharomyces cerevisiae.* Genes for the small and large ribosomal RNAs, cytochrome oxidase subunits I, II and III and apocytochrome *b* were positioned relative to the mitochondrial DNA fragments generated with 6 different restriction endonucleases utilized singly or in combination. Results show that these genes are in the same relative positions as *Drosophila* genes.

Africanized honey bee / mitochondrial gene / gene mapping / restriction endonuclease / molecular hybridization

INTRODUCTION

Mitochondrial DNA (mtDNA) has recently been the focus of a number of molecular, genetic and evolutionary studies (Wilson *et al,* 1985; Tzagoloff and Myers, 1986; Avise *et al,* 1987; Moritz *et al,* 1987). In multicellular animals, mtDNA is a circular molecule of approximately 16 kb carrying a set of highly conserved genes throughout the species and phyla studied so far (Wilson *et al,* 1985). The complete determination of nucleotide sequences of mtDNA in man (Anderson *et al,* 1981), mice (Bibb *et al,* 1981), cattle (Anderson *et al,* 1982), *Xenopus* (Roe *et al,* 1985) and *Drosophila yakuba* (Clary and Wolstenholme, 1984) revealed genes for 2 ribosomal RNAs, 22 tRNAs, 3 subunits of cytochrome oxidase, 2 subunits of ATPase, apocytochrome *b* and 7 coding regions for different subunits of the NADH dehydrogenase (Mariottini *et al,* 1986).

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Abbreviations: mitochondrial gene abbreviations are explained in the legend to figure 3.

These studies showed conservation of gene content but revealed a distinct gene order among phyla and different tRNA positions (Clary and Wolstenholme, 1985; Dubin *et al*, 1986). Partial sequencing work in honey bee mtDNA has been completed by Crozier *et al* (1989), encompassing the COI and COII genes. Here we report on the location of 6 mitochondrial genes in the honey bee determined by heterologous hybridization with mtDNA gene probes from *Saccharomyces cerevisiae.*

MATERIALS AND METHODS

Samples of ≈ 300 white-eyed pupae, 11 days old, were collected from 2 Africanized colonies kept at the Laboratório de Abelhas, Depto de Ecologia Geral, USP, São Paulo. Mitochondrial DNA was isolated from purified organelles according to the procedure of Moritz *et al* (1986).

Restriction enzyme digestion, agarose gel electrophoresis, Southern transfer of DNA fragments to Hybond-N filters (Amersham), preparation of nick-translated radioactive DNA probes and hybridization conditions were as described in Sambrook *et al* (1989). Mitochondrial DNA from yeast petite mutants was prepared as described by Nobrega and Nobrega (1986). The yeast petite strains used are described in table I.

Hybridization with heterologous yeast probes was performed overnight in 6 x SSC, 0.1% Sarkosyl, 50 μg/ml denatured carrier DNA at 40 °C. The filters were initially washed in 2 x SSC, 0.1% Sarkosyl at room temperature prior to autoradiography. After the first exposure the filters were washed in more stringent conditions by incubation in the wash solution for 1 h at 45, 50, 55 or 60 °C with autoradiography after each wash to determine the location of the DNA bands most homologous to the probe utilized.

Rehybridization of filters to new probes was carried out after removal of the radioactive probe by incubation at 45 °C for 15 min in 0.4 N NaOH followed by washes in 2 x SSC. The filters were exposed to X-ray film to ensure complete removal of label before re-use.

RESULTS AND DISCUSSION

Single and double digestions used for hybridization studies are shown in figure 1 and table II and results of a typical hybridization experiment are shown in figure 2. Hybridizations clearly indicated gene position except for the COIII gene. In this case (fig 2, lanes 9 and 10), there is a strong labeling of the second *Eco*RI mtDNA band (3 950 bp) but there is always a weaker labeling of the first (9 400 bp) and third (3 200 bp) band as well. This result was not improved with the increased stringency. We decided to place COIII at the first *Eco*RI fragment (9 400 bp) due to results of double digest with *Eco*RI and *Cla*I that

Table I. *Saccharomyces cerevisiae* petite mutants utilized as probes.

Petite	*Gene*	*Size of mtDNA repeating unit (kbp)*	*References*
DS400/A12	Apocytochrome *b*	7,6	Nobrega and Tzagoloff (1980)
DS6/407	Cytochrome oxidase subunit *I*	3,7	Bonitz *et al* (1980)
DS200/A1	Cytochrome oxidase subunit *II*	4,5	Coruzzi and Tzagoloff (1979)
DS40	Cytochrome oxidase subunit *III*	4,1	Thalenfeld and Tzagoloff (1980)
DS80	Small ribosomal RNA	2,6	Li and Tzagoloff (1982)
DS631	Large ribosomal RNA	3,5	Dujon (1980)

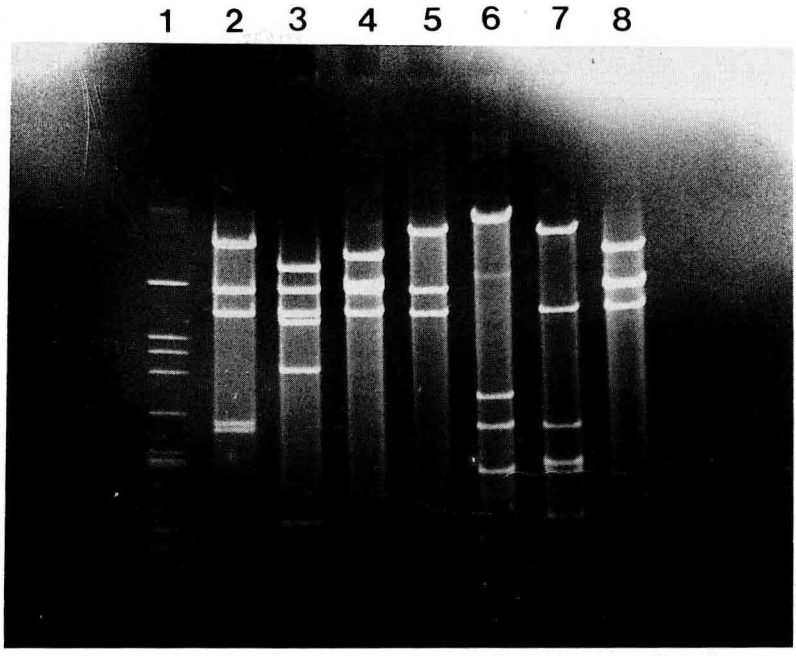

Fig 1. Ethidium bromide stained mitrochondrial DNA of *Apis mellifera scutellata* digested with different restriction enzymes and electrophoresed in 0.9% agarose gels. 1, lambda DNA digested with *Hae*III (the 6 larger DNA fragments are 4.1; 2.4; 2.1; 1.8; 1.3 and 1.0 kb long respectively); 2, *Hin*dIII/*Eco*RI; 3, *Acc*I/*Eco*RI; 4, *Eco*RV/*Eco*RI; 5, *Eco*RI; 6, *Cla*I; 7, *Cla*I/*Eco*RI; and 8, *Xho*I/*Eco*RI.

split the second *Eco*RI fragment (3 950 bp) (Arias *et al,* 1990). Autoradiography of this digest after hybridization with COIII probe showed strong signal at the first *Eco*RI fragment (9 400 bp), no labeling of *Cla*I pieces and a weak signal at the third *Eco* RI fragment (3 200 bp) band. A mitochondrial gene map based on hybridization with yeast probes appears in figure 3.

The introduction of African bees *(Apis mellifera scutellata)* to Brazil in 1956 followed by dispersal of descendents throughout South and Central America stimulated investigators to look for molecular markers to study the process of Africanization. Restriction fragment polymorphism in honey bee mtDNA has been widely used to provide molecular markers for such studies (Moritz *et al,* 1986; Smith, 1988; Smith *et al,* 1989; Hall and Muralidharan, 1989; Smith and Brown, 1990; Sheppard *et al,* 1991).

The size of mtDNA in *Apis mellifera* has been estimated at around 16 kb long (Smith, 1988; Arias *et al,* 1990) and is similar in size to that reported for *Drosophila yakuba* (Clary and Wolstenholme, 1984) and man (Cann *et al,* 1987). Major gene

Table II. *Apis mellifera* mtDNA fragment sizes after restriction endonuclease digestion.

Enzymes	HindIII [a] EcoRI	AccI [a] EcoRI	EcoRV [a] EcoRI	EcoRI	ClaI	ClaI [a] EcoRI	XhoI [a] EcoRI	AccI [a] HindIII
Fragment	7 000	4 530	5 300	9 400	13 000	9 400	5 450	11 000
	3 950	3 950	4 100	3 950	1 480	3 200	3 950	4 400
Sizes	3 200	3 200	3 950	3 200	1 200	1 200	3 950	700
	1 230	2 850	3 200		800	900	3 200	500
(bp)	1 170	1 720				900		500
		500				800		
						500		
Total	16 550	16 750	16 550	16 550	16 480	16 900	16 550	17 100

[a] Double digestion.

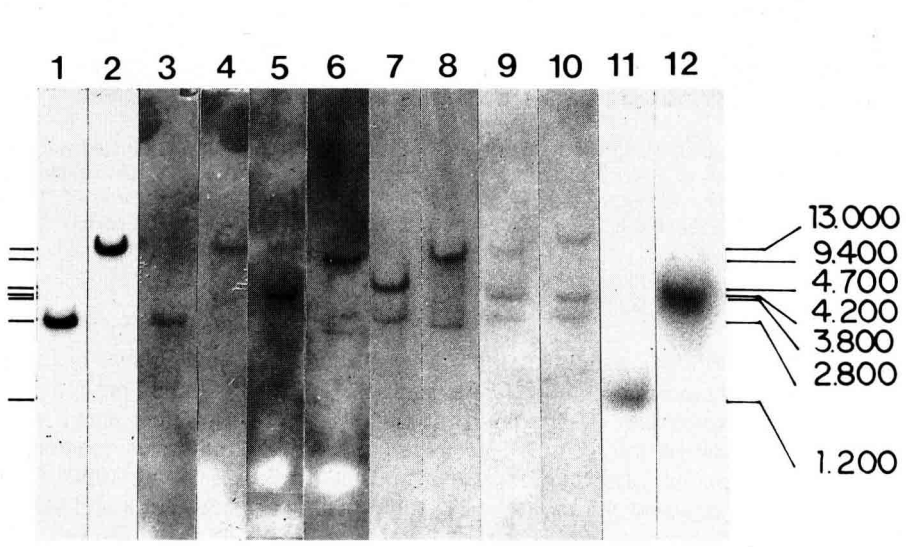

Fig 2. Composed autoradiographs of representative hybridization experiments of yeast mitochondrial gene probes to *Apis mellifera scutellata* mtDNA restriction fragments. Digestion was carried out with *Eco*RI (lanes 1, 3, 6, 8, 10, 12); *Cla*I (lanes 2 and 4); *Acc*I/*Hin*dIII (lane 5); *Eco*RI/*Hin*dIII (lane 9); *Eco*RI/*Eco*RV (lane 7) and *Cla*I/*Eco*RI (lane 11). Probes utilized correspond to the following genes: large rRNA (lanes 1 and 2); small rRNA (lanes 3 and 4); cytochrome oxidase subunit I (lanes 5 and 6); cytochrome oxidase subunit II (lanes 7 and 8); cytochrome oxidase subunit III (lanes 9 and 10) and apocytochrome *b* (lanes 11 and 12). The position and size (base pairs) of the corresponding mtDNA fragments is shown.

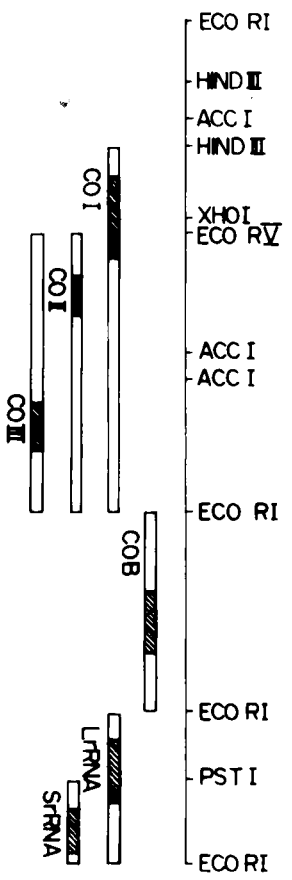

Fig 3. Physical map of mitochondrial genome of presumptive *Apis mellifera scutellata*. The 16 600 bp circular genome has been linearized at the *Eco*RI site located between the rRNA gene and the COI gene. The position of hybridizable regions for 6 mitochondrial gene probes is show as open bars. The shaded areas within correspond to the estimated location of the coding region for each gene, assuming that there is no gene overlap and taking into account published data for the large rDNA gene (Vlasak *et al*, 1987) and the segment that encompasses the cytochrome oxidase subunits I and II (Crozier *et al*, 1989). COI, II and III: cytochrome oxidase subunits 1, 2, and 3 respectively; COB: apocytochrome *b*; SrRNA: small ribosomal RNA and LrRNA: large ribosomal RNA.

order is identical in *Xenopus, Homo, Bos* and *Mus* (Moritz *et al*, 1987), although inversions and transpositions are frequent, chiefly among tRNA genes (Clary and Wolstenholme, 1985; Wolstenholme *et al*, 1987). Studies in fishes suggest that there is conservation of major gene order in the phylum *Chordata* (Moritz *et al*, 1987).

Sequence data reported to date from honey bee mtDNA describe a set of 6 consecutive genes (tRNAs for *Leu*, *Asp*, *Lys* and *Trp*, COI, COII) and the large rRNA gene in *Apis mellifera ligustica* (Crozier *et al*, 1989 and Vlasak *et al*, 1987, respectively). Compared to *Drosophila*, *Apis* gene order displays translocations of 3 of the 4 tRNA genes. Such tRNA transpositions have been documented between most orders (Dubin *et al*,1986; Haucke and Gellissen, 1988) but no transposition affecting respiratory complex subunits have been found.

Conditions of reduced stringency were utilized to map, by hybridization to yeast probes, the corresponding honey bee mitochondrial genes. Most hybrids remained intact after washes in 2 x SSC and 60 °C indicating a considerable degree of similarity. Yeast probes are available for individual mtDNA genes and have been used successfully in mapping organelle genes (Macino, 1980; Spithill *et al*, 1983; Borkhardt and Olson, 1986). Concerning our difficulty in positioning the COIII gene, it is interesting that Spithill *et al* (1983) reported extensive hybridization of the yeast COIII probe to several non-adjacent mtDNA fragments in *Leishmania tarentolae*.

Our results reveal a conserved gene order between *Apis mellifera* and *Drosophila*, and reinforce the inversion detected between invertebrate and vertebrate genomes concerning the large and small ribosomal subunit genes (fig 4).

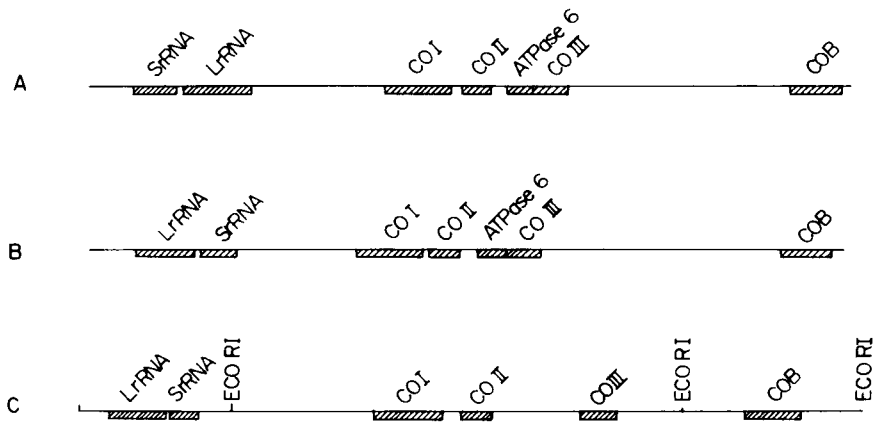

Fig 4. Comparative mitochondrial gene maps. A, mice (Bibb *et al,* 1981), B, *Drosophila yakuba* (Clary and Wolstenholme, 1985); and C, *Apis mellifera scutellata* (present work).

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Résumé — Localisation des gènes sur l'ADN mitochondrial issu d'*Apis mellifera scutellata* chez des abeilles africanisées. L'ADN mitochondrial (ADNmt) est une molécule circulaire longue d'environ 16 à 18 kb. La détermination complète des séquences de nucléotides chez l'homme, la pieuvre, la vache et la drosophile a montré que le nombre de gènes et leur organisation sont restés quasiment inchangés au cours de l'évolution, l'ordre ne changeant qu'entre les phyla et principalement parmi les gènes d'ARN_t. Nous avons déterminé la position de 6 gènes dans la molécule d'ADN_{mt} issue d'abeilles présumées africanisées. Les mitochondries provenant d'environ 300 nymphes aux yeux blancs ont été purifiées par centrifugation dans un gradient de saccharose. L'ADN_{mt} a été extrait et purifié. Les échantillons ont été digérés soit seuls, soit en combinaison avec les endonucléases suivantes : *Acc*I, *Cla*I, *Eco*RI, *Eco*RV, *Hind*III et *Xho*I (fig 1, tableau II). Les fragments ont été séparés sur des gels d'agarose à 0,9% et transférés sur des filtres Hybond-N. Des sondes spécifiques pour les principaux gènes mitochondriaux (COI, COII, COIII, CytB, sous-unité ribosomale petite et grande) (tableau I) ont été obtenues à partir des mutants «petite» de la levure *Saccharomyces cerevisae* et marqués par *nick translation.* L'hybridation a été faite en une nuit à 40 °C dans 6 x SSC, du Sarkosyl à 0,1% et 50 µg/ml d'ADN porteur dénaturé. Nous avons utilisé une température basse (40 °C) afin d'obtenir la formation d'hybride avec les sondes de levure hétérologues.

Après autoradiographie, les filtres ont été lavés successivement dans 2 x SSC, Sarkosyl à 0,1% à 45, 50, 55 et 60 °C avec autoradiographie après chaque lavage pour révéler les bandes ayant la plus grande stabilité d'hybride. La figure 2 donne les résultats. Nous avons construit une carte des gènes pour l'abeille africanisée d'après les résultats de l'hybridation (fig 3). Le gène COIII est placé sur le premier fragment *Eco*RI bien que la sonde montre une homologie avec les 2 autres fragments *Eco*RI. La comparaison avec les cartes du génome de la drosophile et de la souris montre qu'entre les 2 insectes, l'ordre des gènes est conservé et que l'on retrouve l'inversion déjà mise en évidence entre les gènes de l'ARN ribosomal des Vertébrés et ceux des Invertébrés.

abeille africanisée / gène mitochondrial / cartographie de gènes / endonucléase de restriction / hybridation moléculaire

Zusammenfassung — Lage von Genen in der von *Apis mellifera scutellata* abstammenden mitochondrialen DNA afrikanisierter Bienen. Die mitochondriale DNA (mtDNA) ist ein ringförmiges Molekül von etwa 16 bis 18 kb Länge. Die vollständige Bestimmung der Nukleotid-Sequenz bei Mensch, *Xenopus*, Rind und *Drosophila* haben gezeigt, daß Gengehalt und ihre Organisation während der Evolution in hohem Maße unverändert geblieben sind; sie zeigen nur beim Vergleich zwischen Tierstämmen Unterschiede in der Anordnung, und zwar vorwiegend bei Genen der tRNA. Wir bestimmten die Lage von sechs Genen des mitochondrialen DNA-Moleküls der Afrikanisierten Biene, die vermutlich von *Apis mellifera scutellata* abstammt. Von ungefähr 300 weißäugigen Puppen wurden die Mitochondrien durch Zentrifugierung im Sucrosegradienten gereinigt. Die mtDNA wurde aus den Organellen extrahiert und gereinigt. Die Proben wurden für sich oder gemeinsam mit folgenden Endonukleasen abgebaut: *Acc*I, *Cla*I, *Eco*Ri, *Eco*RV, *Hin*dIII, und *Xho*I (Abb 1 und Tabelle II). Die Fragmente wurden in 0.9% Agarosegel getrennt und auf Hybond-N-Filter übertragen. Spezifische Untersuchungen auf die mitochondrialen Hauptgene (COI, COII, COIII, CytB, kleine und große ribosomale Untereinheit; Tab I) wurden mittels geeigneter "petite" Hefemutanten durchgeführt und mit Nick-Translation markiert. Die Hybridisierung wurde über Nacht in 6 x SSC, 0.1% Sarcosyl und 50 µg/ml denaturierter Träger-DNA bei 40 °C durchgeführt. Wir benutzten niedrigere Temperaturen (40 °C), um Hybridbildungen mit den heterologen Hefesonden zu ermöglichen. Nach der Autoradiographie wurden die Filter sukzessive in 2 x SSC, 0.1% Sarkosyl bei 45, 50, 55 und 60 °C gewaschen, mit Autoradiographie nach jedem Auswaschen; damit sollten die Bande mit der höchsten Hybrid-Stabilität aufgedeckt werden. Die Ergebnisse werden in Abbildung 2 gezeigt. Wir haben nach den Hybridisierungsresultaten eine Genkarte für die afrikanisierte Honigbiene konstruiert (Abb 3). Das COIII-Gen kam auf dem ersten *Eco*RI-Fragmen zu liegen, obwohl die Sonde eine Homologie mit den anderen beiden *Eco*RI-Fragmenten zeigte. Diese Karte zeigt bei Vergleich mit den Genkarten von *Drosophila* und Maus (Abb 4) eine konservative Genanordnung bei den beiden Insekten und deckt dieselbe Inversion auf, die schon früher bei den ribosomalen RNA-Genen zwischen Vertebraten und Invertebraten gefunden wurde.

afrikanisierte Biene / mitochondriales Gen / Genkarte / Endonuklease / molekulare Hybridisierung

REFERENCES

Anderson S, Bankier AT, Barrell GT, De Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence organization of the human mitochondrial genome. *Nature (Lond)* 290, 457-465

Anderson S, De Bruijn MHL, Coulson AR, Eperon IC, Sanger F, Young IG (1982) Complete sequence of bovine mitochondrial DNA. Conserved features of the mammalian mitochondrial genome. *J Mol Biol* 156, 683-717

Arias MC, Soares AEE, Nobrega FG (1990) Improvements to the mitochondrial restriction maps for italian and africanized honey bees. *Braz J Genet* 13, 501-507

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Niegel JE, Reeb CA, Saunders NC (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Ann Rev Ecol Syst* 18, 489-522

Bibb MJ, Van Etten RA, Wright CT, Walberg MW, Clayton DA (1981) Sequence and organization of mouse mitochondrial DNA. *Cell* 26, 167-180

Borkhardt B, Olson LW (1986) The mitochondrial genome of the aquatic phycomycete *Blastocladiella emersoni. Curr Genet* 11,139-141

Bonitz SG, Coruzzi G, Thalenfeld BE, Tzagoloff A (1980) Assembly of mitochondrial membrane system. Structure and nucleotide sequence of the gene coding for subunit I of yeast cytochrome oxidase. *J Biol Chem* 255, 11927-11941

Cann RL, Stoneking M, Wilson AC (1987) Mitochondrial DNA and human evolution. *Nature (Lond)* 325, 31-36

Clary DO, Wolstenholme DR (1984) The *Drosophila* mitochondrial genome. *In: Oxford Surveys on Eukaryotic Genes* (Maclean N, ed) Oxford University Press, vol 1, 1-35

Clary DO, Wolstenholme DR (1985) The mitochondrial DNA molecule of *Drosophila yakuba* : nucleotide sequence, gene organization and genetic code. *J Mol Evol* 22, 252- 271

Coruzzi G, Tzagoloff A (1979) Assembly of the mitochondrial membrane system. DNA sequence of subunit II of yeast cytochrome oxidase. *J Biol Chem* 254, 9324-9330

Crozier RH, Crozier YC, Mackinlay AG (l989) The CO*I* and CO*II* region of honeybee mitochondrial DNA: evidence for variation in insect mitochondrial evolutionary rates. *Mol Biol Evol* 6, 399-411

Dubin DT, HsuChen CC, Tillotson LE (1986) Mosquito mitochondrial transfer RNAs for valine, glycine and glutamate:RNA and gene sequences and vicinal genome organization. *Curr Genet* 10, 701-707

Dujon B (1980) Sequence of the intron and flanking exons of the mitochondrial *21S* rRNA gene of yeast strains having different alleles at the w and rib-1 loci. *Cell* 20, 185-197

Hall HG, Muralidharan K (1989) Evidence from mitochondrial DNA that African honey bees spread as continuous maternal lineages. *Nature (Lond)* 339, 211-213

Haucke HR, Gellissen G (1988) Different mitochondrial gene order among insects: exchanged tRNA gene position in the CO*II* / CO*III* region between an orthopteran and a dipteran species. *Curr Genet* 14, 471-476

Li M, Tzagoloff A (1982) Identification of the paramomycin-resistance mutation in the *15S* rRNA gene of yeast mitochondria. *J Biol Chem* 257, 5921-5928

Macino G (1980) Mapping of mitochondrial structural genes in *Neurospora crassa. J Biol Chem* 255,10523-10565

Mariottini P, Chomyn A, Riley M, Cottrell B, Doolittle RF, Attardi G (1986) Identification of the polypeptides encoded in the unassigned reading frames 2,4,4L and 5 of human mitochondrial DNA. *Proc Natl Acad Sci USA* 83, 1563-1567

Moritz C, Dowling TE, Brown WM (1987) Evolution of animal mitochondrial DNA. Relevance for population biology and systematics. *Ann Rev Ecol Syst* 18, 269-292

Moritz RFA, Hawkins CF, Crozier RH, Mackinlay AG (1986) A mitochondrial DNA polymorphism in honeybee *(Apis mellifera). Experientia* 42, 322-324

Nobrega FG, Tzagoloff A (1980) Assembly of the mitochondrial membrane system. DNA sequence and organization of the cyto-

chrome *b* gene in *Saccharomyces cerevisiae* D273-10B. *J Biol Chem* 255, 9828-9837

Nobrega MP, Nobrega FG (1986) Mapping and sequencing of the wild-type and mutant (G116-40) alleles of the tyrosyl-tRNA mitochondrial gene in *Saccharomyces cerevisiae*. *J Biol Chem* 261, 3054-3059

Roe BA, Ma DP, Wilson RK, Wong JHF (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. *J Biol Chem* 260, 9759-9774

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sheppard WS, Rinderer TE, Mazzoli JA, Stelzer JA and Shimanuki H (1991) Gene flow between African- and European-derived honey bee populations in Argentine. *Nature (Lond)* 349, 782-784

Smith DR (1988) Mitochondrial DNA polymorphisms in five Old World subspecies of honey bees and New World hybrids. *In: Africanized Honey Bees and Bee Mites* (Needham GR, Page RE JR, Delfinado-Baker M, Bowman CL, eds) John Wiley and Sons, New York, 303-312

Smith DR, Brown WM (1990) Restriction endonuclease cleavage site and length polymorphisms in mitochondrial DNA of *Apis mellifera mellifera* and *A m carnica* (Hymenoptera: Apidae). *Ann Entomol Soc Am* 83, 81-88

Smith DR, Taylor OR, Brown WM (1989) Neotropical Africanized honey bees have African mitochondrial DNA. *Nature (Lond)* 339, 213-215

Spithill TW, Simpson AM, Simpson L (1983) Identification of sequence homologies between maxicircle DNA of *L tarentolae* and specific yeast mitochondrial structural genes. *In: Manipulation and Expression of Genes in Eukaryotes* (Nagley P, Linnane AW, Peacock NJ, Pateman JA, eds) Academic Press, New York, 307-311

Thalenfeld BE, Tzagoloff A (l980) Assembly of mitochondrial membrane system. Sequence of the *oxi* 2 gene of yeast mitochondrial DNA. *J Biol Chem* 255, 6173-6180

Tzagoloff A, Myers AM (1986) Genetics of mitochondrial biogenesis. *Ann Rev Biochem* 55, 249-285

Vlasak I, Burgschwaiger S, Kreil G (1987) Nucleotide sequence of the large ribosomal RNA of honeybee mitochondria. *Nucleic Acids Res* 15, 2388

Wilson AC, Cann RL, George M, Gyllensten UB, Helm-Bychowski KM, Higushi RG, Palumbi SR, Prager EM, Sage RD, Stoneking M (1985) Mitochondrial DNA and two perspectives on evolutionary genetics. *Biol J Linn Soc* 26, 375-400

Wolstenholme DR, Macfarlane JL, Okimoto R, Clary DO, Wahleitner JA (1987) Bizarre tRNAs inferred from DNA sequences of mitochondrial genomes of nematode worms. *Proc Natl Acad Sci USA* 84, 1324-1328