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Original article

An oligonucleotide primer set for PCR amplification of the complete honey bee mitochondrial genome*

Maria Cristina ARIAS[1], Daniela SILVESTRE[1], Flávio de Oliveira FRANCISCO[1], Ricardo WEINLICH[1], Walter Steven SHEPPARD[2]

[1] Departamento de Genética e Biologia Evolutiva, Instituto de Biociências,Universidade de São Paulo, São Paulo, SP, 05508-090, Brazil
[2] Department of Entomology, Washington State University, Pullman, WA, 99164-6382, USA

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Abstract – Mitochondrial DNA markers have been widely used to address population and evolutionary questions in the honey bee *Apis mellifera*. Most of the polymorphic markers are restricted to few mitochondrial regions. Here we describe a set of 24 oligonucleotides that allow PCR amplification of the entire mitochondrial genome of the honey bee *A. mellifera* in 12 amplicons. These fragments have important applications for the study of mitochondrial genes in different subspecies of *A. mellifera* and as heterospecific probes to characterize mitochondrial genomes in other bee species.

mitochondrial DNA / primer set / PCR / honey bee

1. INTRODUCTION

The honey bee *Apis mellifera* L. is one of the most-studied invertebrates. This species has a wide range distribution in the Old World and has been introduced by humans to numerous countries worldwide. Its ecological and economic importance and, moreover, its social organization, have stimulated research in a wide variety of fields. Recently, the nuclear genome of *A. mellifera* was published (HGSC, 2006) leading to several fundamental inferences about its biology: the rate of genome evolution is slower than in Diptera; the honey bee has more genes related to odorant receptors and nectar and pollen utilization than *Drosophila* and *Anopheles*; and population genetic analysis based on assessment of variation in single nucleotide polymorphisms suggests an African origin for the species (Whitfield et al., 2006).

A. mellifera was the first hymenopteran for which the mitochondrial DNA sequence was published (GenBank accession number L06178, Crozier and Crozier, 1993). The mitochondrial genome has been a very useful molecule for population genetic studies of *A. mellifera* and phylogenetic studies in *Apis*, as it contains regions with variable evolutionary rates. Most of the honey bee population genetic and evolutionary studies to date have been based on only a few mtDNA regions, with the primers used for amplification normally anchored in conservative regions of the genome (Garnery et al., 1992, 1995; Arias and Sheppard, 1996; De la Rúa et al., 1998; Meixner et al., 2000; Arias and Sheppard, 2005; Collet et al., 2006, 2007).

A list of primers based on the mitochondrial DNA sequences of several insects has been published and have proven widely useful (Simon et al., 1994). However, some

Corresponding author: Maria Cristina Arias, mcarias@ib.usp.br
Departamento de Genética e Biologia Evolutiva, Instituto de Biociências,Universidade de São Paulo, São Paulo, SP, 05508-090, Brazil.
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Table I. Primer sequences, their length, and genome location in *Apis mellifera*.

Name	Sequence 5'→3'	Length	Genome Location*
AMB1[a]	TGATAAAAGAAATATTTTGA	20	444
AMB2	CATGATCCTGGGGTACTTAA	20	1927
AMB3	TTTAAAAACTATTAATCTTC	20	1739
AMB4	GAAAGTTAGATTTACTCC	18	3062
AMB5	CAATAGGTGCAGTATTTGC	19	2932
AMB6	TATACTATATTTGATTTAAA	20	4420
AMB7	ATAATATAACATTAGTTTGT	20	4307
AMB8	GAGTATTCAATTGTTTGAA	19	5826
AMB9	TTTATTCTTGTATCATCAGG	20	5684
AMB10	GTACAATTTTTACTGTATCC	20	7427
AMB11	AATTATTATTATCATCATAG	20	7300
AMB12	TTAATTGGGATAATTTCGTG	20	8862
AMB13	TTCAACTAAAATTCCATTTT	20	8710
AMB14	TTTAAAAATGGTAATTTTGG	20	10417
AMB15	ATTTAAAAACATTAATTTTG	20	10283
AMB16[b]	ATTACACCTCCTAATTTATTAGGAAT	26	11884
AMB17[b]	TATGTACTACCATGAGGACAAATATC	26	11400
AMB18	ATTCAGGATCGTAAAGGTCC	20	13126
AMB19	AAATCCAAATCAAGGATACA	20	12941
AMB20[c]	TTTTGTACCTTTTGTATCAGGGTTG	25	14447
AMB21	GCTCCCTTATTTTCGAGATA	20	14377
AMB22	CTGAAACAATGAATGAAAGT	20	15399
AMB23	ACTTTCATTCATTGTTTCA	19	15380
AMB24	TCAAAATATTTCTTTTATCA	20	463

* Position of the 5' end of each primer in *A. mellifera*, according to Crozier and Crozier (1993); [a] Arias and Sheppard (1996); [b] Crozier et al. (1991); [c] Hall and Smith (1991).

inconsistencies exist in the ability of a number of these primer pairs to amplify honey bee mtDNA (Arias et al., unpubl. data). Difficulties in PCR amplification of hymenopteran DNA have been noted (Roehrdanz and DeGrugillier, 1998) likely due, in part, to the high level of interspecific variability (Crozier and Crozier, 1993). Thus the possibility to access other mtDNA regions or amplify the complete molecule led us to design a set of primers that, in combination with others from the literature, readily permits amplification of the entire mitochondrial genome of *A. mellifera*.

2. MATERIALS AND METHODS

Adult worker samples of Africanized honey bees were collected from flowers in São Paulo, SP, Brazil. Comparable samples of an Italian honey bee "strain" were collected from colonies maintained at the Genetic Department of São Paulo University in Ribeirão Preto, SP, Brazil. The honey bee samples were stored at –80 °C. Total DNA extraction was performed using a single thorax according to the procedure described by Sheppard and McPheron (1991).

Specific primers (Tab. I) for amplifying the whole mitochondrial genome in 12 overlapping fragments were designed based on the published honey bee mtDNA sequence (Crozier and Crozier, 1993). To design them we considered the length of each fragment, with the last base position being preferentially a second codon position and an overlap of amplified regions of approximately 100 bp (Fig. 1).

Each PCR reaction was set up with 1 μL of DNA, 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 20 μM of each primer, 2 mM each dNTPs, 2.5 U of *Taq* DNA polymerase (Invitrogen), and sterile water to achieve a final volume of 50 μL. The PCR amplifications (using a Perkin Elmer 9700 thermocycler) consisted of an initial denaturation of 94 °C for 5 min, followed

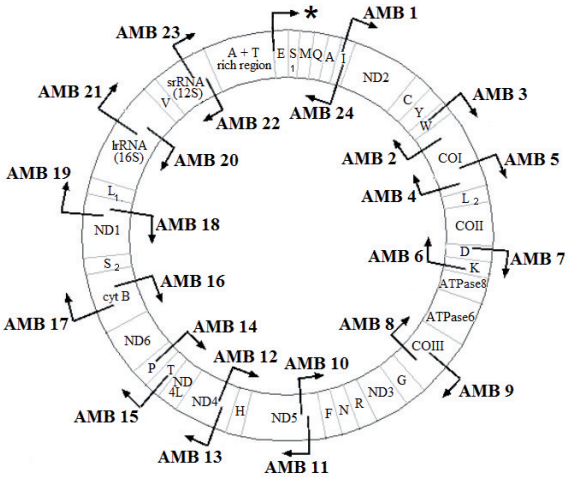

Figure 1. Circular map of *Apis mellifera* mtDNA (not in scale) indicating the primer location and direction, and gene order: E - tRNAGlu; S1 - tRNASer^{AGN}; M - tRNAMet; Q - tRNAGln; A - tRNAAla; I - tRNAIle; ND2 - NADH dehydrogenase subunit 2; C - tRNACys; Y - tRNATyr; W - tRNATrp; COI - cytochrome c oxidase subunit I; L2 - tRNALeu^{UUR}; COII - cytochrome c oxidase subunit II; D - tRNAAsp; K - tRNALys; ATPase8 - ATP synthase subunit 8; ATPase6 - ATP synthase subunit 6; COIII - cytochrome c oxidase subunit III; G - tRNAGly; ND3 - NADH dehydrogenase subunit 3; R - tRNAArg; N - tRNAAsn; F - tRNAPhe; ND5 - NADH dehydrogenase subunit 5; H - tRNAHis; ND4 - NADH dehydrogenase subunit 4; ND4L - NADH dehydrogenase subunit 4 (light chain); T - tRNAThr; P - tRNAPro; ND6 - NADH dehydrogenase subunit 6; cytB - cytochrome B; S2 - tRNASer^{UCN}; ND1 - NADH dehydrogenase subunit 1; L1 - tRNALeu^{CUN}; lrRNA(16S) - rRNA subunit16S; V - tRNAVal; srRNA(12S) - rRNA subunit 12S; A+T rich region - control region. * Indicates the base pair position number 1 according to Crozier and Crozier (1993).

by 15 to 35 cycles of 94 °C for 60 s for denaturing the DNA, 30 or 80 s at the appropriate temperature for annealing (Tab. II) and an extension step of 64 °C for 120 s. After the amplication cycles were completed, an additional final extension step of 64 °C for 10 min was performed. The PCR products were electrophoresed in 0.8% agarose gels, stained with ethidium bromide, and visualized and photographed under UV light.

3. RESULTS AND DISCUSSION

The described primer set (Tabs. I, II) successfully amplified the complete honey bee mitochondrial genome in 12 overlapping fragments. Although some papers have described the possibility of amplifying arthropodan mtDNA in one or 2 amplicons (Hwang et al., 2001; Barau et al., 2005), we were unsuccessful in applying these methods to the honey bee. The amplicon sizes ranged from 1022 (AMB21 + AMB22) to 1743 bp (AMB9 + AMB10) for both honey bee strains (Italian and Africanized). One concern was that the use of 12 amplicons could lead to inadvertent pseudogene amplification. Recent analyses of the honey bee genome have revealed the widespread dispersal of nucleotide sequences of mitochondrial origin throughout the nuclear DNA (Pamilo et al., 2007; Behura, 2007). These nuclear copies of mitochondrial sequences (numts) were estimated to comprise 0.08% of the nuclear genome of the honey bee and ranged in length from 39 to 926 bp, with a mean length of 134 bp (*ibid*). This size range for honey bee numts is below the minimum amplicon length produced by any of the current mtDNA primer set pairs (1022 bp). In addition, as adjacent primer pairs amplify overlapping sequence regions of the mtDNA that serve as checks for contiguity, it is not likely that nuclear copies of mitochondrial genes were inadvertently amplified.

Table II. PCR conditions for each primer pair and amplicon size.

Primer pair	Number of cycles	Annealing Temperature (°C)	Annealing Time (s)	Amplicon size
AMB1 + AMB2	35	42	80	1483
AMB3 + AMB4	35	42	80	1323
AMB5 + AMB6	35	42	80	1488*
AMB7 + AMB8	35	42	80	1519
AMB9 + AMB10	15	44	30	1743
AMB11 + AMB12	35	42	80	1562
AMB13 + AMB14	15	42.5	30	1707
AMB15 + AMB16	35	42	80	1601
AMB17 + AMB18	35	42	80	1726
AMB19 + AMB20	35	42	80	1506
AMB21 + AMB22	25	42	80	1022
AMB23 + AMB24	15	44	30	1470

* This amplicon comprises the COI-COII intergenic region, thus its size varies according to the honey bee subspecies.

Table III. AMB primer pairs or their combination with other primers and bee genera which gave successful PCR amplification.

Primer pair	Bee genera or species	Amplicon size (bp)	Reference
AMB17 + AMB18	*Plebeia* – 5 species	1700	Francisco et al., 2001
	Melipona – 7 species		Weinlich et al., 2004
AMB3 + AMB4	*Centris*	1300	Silvestre and Arias, unpubl. data
	Melipona bicolor		
AMB3 + mtD9[a]	*Mesocheira*	800	Silvestre and Arias, unpubl. data
	Eufriesia		
	Xylocopa		
AMB1 + mtD9[a]	*Bombus morio*	2100	Silvestre and Arias, unpubl. data
AMB1 + seq41[b]	*Melipona bicolor*	1700	Silvestre and Arias, unpubl. data

[a] Simon et al., 1994.
[b] Silvestre and Arias, unpubl.

Using these primers, specific mtDNA regions can be more rigorously investigated, which should permit the development of new molecular markers for population and evolutionary studies. Of special interest will be the application of these findings to address the process of Africanization in the New World and the nature of subspecific hybrid zones in the Old World (Lobo et al., 1989; Meixner et al., 1993; Collet et al., 2006, 2007). The 12 amplicons produced from this primer set also can be used as probes in Southern blot experiments, avoiding the requirement of salt gradient ultracentrifuge to purify probe mtDNA. The feasibility of using the amplicons as heterospecific probe was demonstrated by using this pool of fragments to determine mtDNA restriction maps for 17 species of Meliponini, where polymorphic restriction sites at both intra- and inter-specific levels were mapped (Arias et al., 2006).

The original AMB primer pairs, especially AMB 1, 2, 3, 4, 17 and 18, and others in combination with those described by Simon et al. (1994) permitted amplification of specific regions for RFLP analysis and sequencing in population genetic and phylogenetic studies of several non-*Apis* bee species (Tab. III). Primers AMB 1, 3 and 4 have been employed successfully in a mitochondrial genome sequencing project for *Melipona bicolor* (Silvestre et al., 2008). As these primers amplify specific overlapped regions encompassing genes and intergenic regions, some

amplicons have provided indirect evidence of gene order alterations by presenting sizes different than expected, compared to *A. mellifera* amplicons. These findings led to the recent detection of a high level of tRNA translocations in the bee mtDNA genome (Silvestre et al., 2002; Silvestre and Arias, 2006). The set of primers presented in this study, although specially designed for the honey bee *A. mellifera*, has proven highly useful to detect restriction site polymorphism in other bees. We expect that these primers will be useful also to investigate the mitochondrial genome of other taxa within the family Apidae and to further improve our understanding of honey bee population dynamics.

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Un kit d'amorces oligonucléotides pour amplification par PCR du génome mitochondrial complet de l'Abeille domestique, *Apis mellifera*.

***Apis mellifera* / ADN mitochondrial / kit d'amorces / PCR / génome**

Zusammenfassung – Ein Oligonukleotidprimer-Satz zur PCR-Amplifikation des kompletten mitochondrialen Genoms der Honigbiene. Der Informationsgehalt des mitochondrialen Genoms (mtDNA) ist in weitem Mass von Bedeutung für populationsgenetische und evolutive Studien. Bei der Honigbiene, *Apis mellifera*, trug die Aufdeckung von Polymorphismen in diesem Molekül zu wichtigen Einsichten in die Populationsstruktur und zur Phylogenie bei. Obwohl *A. mellifera* die Hymenopterenart ist, für die als erste das mitochondriale Genom komplett sequenziert wurde, beschränken sich selbst bei dieser Art die meisten Populations- und Evolutionsstudien auf nur einige wenige mitochondriale Regionen. Für die Nutzung weiterer mtDNA Regionen ist die Entwicklung geeigneter Primer von zentraler Bedeutung für erfolgreiche PCR-Amplifikationen. Die Verwendung genereller Oligonukleotidprimer (universelle Primer) für das mitochondriale Genom von Insekten führte bei der Honigbiene oft zu Fehlschlägen oder lieferte keine hochwertigen PCR-Produkte. Wir beschreiben in der vorliegenden Arbeit einen Satz bestehend aus 24 Oligonukleotiden, mit denen es möglich war, das gesamte mitochondriale Genom der Honigbiene *A. mellifera* in Form von 12 Fragmenten zu amplifizieren (Abb. 1, Tab. I). Die Amplifikationsbedingungen für diese Primerkombinationen wurden etabliert und sind in Tabelle II zusammengestellt. Diese Fragmente können von Wichtigkeit sein für die Untersuchung mitochondrialer Gene bei verschiedenen Unterarten von *A. mellifera* und ebenso als heterospezifische Sonden zur Charakterisierung des mitochondrialen Genoms anderer Bienenarten dienen. Ausserdem kann die Verwendung eines Teilsatzes dieser Primer für die Amplifizierung homologer Regionen bei anderen Bienen genutzt werden (Tab. III) und damit künftige Populationsuntersuchungen und evolutive Studien innerhalb der Hymenopteren vorantreiben.

Mitochondriale DNA / Primer-Satz / PCR / Honigbiene

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