

PCR primers for the amplification of four insect mitochondrial gene fragments

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Abstract

Insect mitochondrial genome (mtDNA) analysis is a powerful tool for the study of population genetics and phylogenetics. In the past few years primer sequences for the PCR amplification of various insect mtDNA genes have been published. The objectives of this study were (1) present new primer sequences for six insect mitochondrial genes and (2) test primers designed in our laboratory and some previously published primers on a wide range of insects to determine if amplification of the target fragment could be obtained. The primers for the amplification of the two ribosomal RNA gene (16S and 12S rRNA) fragments are universal for insects and related groups; the primers for NADH5 and NADH4 dehydrogenase gene fragments and cytochrome c oxidase I gene fragment are applicable broadly.

Keywords: mtDNA, ribosomal RNA genes, cytochrome c oxidase gene, NADH dehydrogenase genes.

Introduction

Animal mitochondrial DNA (mtDNA) is a small circular molecule ranging in size from 15 to 18 kilo basepairs (bp) (Wilson *et al.*, 1985). Insect mtDNA consists of thirty-seven genes including two ribosomal RNA (rRNA) genes, twenty-two transfer RNA (tRNA) genes and thirteen protein coding genes (Clay & Wolstenholme, 1985; Crozier & Crozier, 1993; Mitchel *et al.*, 1993). With rare exceptions, insect mtDNA is inherited exclusively maternally. These and other features have made mtDNA a powerful tool for population genetic and phylogenetic studies of a variety of organisms (see for reviews Avise, 1994; Wilson *et al.*, 1985; Simon *et al.*, 1994).

With the advent of polymerase chain reaction (PCR; Saiki *et al.*, 1985), primers for the amplification of mitochon-

drial genes have become available (Kocher *et al.*, 1989; Simon *et al.*, 1994). The quest has been for 'universal primers' applicable to a broad range of taxa (Kocher *et al.*, 1989). Most recently, Simon *et al.* (1994) provided a comprehensive compilation of primer sequences for the amplification of virtually every gene encoded in insect mtDNA. In this paper we provide DNA sequence for four new primers for amplification of insect mtDNA gene fragments to supplement those presented by Simon *et al.* (1994). We also present the results of PCR amplification of portions of six mitochondrial genes from ten orders of insects and a tick using the four new primers in combination with previously published primers. The primers used in this study amplify portions of the two rRNA genes, cytochrome c oxidase I gene, NADH4 and NADH5 dehydrogenase genes and the entire tRNA^{His} gene.

Results and Discussion

Large ribosomal subunit (16S rRNA) primers

The forward primer (*LR-J-13017*) for the amplification of a portion of the 16S rRNA gene was designed in our laboratory; the reverse primer (*LR-N-13398*) was reported by Simon *et al.* (1994). PCR using these primers resulted in the amplification of a ~415 bp fragment from insects of all ten orders and the tick included in our analysis (Fig. 1a). In addition, the 16S primer set has been used to amplify and sequence a homologous fragment from cockroaches, termites and a mantid (Kambhampati, 1995), sand flies (Diptera: Psychodidae), *Aedes* spp. (Diptera: Culicidae), aphids of the family Lachnidae, dragonflies (Odonata) and spiders (S. Kambhampati, unpubl. data). The fragment was also amplified from five species of thrips (Thysanoptera; B. Crespi, pers. comm.). The sequence of an alternative forward primer, *LR-J-12887*, varies among insects of different orders and families (see Simon *et al.*, 1994). Therefore the primer *LR-J-12887* when combined with *LR-N-13398* fails to amplify the target region from certain insects (e.g. Aphidiidae; S. Kambhampati, unpubl. data). In contrast, the sequence of *LR-J-13017* is conserved among insects of at least ten orders and when combined with *LR-J-13398* is more effective in amplifying the target region from many insects and related organisms. A primer modified by moving *LR-J-12887* four bases upstream was used

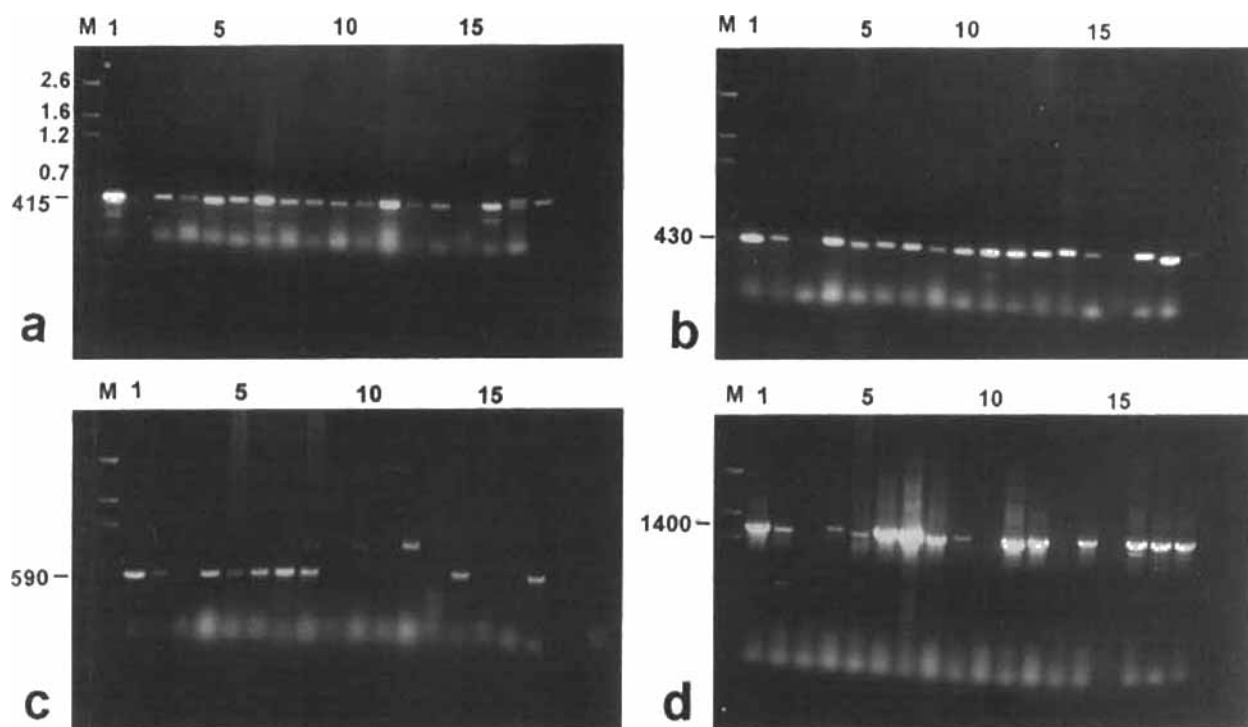


Figure 1. Photographs showing the amplification of (a) ~415 bp fragment of the 16S rRNA gene (b) ~430 bp fragment of the 12S rRNA gene, (c) ~570 bp fragment of the COI gene, and (d) ~1400 bp fragment of the NADH5 and NADH4 dehydrogenase and the tRNA^{His} genes. The lane marked *M* is the molecular weight standard (pGEM digested with *Hinf* I, *Rsa* I and *Sin* I; Promega Corp.; molecular weights in kbp.). The lane order is as indicated in Table 1 with the following exceptions. In (c) and (d), *N. mona* is not included; in (d) lane 19 contains the amplified fragment from *Lysiphlebus testaceipes* (Hymenoptera: Aphidiidae). Lanes 2 and 15 in panel (a), 3 and 19 in panel (b) and 2 and 10 in panel (c) are relatively faint.

by Xiong & Kocher (1991) to amplify the target fragment from black flies.

Small ribosomal subunit (12S rRNA) primers

The forward primer (*SR-J-14199*) for the amplification of a portion of the 12S rRNA gene was designed in our laboratory. The reverse primer (*SR-N-14594*) is similar to *SR-J-14588* of Simon *et al.* (1994), the former being six nucleotides shorter than the latter. These primers resulted in the amplification of a ~430 bp fragment from all insects and the tick included in this study, with the exception of *M. sanguinipes* (Fig. 1b). In addition, this fragment has been amplified and sequenced from cockroaches, termites and a mantid (Kambhampati, 1995), sand flies (D. Rickard and S. Kambhampati, unpubl. data) and *Aedes* spp. (S. Kambhampati, unpubl. data). Therefore the 12S rRNA primer set is also applicable across a broad range of insect taxa as well as ticks.

Cytochrome *c* oxidase I primers

The COI forward primer (*CI-J-1632*) was designed in our laboratory. The *CI-N-2191* primer is three nucleotides shorter on the 5' end than the one reported by Simon *et al.* (1994) under the same name. Amplification of a ~590 bp

fragment of the COI gene using these primers was obtained from most insects we tested (Fig. 1c). The fragment we obtained from *H. virescens* and *S. graminum* is considerably larger (~800 bp) than that from other insects. At present we cannot confirm whether the fragment amplified from *H. virescens* and *S. graminum* is part of the COI gene or if it is a non-target fragment. The COI fragment has been sequenced from several wasp genera of the family Aphidiidae (S. Kambhampati, unpubl. data).

NADH dehydrogenase primers

The *N5-J-7502* primer was designed in our laboratory. A complement of the primer *N4-N-8925* that is three nucleotides longer was reported as *N4-J-8944* by Jermini & Crozier (1994). The amplified fragment consists of ~620 bp of the NADH5 dehydrogenase gene, the entire tRNA^{His} gene (67 bp in *Apis mellifera*) and about ~700 bp of the NADH4 dehydrogenase gene for a total of ~1400 bp. The NADH primers resulted in successful amplification in fourteen of the eighteen taxa tested (Fig. 1d). The amplified fragment has been partially sequenced from three wasp genera of the family Aphidiidae (P. T. Smith and S. Kambhampati, unpubl. data). The amplified fragment is sufficiently large to enable RFLP analyses to detect sequence variation among individuals, populations and species.

Table 1. List of taxa used in this study.

Species	Family	Order
1. <i>Apis mellifera</i>	Apidae	Hymenoptera
2. <i>Aphidius ervi</i>	Aphidiidae	Hymenoptera
3. <i>Comptonotus</i> sp.	Formicidae	Hymenoptera
4. <i>Polistes dominulus</i>	Vespidae	Hymenoptera
5. <i>Ceratogastra</i> sp.	Ichneumonidae	Hymenoptera
6. <i>Drosophila melanogaster</i>	Drosophilidae	Diptera
7. <i>Stomoxys calcitrans</i>	Muscidae	Diptera
8. <i>Callosobruchus maculatus</i>	Curculionidae	Coleoptera
9. <i>Tribolium castaneum</i>	Tenebrionidae	Coleoptera
10. <i>Schizaphis gaminum</i>	Aphididae	Homoptera
11. <i>Acyrtosiphon pisum</i>	Aphididae	Homoptera
12. <i>Heliothis virescens</i>	Noctuidae	Lepidoptera
13. <i>Lepisma saccharina</i>	Lepismatidae	Thysanura
14. <i>Blattella germanica</i>	Blattellidae	Dictyoptera
15. <i>Melanoplus sanguinipes</i>	Acrididae	Orthoptera
16. <i>Ctenocephalides felis</i>	Pulicidae	Siphonaptera
17. <i>Dermacentor andersoni</i>	Ixodidae	Acari
18. <i>Neotermes mona</i>	Kalotermitidae	Isoptera

Experimental procedures

Insects

Representatives of ten orders of insects and one tick of the family Ixodidae were used in this study (Table 1). The insects used were either frozen specimens, preserved in ethanol or were collected live.

DNA extraction

DNA was extracted from individual insects as described by Kambhampati & Rai (1991). A single insect (or a portion in case of larger insects) was homogenized in 50 µl of buffer (0.1 M, NaCl, 0.2 M

sucrose, 0.01 M EDTA, 0.03 M Tris-HCl, pH 8.0) using a hand-held homogenizer. An additional 50 µl of the homogenization buffer was added followed by 30 µl of lysis buffer (0.25 M EDTA, 2.5% (w/v) SDS, 0.5 M Tris-HCl, pH 9.2). Following incubation at 65°C for 40 min, 50 µl of 8 M potassium acetate was added and the mixture incubated on ice for 30 min. The tubes were centrifuged at 14,000 g for 15 min. The supernatant was transferred to a fresh 1.5 ml tube, followed by the addition of 1/10 vol of 2 M potassium acetate and 2 vol of chilled 95% ethanol. The tubes were incubated at -20°C overnight and centrifuged for 15 min at 14,000 g. The resulting DNA pellet was washed in 70% ethanol followed by 100% ethanol and dissolved in 40–50 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). 2–3 µl of this solution was used as a template in the PCR mixture. In the case of *D. andersoni*, 0.5 µl of CsCl-purified total genomic DNA (provided by W. C. Black IV) was used as template.

Polymerase chain reaction

PCR was set up in 50 µl volume as described by Kambhampati *et al.* (1992) in a thermal cycler (MJ Research; Model PTC-100-60). The temperature profile for the amplification of the two rRNA gene fragments was 95°C for 30 s, 40°C for 1 min and 72°C for 1 min for ten cycles followed by twenty-five cycles of 95°C for 30 s, 50°C for 1 min and 72°C for 1 min. For the amplification of the NADH dehydrogenase and the COI gene fragments, the temperature profile was 95°C for 30 s, 45°C for 1 min and 72°C for 1 min for thirty-five cycles. For both temperature profiles, an initial denaturation step of 95°C for 3 min and a final extension step of 72°C for 7 min were added. After amplification, the products were electrophoresed on 1% agarose gels containing 50 µl per litre of 10 mg/ml ethidium bromide. No contamination was detected in negative controls.

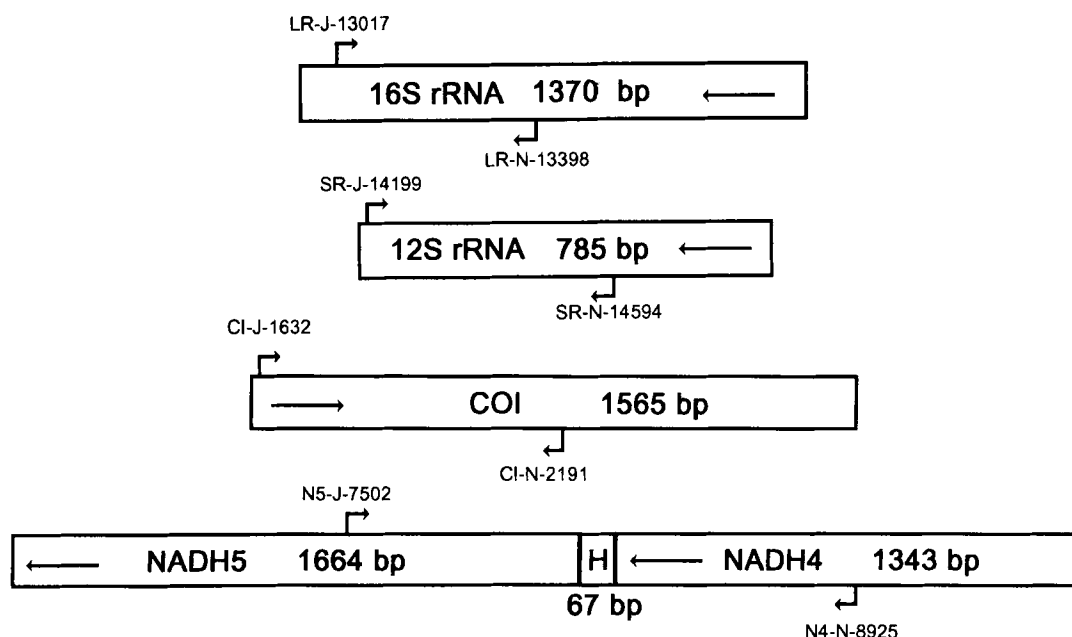


Figure 2. A schematic diagram of the locations of the primers used in this study in relation to the *D. yakuba* mitochondrial genome. Arrows inside the boxes indicate the direction of transcription. See Table 2 for primer sequences.

Table 2. DNA sequences for primers used for amplification of insect mitochondrial genes in this study. In cases of previously published sequences, the reference is provided. The locations of the primers refer to *D. yakuba* sequence. The approximate size of the amplified fragment is also shown.

Primer sequence (5'-3')	Location	Reference
16S rRNA gene (~ 415 bp)		
LR-J-13017: TTACGCTGTTATCCTAA	13000-13017	
LR-N-13398: CACCTGTTAACAAAAACAT	13398-13417	Simon <i>et al.</i> (1994)*
12S rRNA gene (~ 430 bp)		
SR-J-14199: TACTATGTTACGACTTAT	14182-14199	
SR-N-14594: AAACCTAGGATTAGATACCC	14594-14612	Simon <i>et al.</i> (1994)*
Cytochrome c oxidase I (~ 570 bp)		
CI-J-1632: TGATCAAATTTATAAT	1617-1632	
CI-N-2191: GGTAATAATTAATAAACTTC	2191-2213	Simon <i>et al.</i> (1994)*
NADH 4 + NADH 5 dehydrogenase (~ 1400 bp)		
N5-J-7502:		
CTAAAGTTGATGAATGAACCTAAAG	7479-7502	
N4-N-8925: GCTCATGTTGAAGCTCC	8925-8941	Jermiin & Crozier (1994)

*Primers modified slightly from those in Simon *et al.* (1994). See text for details.

Oligonucleotide primers

DNA sequences for the primer sets used for the amplification of insect mtDNA genes are given in Table 2 and their locations shown in Fig. 2. The primer sequences were derived from the mtDNA sequences of *Drosophila yakuba* (Clary & Wolstenholme, 1985), *Apis mellifera* (Crozier & Crozier, 1993) and *Anopheles quadrimaculatus* (Mitchell *et al.*, 1993). Primers were synthesized by Operon Technologies (Alameda, Calif.) or Cruachem Inc. (Sterling, Va.).

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