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Short Communication

# Exploitation of mitochondrial *nad6* as a complementary marker for studying population variability in Lepidoptera

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## Abstract

The applicability of mitochondrial *nad6* sequences to studies of DNA and population variability in Lepidoptera was tested in four species of economically important moths and one of wild butterflies. The genetic information so obtained was compared to that of *cox1* sequences for two species of Lepidoptera. *nad6* primers appropriately amplified all the tested DNA targets, the generated data proving to be as informative and suitable in recovering population structures as that of *cox1*. The proposal is that, to obtain more robust results, this mitochondrial region can be complementarily used with other molecular sequences in studies of low level phylogeny and population genetics in Lepidoptera.

*Key words: cytochrome* c *oxidase* I, *Diatraea saccharalis*, DNA polymorphism, *Hermeuptychia atalanta*, Noctuidae.

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Lepidoptera is the best-known order among insects, with relatively well-established systematic for most groups (Freitas *et al.*, 2006). It presents a number of monophagous and polyphagous moth species, capable of inflicting severe losses in several of the major agricultural commodities worldwide (Barros *et al.*, 2010; Molina-Ochoa *et al.*, 2010). On the other hand, a rising number of butterfly species have been targeted in conservation programs, leading these insects to be considered flagship taxa for conservation (New, 1997). Knowledge on species genetic relationships, population structures and patterns of gene flow among populations is a key, not only to the development of pest-management programs (Krafsur, 2005), but also to the selection and use of organisms for conservation initiatives (Dale and Beyeler, 2001).

The usefulness of animal mitochondrial DNA (mtDNA), as a molecular marker for studies of population structure, is well-known on account of ease in manipulation, rapid mutation rate, supposed lack of significant recombination, and availability of universal primers (Avise, 1986; Moritz *et al.*, 1987; Simon *et al.*, 1994). Recently, the use of the *cytochrome* c *oxidase* I gene (*cox1*) has largely replaced that of other mitochondrial regions in studies with animals, including many Lepidoptera (Silva-Brandão *et al.*, 2009), ever since its proposal as a "DNA barcode" for species diagnosis and delimitation (Hebert *et al.*, 2004), as well as its historical application in population genetics and phylogeographic studies (Avise, 2000). Notwithstanding, the recent availability of complete mitochondrial genomes of several Lepidoptera species (Cameron and Whiting, 2008; Yang *et al.*, 2009), has facilitated the evaluation and establishment of new genes for studying population genetics within the group. Subunits of *nicotinamide adenine dinucleotide dehydrogenase* (NADH), such as *nad1* (Miller *et al.*, 2009), *nad4* (Gomez *et al.*, 2009) and *nad5* (Meraner *et al.*, 2008), are beginning to be exploited in studies of population structure. These genes have already been widely used in studies at higher taxonomic levels (Weller *et al.*, 1994; Morinaka *et al.*, 1999; Yagi *et al.*, 1999), subunits of *nad* having proved to be more variable than the other mitochondrial regions frequently used in such instances (Cameron and Whiting, 2008).

The subunits of both *cox* and *nad* are related to the oxidative phosphorylation complexes encoded by the mito-

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chondrial genome (Montooth *et al.*, 2009). The gene that codifies subunit 6 of NADH (*nad6*) provides instructions for making a protein, NADH dehydrogenase 6, officially named "mitochondrially encoded NADH dehydrogenase 6", which is part of a large enzymatic machinery known as Complex I (Genetics Home Reference, 2011). The *nad6* gene ranges from 480 to 540 bp within the mitochondrial genomes of the 32 species of Lepidoptera available in GenBank.

Both the applicability of primers designed to amplify the mitochondrial gene *nad6*, and the efficacy of this region in differentiating populations, were tested with four species of moths considered economically important in Brazil, as well as one wild butterfly species. The genetic information so obtained was also compared with information provided by *cox1* on two of these species, the sugarcane borer *Diatraea saccharalis* (F.), the main pest of sugarcane (*Saccharum officinarum* L.) and an important one of corn (*Zea mays* L.), as well as *Hermeuptychia atalanta* Butler, a widely distributed Nymphalidae butterfly.

A total of 107 specimens from five species of Lepidoptera were sampled from distinct populations (Table 1). Total genomic DNA was obtained from the thoracic tissues of each, according to the Invisorb Spin Tissue kit (Uniscience) protocol. Extracted DNA was stored in a TE buffer at -20 °C. Primers for *nad6* gene amplification were designed, based on the alignment of complete mitochondrial genomes of all the Lepidoptera species available in GenBank (alignment available upon request). Forward and reverse primers were named according to their reference positions on the mitochondrial genome of *Manduca sexta* (L.) (GenBank accession number NC_010266), the forward primer thus beginning at 10090 (tPro-J10090-5ATCWATAATCTCCAAAATTAT 3), and the reverse at 10624 (ND6-N10624-5 GGNCCATAAAAAATATTWGT 3), thereby totaling 534 bp. Complete (for *D. saccharalis*) or partial (for *H. atalanta*) *cox1* fragments were amplified according to Silva-Brandão *et al.* (2008).

The *nad6* gene was amplified using 1 μL of total DNA, 2.0 mM of $MgCl_2$, 40 μM of dNTPs, 0.2 mM of each primer, 1U of GoTaq DNA Polymerase (Promega), and 10% of 10X *Taq* buffer, in 25 μL of final volume. The amplification protocol was as follows: an initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 45 °C for 45 s, and elongation at 60 °C for 1.5 min, followed by an extension step at 60 °C for 5 min. Aliquots were then analyzed by electrophoresis in 1% agarose gel. After purifying from primers and deoxynucleotides with ExoSAP-IT (GE Healthcare), the PCR products were then sequenced by an ABI Prism BigDye Kit protocol in an ABI 3700 automated sequencer (Applied Biosystems), with the forward primer used for amplification. Sequences were analyzed with the FinchTV 1.4.0 program (Geospiza Inc.), and manually aligned with BioEdit 7.0.5.3 (Hall, 1999).

Sequence divergence was quantified with the *p*-distance model of nucleotide substitution (Nei and Kumar, 2000), implemented into the MEGA v.5.0 program (Tamura *et al.*, 2011). Employing the same model and program, the Neighbor-Joining (NJ) clustering algorithm (Saitou and Nei, 1987) was applied for graphically obtaining phenetic distances among *D. saccharalis* and *H. atalanta* individuals. Robustness of each branch was defined with the non-parametric bootstrapping procedure (Felsenstein, 1985), with 1,000 replicates. Standard parameters of DNA polymorphism were estimated in DnaSP v.5.10 (Librado and Rozas, 2009) and MEGA v.5.0 (Tamura *et al.*, 2011).

The primers proposed here adequately served for amplifying the *nad6* region in all the species tested (GenBank accession numbers are shown in Table 2). The reported sequence length variation was due to the quality of the last bases sequenced. DNA polymorphism was low throughout (Table 2), although low genetic variability is the general rule for lepidopteran pest species (Coates *et al.*, 2004; Saw *et al.*, 2006; Behere *et al.*, 2007). Genetic distances for *Alabama argillacea* (Hübner) and *Heliothis virescens* (F.) populations ranged from 0.0 to 0.006, and from 0.0 to 0.032 for *Spodoptera frugiperda* (J.E. Smith). DNA polymorphism and pairwise genetic distances were higher in *S. frugiperda* populations than in all the other species, with most nucleotide substitutions being non-synonymous (Table 2). Worthy of note, these populations were sampled on two different crops (corn and cotton), even though no difference was found between populations collected in these two host plants in a previous study that applied RAPD markers (Martinelli *et al.*, 2006). Nonetheless, corn and rice biotypes of *S. frugiperda* have already been recorded in Brazil, when using AFLP markers (Busato *et al.*, 2004).

As regards *D. saccharalis* populations, the analysis of information provided by *nad6* and *cox1* showed the same amount of DNA variation for the two (Table 2). Genetic distances based on the two regions ranged from 0.0 to 0.004. However, the general pattern of genetic divergence was different, for with the overall increase, *cox1* divergence becoming more pronounced at the 3rd codon position (Figure 1 A and B). Both regions presented similar results in recovering population structure (Figure S1). Pairwise genetic distances of concatenated data also ranged from 0.0 to 0.004, NJ analysis resulting in a topology similar to that based only on *cox1* sequences (Figure 2 A).

The 5' end of *cox1* (the proposed "barcode") and *nad6* yielded almost the same results for *H. atalanta*, with similar values for general parameters of DNA polymorphism (Table 2). Pairwise genetic distances among *nad6* sequences ranged from 0.0 to 0.007, and among *cox1* from 0.0 to 0.006. Divergences, which occurred mainly at the 3rd codon position (Table 2), became progressively greater together with the overall increase (Figure 1 C and D). Phenetic relationships obtained with the two datasets were different, although both regions recovered a cluster com-

**Table 1** - Species of Lepidoptera, populations and number of specimens used to test the primers designed to amplify the mitochondrial region *nad6*.

| Species (Family) | Host plant | Populations: Locality, State (Code*; number of specimens) | Latitude | Longitude |
|---|---|---|---|---|
| *Alabama argillacea* | Cotton | Campina Grande, PB (2) | 7°13'52" S | 35°52"55.1"W |
| (Noctuidae) | | Campo Verde, MT (2) | 15°32'42.7" S | 55°9"55.6"W |
| | | Chapadão do Sul, MS (3) | 18°46'44" S | 52°36"59.4"W |
| | | Cristalina, GO (2) | 16°46'0.9" S | 47°36'29.9"W |
| | | Luis Eduardo Magalhães, BA (2) | 12°51'57.9" S | 45°47'53.7"W |
| | | Montividiu, GO (2) | 17°47'49.1" S | 50°54'0.2"W |
| | | Primavera do Leste, MT (1) | 15°33'32.7" S | 54°17'51.2"W |
| | | Roda Velha, BA (2) | 12°41'48.6" S | 45°49'57.6"W |
| | | São Disidério, BA (2) | 12°21'7.6" S | 44°59'3.2"W |
| *Diatraea saccharalis* | Corn | Bambuí, MG (Ds_MG_Zm; 3) | 20°1'8.7" S | 45°57'37.8"W |
| (Crambidae) | | Catalão, GO (Ds_GO_Zm; 3) | 18°9'43.6" S | 47°56'38.2"W |
| | | Itaberá, SP (Ds_SP_Zm; 3) | 23°51'20.7" S | 49°8'8.7"W |
| | | Passo Fundo, RS (Ds_RS_Zm; 3) | 28°15'38.5" S | 52°24'28.8"W |
| | | Ponta Grossa, PR (Ds_PR_Zm; 3) | 25°5'40.4" S | 50°9'47.9"W |
| | | Primavera do Leste, MT (Ds_MT_Zm; 3) | 15°33'32.7" S | 54°17'51.2"W |
| | Sugarcane | Jaboticabal, SP (Ds_SP1_So; 3) | 21°15'21.7" S | 48°19'22"W |
| | | Maringá, PR (Ds_PR_So; 3) | 23°25'34.6" S | 51°56'8.8"W |
| | | Monte Alegre, MG (Ds_MG_So; 3) | 21°24'4.4" S | 46°15'12.4"W |
| | | Pradópolis, SP (Ds_SP2_So; 3) | 21°21'35.3" S | 48°4'21.5"W |
| *Heliothis virescens* | Cotton | Chapadão do Sul, MS (3) | 18°46'44" S | 52°36"59.4"W |
| (Noctuidae) | | Luis Eduardo Magalhães, BA (3) | 12°51'57.9" S | 45°47'53.7"W |
| | | Palmeiras, GO (3) | 16°47'23" S | 49°55'58.5"W |
| | | Primavera do Leste, MT (3) | 15°33'32.7" S | 54°17'51.2"W |
| | | Riachão das Neves, BA (3) | 11°44'49" S | 44°54'25.5"W |
| | | Rio Verde, GO (3) | 16°46'0.9" S | 47°36'29.9"W |
| | | Sapezal, MT (3) | 12°59'21.8" S | 58°45'52"W |
| | | Sinop, MT (3) | 11°52'31.3" S | 55°30'17.7"W |
| *Hermeuptychia atalanta* | - | Campinas, SP (Ha_SP; 3) | 22°54'25.4" S | 47°3'47.8"W |
| (Nymphalidae) | | Catuípe, RS (Ha_RS; 3) | 28°14'59.8" S | 54°0'20.3"W |
| | | Jundiaí, SP (Ha_SP2; 3) | 23°11'15.1" S | 46°53'9.3"W |
| | | Paranaíta, MT (Ha_MT; 3) | 9°40'22.7" S | 56°28'50.3"W |
| | | Porto Mauá, RS (Ha_RS2; 3) | 27°34'15.7" S | 54°40'13.4"W |
| | | Santa Teresinha, BA (Ha_BA; 3) | 12°44'59.4" S | 39°31'6.1"W |
| | | Sousas, SP (Ha_SP3; 3) | 22°52'52" S | 46°57'57"W |
| *Spodoptera frugiperda* | Cotton | Acreúna, GO (2) | 17°23'41.7" S | 50°22'57"W |
| (Noctuidae) | | Barreiras, BA (2) | 12°8'55.22" S | 44°59'45.78"W |
| | | Primavera do Leste, MT (2) | 15°33'32.7" S | 54°17'51.2"W |
| | | Unaí, MG (2) | 16°20'38.11" S | 46°54'30.04"W |
| | Corn | Douradina, MS (1) | 22°2'12.59"S | 54°36'42.07"W |
| | | Luis Eduardo Magalhães, BA (1) | 12°51'57.9" S | 45°47'53.7"W |
| | | Passo Fundo, RS (1) | 28°15'38.5" S | 52°24'28.8"W |
| | | Ponta Grossa, PR (2) | 25°5'40.4" S | 50°9'47.9"W |
| | | Sapezal, MT (1) | 12°59'21.8" S | 58°45'52"W |
| | | Uberlândia, MG (2) | 18°54'52.88" S | 48°16'8.43"W |

*Code was applied only for populations analyzed with both markers (*cox1* and *nad6*).

**Table 2** - Standard genetic parameters of DNA polymorphism of five species of Lepidoptera. GenBank accession numbers; Number of polymorphic nucleotides and amino acids; Number of haplotypes; Mean pairwise divergence based on *p*-distance for all data and by codon position; $\pi$ = average number of nucleotide differences per site between two sequences (nucleotide diversity; Nei, 1987); k = average number of nucleotide differences (Tajima, 1983).

| | | Species | | | | |
|---|---|---|---|---|---|---|
| | | *Alabama argillacea* | *Diatraea saccharalis* | *Heliothis virescens* | *Hermeuptychia atalanta* | *Spodoptera frugiperda* |
| GenBank accession numbers | *cox1* | - | JN108957-JN108986 | - | JN109039- JN109059 | - |
| | *nad6* | JN108939-JN108956 | JN108987-JN109016 | JN109017- JN109038 | JN109060- JN109080 | JN109081- JN109096 |
| Number of polymorphic sites | *cox1* | - | 16/1486 | - | 7/660 | - |
| | *nad6* | 3/501 | 4/504 | 3/501 | 3/436 | 20/501 |
| Number of polymorphic amino acids | *cox1* | - | 4/495 | - | 0/220 | - |
| | *nad6* | 0/167 | 2/168 | 1/167 | 1-145 | 6/167 |
| Number of haplotypes | *cox1* | - | 9 | - | 4 | - |
| | *nad6* | 3 | 3 | 3 | 4 | 8 |
| Mean pairwise divergence (%) | *cox1* - All data | - | 0.1 | - | 0.2 | - |
| | *cox1* - $1^{st}/2^{nd}/3^{rd}$ | - | 0/0/0.2 | - | 0/0/0.7 | - |
| | *nad6* - All data | 0.1 | 0.1 | 0.1 | 0.3 | 0.8 |
| | *nad6* - $1^{st}/2^{nd}/3^{rd}$ | 0/0/0.3 | 0/0/0.1 | 0.1/0.1/0.1 | 0.3/0/0.6 | 0.5/0.1/1.7 |
| $\pi$ | *cox1* | - | 0.00079 | - | 0.00224 | - |
| | *nad6* | 0.00086 | 0.00050 | 0.00117 | 0.00296 | 0.00758 |
| k | *cox1* | - | 1.154 | - | 1.390 | - |
| | *nad6* | 0.431 | 0.505 | 0.584 | 1.286 | 3.792 |




**Figure 1** - Percent of overall pairwise divergence, based on a *p*-distance model of nucleotide substitution, plotted as a function of divergence by the codon position of A. *cox1* and B. *nad6* of *Diatraea saccharalis*, and C. *cox1* and D. *nad6* of *Hermeuptychia atalanta*.

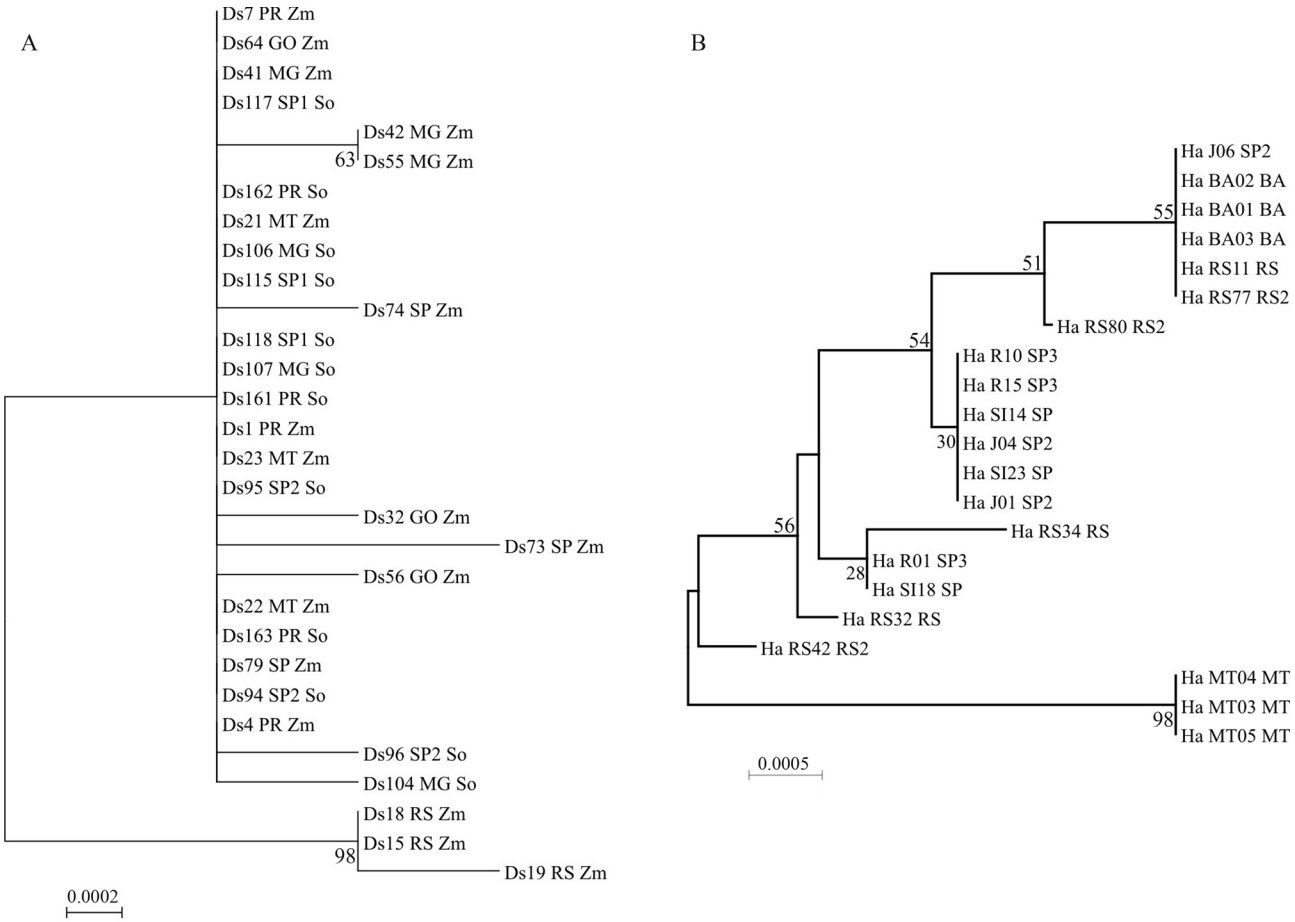


**Figure 2** - Neighbor-Joining phenetic relationships among specimens of A. *Diatraea saccharalis* and B. *Hermeuptychia atalanta*, based on concatenated data of *cox1* + *nad6* sequences and a *p*-distance model of nucleotide substitution. Numbers above the branches are bootstrap values of 1,000 replicates (when values are higher than 50%). (Ds = *D. saccharalis*, SP = São Paulo, MG = Minas Gerais, PR = Paraná, MT = Mato Grosso, RS = Rio Grande do Sul; Zm = corn, So = sugarcane; Ha = *H. atalanta*; SP = São Paulo, BA = Bahia, MT = Mato Grosso, RS = Rio Grande do Sul).

prising samples from Paranaíta, MT (Ha_MT) (Figure S2). Genetic distances of concatenated data ranged from 0.0 to 0.007. The combined analysis of *cox1* and *nad6* resulted in the retrieval of a NJ tree with improved overall branch resolution (Figure 2 B).

Mitochondrial regions are capable of revealing distinct rates of mutation, as well as pronounced heterogeneity at different parts of the molecule (Ballard, 2000; Montooth *et al.*, 2009). In fact, a comparison between genes that codify the subunits of *cytochrome oxidase* (*cox*) and *nad* revealed that, across insect taxa, *nad* accumulates many more amino acid substitutions than *cox*, possibly due to a different functional constraint (Montooth *et al.*, 2009). The availability of several mitochondrial genomes of Lepidoptera is now making all these regions accessible for consideration as markers at every taxonomic level. The use of similar and widely tested regions is appealing, since the study of comparable gene regions can contribute synergistically to a broader idea of the evolution of any group of organisms (Caterino *et al.*, 2000). However, for many groups of animals, new regions can be as, or more informative than, the currently used *cox1-cox2* sequences (Cameron and Whiting, 2008), specially for exploring variation at the intraspecific level.

Furthermore, *nad6* sequences worked as well as *cox1* in recovering DNA variation and genetic relationships among populations of *D. saccharalis* and *H. atalanta*. In this way, *nad6* might offer additional information, when complementarily used with other regions in population-genetics studies, since the combination of multiple genes with variable mutation rates could facilitate the investigation of the complex evolutionary history of a group of organisms (Cameron and Whiting, 2008). The easy amplification of the region presumes the applicability of the proposed designed primers to other Lepidoptera species, manifest through their successful amplification of target DNA of all the species tested, in families as diverse and taxonomically distant as Nymphalidae and Crambidae. Thus, the *nad6* region itself can be applied to low level phylogeny and population genetic studies, since the usual inclusion of more than one molecular marker to generate more robust data (Wahlberg and Wheat, 2008), would contribute to-

wards a more comprehensive view of the evolution of lepidopterans, through facilitating the analysis of comparable gene regions.

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## Internet Resources

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## Supplementary Material

The following online material is available for this article:

Figure S1 - Neighbor-Joining phenetic relationships among specimens of *D. saccharalis* based on A. *cox1* and B. *nad6* sequences.

Figure S2 - Neighbor-Joining phenetic relationships among specimens of *H. atalanta* based on A. *cox1* and B. *nad6* sequences.

This material is available as part of the online article form http://www.scielo.br/gmb.

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