

# The speciation of *Noctua atlantica* (Lepidoptera, Noctuidae) occurred in the Azores as supported by a molecular clock based on mitochondrial COI sequences

RAFAEL MONTIEL, V. VIEIRA, T. MARTINS, N. SIMÕES & M.L. OLIVEIRA



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The complete sequence of the cytochrome c oxidase subunit I (COI) gene of *Noctua atlantica* (Warren, 1905) has been determined and deposited in the NCBI GenBank under the Accession number AY600452. Complete and partial COI sequences of other Lepidoptera have been collected and used to reconstruct a phylogeny with both the Neighbor-Joining and the Maximum Likelihood methods. A molecular clock calibrated for our models indicate a divergence time between *Noctua atlantica* and *Noctua pronuba* of 4.7-5.9 Million years, consistent with the geological age of the Azores and suggesting that the speciation process of *N. atlantica* occurred in this archipelago.

Key words: divergence, endemic, Neighbor-Joining tree, *Noctua pronuba*, sequences

Rafael Montiel (e-mail: montiel@uac.pt), V. Vieira, T. Martins, N. Simões & L. Oliveira. Universidade dos Açores, Universidade dos Açores, Departamento de Biologia, CIRN, Rua da Mãe de Deus, PT- 9501-801 Ponta Delgada, Açores, Portugal.

## INTRODUCTION

The Azorean archipelago consists of nine volcanic islands of variable geological ages ranging from 0.037 to 8.12 million years (Queiroz 1990). It is situated in the Atlantic Ocean at 1500 km from mainland Portugal. These characteristics make it very interesting model systems to study colonization, radiation and even speciation process of invertebrate and vertebrate taxa. In the Azores there have been identified 38 Lepidoptera endemic species, eight of which are Noctuidae (Karsholt & Vieira 2005).

*Noctua atlantica* (Warren, 1905) is an endemic species of Azores (Warren 1905; Meyer 1991; Vieira & Silva 1994; Hacker & Schmitz 1996; Vieira 1997) that inhabit all the Azores islands, excepting Santa Maria Island (Carvalho et al. 1999; Karsholt & Vieira 2005). It is found in natural ecosystems of "Laurisilva" (ancient native

evergreen forest found in Macaronesia) (Oliveira et al. 2004) and has been catalogued as an endangered species, affected mainly by the specificity of its habitat. On the other hand, *N. pronuba* (Linnaeus, 1758), of palaeartic origin and also found in the Azores, has a less specific habitat and a more diverse geographic distribution. *Noctua atlantica* is morphologically very similar to *N. pronuba*, having a smaller wingspan and wings with a less shining color (Carvalho et al. 1999). Thus, it is very interesting to analyze the genetic relationship of these two species in the context of lepidopteran genetic variability to address evolutionary hypotheses and to help in developing conservation planning.

Sequences from the cytochrome c oxidase subunit I (COI) have been used to address phylogenetic problems at a wide range of hierarchical levels, from species to orders (Caterino & Sperling 1999). Hebert et al. (2003a)

proposed that a DNA barcoding system for animal life could be based upon sequence diversity in COI and have applied this method to lepidopterians successfully (Hebert et al. 2003a; 2003b; although see also Will & Rubinoff 2004). Furthermore, Gaunt & Miles (2002) found that the COI is better suited to conduct studies based on the molecular clock assumption than other gene sequences, such as 16S, 18S, cytochrome b (*cob*) or elongation factor 1 $\alpha$  (EF-1 $\alpha$ ). The molecular clock hypothesis is the basis of the modern molecular phylogenetic approach for dating and resolving evolutionary divergence (Gaunt & Miles 2002).

This is the first report on a DNA sequence of *N. atlantica*. We present its complete COI gene sequence that is compared with COI sequences of other lepidopterians, including *N. pronuba*. Also, by calibrating a molecular clock, we determine an approximate time of divergence for these species and its relationship with the geological history of the archipelago. This represent a first step to develop studies aimed to understand how does genetic variation in *N. atlantica* compare with levels observed in other non-endemic Azorean lepidoptera.

## MATERIAL AND METHODS

*Noctua atlantica* specimens were collected at Monte Escuro (800 m altitude) in São Miguel Island. Specimens were reproduced in laboratory and a 3rd-instar larva (from first generation) was selected for analysis.

For DNA extraction, larva was grinded under liquid nitrogen, re-suspended in 500  $\mu$ l of digestion buffer (100 mM Tris HCl pH 8.0, 80 mM EDTA, 1% SDS, 160 mM sucrose) and digested overnight at 37 °C with 25  $\mu$ g proteinase K. After centrifugation, the supernatant was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was ethanol precipitated and re-suspended in 100  $\mu$ l of H<sub>2</sub>O.

Conserved primers TY-J-1460 and TL2-N-3014 (Simon et al. 1994) were used to PCR amplify a segment between genes for tRNA-Tyr

and tRNA-Leu encompassing the complete gene for the mitochondrial cytochrome oxidase I (COI). The amplified fragment was cloned into a TOPO-TA vector (Invitrogen) and three clones were selected for direct sequencing. Universal primers T3 and T7 were used for ends sequencing, and primers NaCOXI-F104 (5'-ATTTTGGGAATTTGAGCTGG-3') and NaCOXI-R777 (5'-ATATAAACTTCTGGATGACC-3') were used for internal sequencing.

Fifteen COI sequences from species representing 4 Lepidopteran families, taken from the NCBI GenBank, were used for comparison (Table 1). For some species (as *N. pronuba*) the complete COI sequence was not available. For these reason our alignment was limited to 615 pb of the 5' end. Ten out of these 15 sequences were from Noctuidae (seven Noctuinae). Sequences were aligned with Clustal W (Thompson et al. 1994) and used to construct Maximum Likelihood (ML) trees with Treepuzzle (Schmidt et al., 2002) and Neighbor-Joining trees using the Tamura-Nei gamma distance with Mega2 (Kumar et al. 2001). Treepuzzle was used to estimate the gamma parameter alpha. The standard errors for the Tamura-Nei distances were estimated analytically, and for the ML distances they were derived from the standard errors of the ML branch lengths. The robustness of the resulting trees was assessed by bootstrapping (1000 resamplings) and by quartet puzzling (10,000 puzzling steps) for the Neighbor-Joining and ML trees, respectively. The divergence time between *Tegeticula synthetica* and *T. yuccasella* (32.4 My), estimated from COI sequence data by Gaunt & Miles (2002), was used to calibrate the molecular clock for our data.

## RESULTS

After remove the primer sequences, we obtained 1579 base pairs (bp) of good quality sequence (without ambiguities) from the three clones analyzed. Blast search (Altschul et al. 1997) at the NCBI GenBank allowed us to identify the complete gene for the cytochrome c oxidase subunit I (COI) of *Noctua atlantica*, from position 11 to 1555 and comprising 1545 bp. The

Table 1. COI sequences used in this study. <sup>a</sup> NCBI GenBank. <sup>b</sup> Economically important pest according to Zhang (1994). <sup>c</sup> Present in the Azores archipelago.

Taxa	Accession No. <sup>a</sup>	Pest <sup>b</sup>	Distribution
<b>Family NOCTUIDAE</b>			
<b>Subfamily Noctuinae</b>			
<i>Agrotis ipsilon</i> <sup>c</sup>	AF549736	yes	Cosmopolitan
<i>Agrotis volubili</i>	AF549702	yes	Nearctic
<i>Anaplectoides prasina</i>	AF549765	yes	Palearctic
<i>Feltia jaculifera</i>	U60990	yes	Nearctic
<i>Feltia tricola</i>	AF549766	no	Nearctic
<i>Noctua atlantica</i> <sup>c</sup>	AY600452	no	Azores (endemic)
<i>Noctua pronuba</i> <sup>c</sup>	AF549752	yes	Palearctic
<b>Subfamily Plusiinae</b>			
<i>Allagrapha aerea</i>	AF549755	yes	Nearctic
<i>Diachrysia chrysitis</i>	AJ420361	yes	Palearctic
<i>Diachrysia tutti</i>	AJ420369	no	Palearctic
<b>Family NYMPHALIDAE</b>			
<b>Subfamily Nymphalinae</b>			
<i>Nymphalis californica</i>	AY248789	yes	Nearctic
<i>Vanessa cardui</i> <sup>c</sup>	AY248782	yes	Cosmopolitan
<i>Vanessa virginiensis</i> <sup>c</sup>	AY248783	no	Nearctic
<b>Family HESPERIIDAE</b>			
<b>Subfamily Pyrginae</b>			
<i>Pyrgus communis</i>	AF170857	no	Nearctic
<b>Family PRODOXIDAE</b>			
<i>Tegeticula sintetica</i>	AY327144	no	Nearctic
<i>Tegeticula yuccasella</i>	AY488835	no	Nearctic

10 bp located upstream correspond to the 3' end of the gene for tRNA-Tyr and the 24 bp downstream comprise the 5' end of the tRNA-Leu gene. The COI start codon is ATA and the stop codon is TAA. As expected for insect mitochondrial genomes this gene has a high A+T content (73.07%). The *N. atlantica* sequence data has been deposited in the NCBI GenBank, under the Accession number AY600452.

We estimated Tamura-Nei and ML distances from the sequences used for comparison (Table 2, see the end of manuscript). Figure 1 shows the

unrooted Neighbor-Joining tree obtained from the Tamura-Nei distances.

## DISCUSSION

We have shown that *Noctua pronuba* is the closest species to *N. atlantica*, showing little divergence between them (Fig. 1). Assuming that the genetic distances observed between *T. synthetica* and *T. yuccasella*, were produced in 32.4 My, the divergence time between *Noctua atlantica* and *N. pronuba* is 4.9 ( $\pm$  2.1) My for the

Table 2. Neighbor-Joining gamma distances (below diagonal) and Maximum Likelihood distances (above) estimated from COI sequences; <sup>a</sup> Standard error = 0.006; <sup>b</sup> Standard error = 0.007.

	<i>A. ipseion</i>	<i>A. volubilis</i>	<i>A. aerea</i>	<i>A. prasina</i>	<i>D. chrysitis</i>	<i>D. tutti</i>	<i>F. jaculifera</i>	<i>F. tricolor</i>	<i>N. atlantica</i>	<i>N. pronuba</i>	<i>N. californica</i>	<i>P. communis</i>	<i>T. synthetica</i>	<i>T. yuccasella</i>	<i>V. cardui</i>	<i>V. virginienensis</i>
<i>Agrotis ipseion</i>		0.048	0.142	0.081	0.149	0.137	0.092	0.083	0.090	0.099	0.177	0.213	0.347	0.284	0.203	0.197
<i>Agrotis volubilis</i>	0.055		0.146	0.080	0.150	0.141	0.081	0.077	0.083	0.089	0.202	0.198	0.311	0.311	0.189	0.208
<i>Allagrapha aerea</i>	0.160	0.148		0.125	0.098	0.097	0.149	0.147	0.144	0.155	0.220	0.247	0.358	0.325	0.216	0.206
<i>Anaplectoides prasina</i>	0.116	0.091	0.141		0.134	0.123	0.092	0.093	0.056	0.064	0.204	0.206	0.358	0.296	0.195	0.201
<i>Diachrysis chrysitis</i>	0.160	0.147	0.104	0.138		0.008	0.167	0.161	0.148	0.153	0.212	0.249	0.319	0.300	0.231	0.204
<i>Diachrysis tutti</i>	0.151	0.141	0.107	0.130	0.009		0.156	0.148	0.140	0.145	0.202	0.241	0.299	0.277	0.218	0.193
<i>Feltia jaculifera</i>	0.100	0.083	0.151	0.098	0.167	0.155		0.031	0.124	0.134	0.198	0.234	0.365	0.331	0.204	0.221
<i>Feltia tricolor</i>	0.094	0.082	0.153	0.108	0.159	0.147	0.036		0.116	0.125	0.169	0.208	0.319	0.297	0.198	0.211
<i>Noctua atlantica</i>	0.106	0.087	0.157	0.064	0.148	0.142	0.129	0.126		0.013 <sup>b</sup>	0.196	0.187	0.304	0.280	0.207	0.219
<i>Noctua pronuba</i>	0.125	0.097	0.178	0.078	0.156	0.149	0.146	0.143	0.014 <sup>a</sup>		0.198	0.209	0.311	0.295	0.223	0.239
<i>Nymphalis californica</i>	0.196	0.204	0.251	0.230	0.220	0.213	0.207	0.171	0.203	0.207		0.239	0.309	0.289	0.135	0.137
<i>Pyrgus communis</i>	0.244	0.201	0.276	0.228	0.254	0.252	0.248	0.217	0.203	0.237	0.281		0.286	0.261	0.225	0.213
<i>Tegeticula synthetica</i>	0.398	0.325	0.392	0.440	0.341	0.326	0.405	0.340	0.320	0.335	0.314	0.317		0.076	0.375	0.366
<i>Tegeticula yuccasella</i>	0.305	0.315	0.361	0.328	0.324	0.301	0.342	0.303	0.288	0.316	0.300	0.274	0.093		0.341	0.343
<i>Vanessa cardui</i>	0.230	0.208	0.242	0.218	0.231	0.223	0.218	0.216	0.225	0.259	0.164	0.246	0.450	0.356		0.056
<i>Vanessa virginienensis</i>	0.208	0.216	0.225	0.221	0.203	0.196	0.229	0.221	0.233	0.274	0.160	0.231	0.425	0.357	0.065	

Tamura-Nei distance, and 5.5 ( $\pm$  3) My for the ML distance. In order to confirm this result we estimated, using the same methods, the distances between *Pachliopta neptunus* and *Papilio garamas* (data not shown), with an estimated divergence time of 89.1 My (Gaunt & Miles 2002). Using this data we obtained divergence times for *N. atlantica* and *N. pronuba* of 5.1 ( $\pm$  2) My and 5.7 ( $\pm$  3.2) My for the Tamura-Nei and ML distances, respectively.

These divergence times were obtained using the most conservative estimations reported by Gaunt & Miles (2002), and therefore can be considered to be near to the upper limit. Also it must be considered that the gene divergence usually predates species divergence (Nei 1987; Nichols 2001). Thus, our estimations are consistent with the Azorean origin of *N. atlantica*. The divergence coincides with the geological age

of the Azorean archipelago either occurring just after formation of the first Island (Santa Maria) or subsequently, following the conformation of the archipelago due to volcanic phenomena. Even, the speciation could be related to the appearing of São Miguel, that occurred 4.01 My ago (Queiroz 1990).

An alternative hypothesis could be that *N. atlantica* occupied a broad geographical range in ancient times and ultimately become confined to the Azores due to ecological changes and niche reduction. This hypothesis could be supported if *N. atlantica* arose as species before the formation of the Azores archipelago, however, if these hypothetical climatic changes occurred more recently, this alternative hypothesis could also be consistent with a recent speciation of *N. atlantica*. In this study we analyzed the divergence between *N. atlantica* and *N. pronuba*, however, further

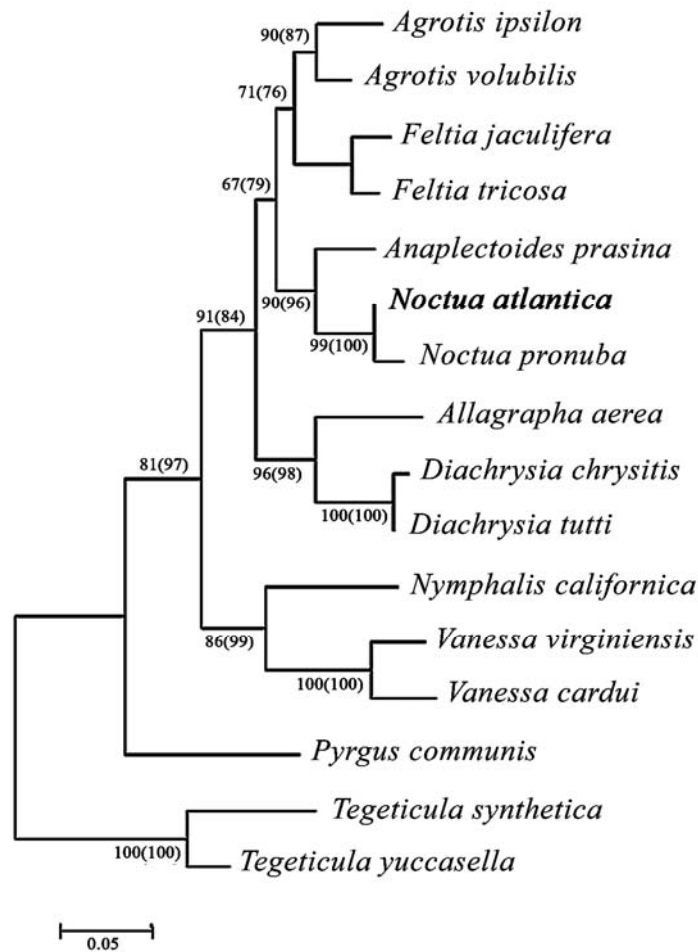


Fig. 1. Unrooted Neighbor-Joining tree constructed from Tamura-Nei gamma distances (alpha parameter = 0.23). The tree topology was identical for the ML tree. Numbers in branches are bootstrap (and quartet puzzling) support values.

insights into the age of these species could be obtained from the analysis of other *Noctua* species from Macaronesia, like *N. carvalhoi* (also an Azorean endemic species), *N. teixeirai* (endemic in Madeira), and *N. noacki* (endemic in Canary islands), and comparing their genetic distances relatively to *N. pronuba*, since this widely distributed species could be the ancestor of all the endemic *Noctua* species from

Macaronesia. Also intra-specific variability in these species should be assessed.

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