Population structure of the *Monocelis lineata* (Proseriata, Monocelididae) species complex assessed by phylogenetic analysis of the mitochondrial Cytochrome c Oxidase subunit I (*COI*) gene

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**Abstract**

*Monocelis lineata* consists of a complex of sibling species, widespread in the Mediterranean and Atlantic Ocean. Previous genetic analysis placed in evidence at least four sibling species. Nevertheless, this research was not conclusive enough to fully resolve the complex or to infer the phylogeny/phylogeography of the group. We designed specific primers aiming at obtaining partial sequences of the mtDNA gene Cytochrome c Oxidase subunit I (*COI*) of *M. lineata*, and have identified 25 different haplotypes in 32 analyzed individuals. The dendrogram generated by Neighbor-Joining analysis confirmed the differentiation between Atlantic and Mediterranean siblings, as well as the occurrence of at least two Mediterranean sibling species. Thus validated, the method here presented appears as a valuable tool in population genetics and biodiversity surveys on the *Monocelis lineata* complex.

**Key words:** microturbellaria, sequencing, sibling species, phylogeography, phylogeny.

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*Monocelis lineata* (O.F. Müller, 1774) (Proseriata: Monocelididae) is a flatworm (Platyhelminthes) characterized by a comparatively “simple” morphology, and a wide distribution along the north Atlantic, Mediterranean and Black Sea coasts, occurring in brackish-water and marine habitats on any kind of substrate (Ax, 1956). Across this range, the species shows a remarkably uniform morphology, except for polymorphism related to the ocular pigment shield, which may be absent in the entire population (Curini-Galletti and Mura, 1998; Casu and Curini-Galletti, 2004). Previous molecular assays, carried out by using allozyme electrophoresis, suggested that the taxon consists of at least four sibling species, three Mediterranean species with a sharp genetic separation between brackish-water (with pigmented eyespots) and marine (unpigmented) populations, and one genetically heterogeneous, as yet unresolved “sibling” in the north Atlantic (Casu and Curini-Galletti, 2004). Previous molecular assays, carried out by using allozyme electrophoresis, suggested that the taxon consists of at least four sibling species, three Mediterranean species with a sharp genetic separation between brackish-water (with pigmented eyespots) and marine (unpigmented) populations, and one genetically heterogeneous, as yet unresolved “sibling” in the north Atlantic (Casu and Curini-Galletti, 2004). However, even though allozymes have proved, in past years, to be a powerful tool in discriminating sibling complexes (Manchenko and Radashevsky, 1998; Klautau et al., 1999; De Matthaeis et al., 2000; Maltagliati et al., 2000, for marine invertebrates), their application is biased by certain technical limitations (among others, the scarce reproducibility across different laboratories), which hinder routine use of the marker in biodiversity surveys. On the contrary, the sequencing of mitochondrial DNA (mtDNA) gene coding for Cytochrome c Oxidase subunit I (*COI*) is usually performed to arrive at inferences on phylogeny and/or phylogeography in different species (Breton et al., 2003, for marine invertebrates). However, so far *COI* sequencing has not been applied to studies on interstitial micro-turbellarians.

We designed specific primers to amplify a partial region of *COI* in *M. lineata*, and validated it, while studying Mediterranean and Atlantic specimens, as a tool for population genetic studies and biodiversity surveys on the *Monocelis lineata* complex.

In a first step, universal primers for marine invertebrates (Folmer et al., 1994) were tested on 160 individuals from 32 populations from the northeastern Atlantic and western to eastern Mediterranean (about five specimens per sampling site) (Figure 1). We aimed at obtaining at least one suitable sequence to use as a base for designing specific primers. Specimens had been stored in 70% alcohol for a period of two to five years. DNA was extracted from the entire individual using the DNeasy® Tissue Kit (QIAGEN Inc.). PCR amplification was carried out in 25 µL total volume, containing about 5 ng/µL of total genomic DNA, 0.4 µM of each primer, 2.5 U of Taq polymerase (Euro Taq®, Euroclone), 1.25 mM MgCl₂, in a reaction mix containing 200 µM of dNTP mix and 1 x buffer. The PCR profile consisted of an initial hot start step (2 min at 94 °C), and 35 cycles, each comprising denaturation for 1 min at 94 °C, annealing for 1 min at 52 °C and extension for 1.30 min at 72 °C, followed by a final extension for 5 min at 72 °C. For
checking products obtained with universal primers, samples were electrophoresed on 2% agarose/0.5 x TBE gels stained with ethidium bromide (10 mg/mL), at 4 V/cm for 20 min. Purified PCR products (ExoSAP-IT®, USB Corporation,) were cycle sequenced using the BigDye Sequencing Kit, Terminator 3.1® (Applied Biosystems), and then analyzed on a 3100 ABI PRISM Avant® (Applied Biosystems) automated sequencer. Cycle-sequencing reactions were carried out in 10 μL total volume, containing 2 to 6 μL of purified PCR product and 0.32 μM of the forward or the reverse primer. An initial hot start step (1 min at 96 °C) was followed by 35 cycles, each comprising denaturation (10 s at 96 °C), annealing (5 s at 50 °C) and extension (4 min at 60 °C). Cycle-sequencing products were purified using the SigmaSpin Post-Reaction Clean-Up Columns® (Sigma Aldrich). Only three from the Mediterranean populations (SGo, MZo, and PPx; Figure 1) among the 160 specimens tested with universal primers showed satisfactory amplification of the target region. Sequences of 577 bp, 568 bp, and 657 bp corresponding to GenBank accession numbers EF583451, EF591057, and EU889254, respectively, were obtained. Using the software Mega 4 (Tamura et al., 2007), these sequences were aligned to those of the Platyhelminthes Nematoplena coelogynoporoides (Proseriata: Nematoplanidae) (GenBank accession number: AJ405985), and Vannuccia sp. (Proseriata: Coelogynoporidae) (GenBank accession number: AJ405986). Nucleotide alignment disclosed a high degree of identity, about 69% for N. coelogynoporoides and 68% for Vannuccia sp., thus pointing to the correct amplification of COI in M. lineata. Specific internal primers have been designed within the most conserved regions using Primer Premier 5.00 software (PREMIER Biosoft International, Palo Alto, CA, Table 1). The above-described PCR conditions, except for the annealing temperature at 51 °C, were used for the designed primers, thus permitting amplification of a 195-199 bp fragment of the COI gene in three specimens of M. lineata. We then studied one individual from each sampling site (Figure 1) in a total of 32 specimens, in order to investigate genetic variability between and within Atlantic and Mediterranean populations.

PCR amplification with the designed primers yielded short fragments (about 200 bp) of the COI gene. This however does not represent a bias, for it has been demonstrated that sequences of about or less than two hundred bp may correctly show the phylogenetic/phylogeographic traits of the species (Tillier et al., 1992; Kirby and Reid, 2001, Bucklin and Allen, 2004, Hajibabaei et al., 2006).

The results obtained by using DNAsp 4.0 software (Rozas et al., 2003) revealed a low mean nucleotide value ($Pi = 0.21$) and elevated mean haplotype diversity ($Hd = 0.97$), distributed on 25 diverse haplotypes out of the 32 sequences analysed (GenBank accession numbers: EU889254-EU889265; EU889268-EU889272; EU889275-EU889276; EU889278-EU889291). The
Neighbor-Joining consensus dendrogram (Figure 2), constructed by means of MEGA4 (Tamura et al., 2007) after 1,000 bootstrap replicates and by applying the Maximum composite likelihood method (MCL), placed in evidence the sharp separation between Atlantic and Mediterranean samples. Furthermore, within the Mediterranean group itself, two main clusters were observed (Figure 2), with individuals from brackish-waters (with the ocular pigment shield) and marine waters (without the ocular pigment shield), respectively. Main nodes were supported by bootstraps higher than 78% (Figure 2). Although preliminary, these findings, consistent with previous allozyme data (Casu and Curini-Galletti, 2004) showing conspicuous genetic differentiation between Atlantic and Mediterranean, and among Mediterranean populations themselves, further support the occurrence of a *M. lineata* sibling species complex.

In this context, the newly designed primers can be adequately used to obtain sequences from individuals of *M. lineata*, with high haplotype diversity and a low level of nucleotide variability. Furthermore, the high reproducibility of the technique allows for increasing the number of individuals/populations over time. The primers here reported may thus be confidently used to resolve the complex of sibling species in *M. lineata*, and to depict phylogeographic patterns within each sibling, on both a re-

| Locus | Primer sequences (5’-3’) | GenBank accession n. | Size (bp) | Sample |
|-------|-------------------------|----------------------|-----------|--------|
| EU889255 | L: GTAATGCGCDTADCCTTTGAGGG | 195 | ALo |
| EU889256 | H: CTACCACCGCCCAATGTAAA | 198 | COo |
| EU889257 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | SGo |
| EU889258 | H: CTACCACCGCCCAATGTAAA | 198 | STo |
| EU889259 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | MMo |
| EU889260 | H: CTACCACCGCCCAATGTAAA | 199 | MZo |
| EU889261 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | CSo |
| EU889262 | H: CTACCACCGCCCAATGTAAA | 198 | LNo |
| EU889263 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | MTo |
| EU889264 | H: CTACCACCGCCCAATGTAAA | 198 | PLo |
| EU889265 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | PPx |
| EU889266 | H: CTACCACCGCCCAATGTAAA | 198 | CPx |
| EU889267 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | SKx |
| EU889268 | H: CTACCACCGCCCAATGTAAA | 198 | VEo |
| EU889270 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | LMo |
| EU889271 | H: CTACCACCGCCCAATGTAAA | 198 | SFo |
| EU889276 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | LFo |
| EU889278 | H: CTACCACCGCCCAATGTAAA | 195 | CNo |
| EU889279 | L: GTAATGCGCDTADCCTTTGAGGG | 195 | DOo |
| EU889280 | H: CTACCACCGCCCAATGTAAA | 195 | HEo |
| EU889281 | L: GTAATGCGCDTADCCTTTGAGGG | 195 | FEo |
| EU889282 | H: CTACCACCGCCCAATGTAAA | 195 | RSx |
| EU889283 | L: GTAATGCGCDTADCCTTTGAGGG | 195 | RSo |
| EU889284 | H: CTACCACCGCCCAATGTAAA | 195 | GTx |
| EU889285 | L: GTAATGCGCDTADCCTTTGAGGG | 195 | ARo |
| EU889286 | H: CTACCACCGCCCAATGTAAA | 195 | ARx |
| EU889287 | L: GTAATGCGCDTADCCTTTGAGGG | 195 | FJo |
| EU889288 | H: CTACCACCGCCCAATGTAAA | 195 | KFo |
| EU889289 | L: GTAATGCGCDTADCCTTTGAGGG | 195 | BMo |
| EU889290 | H: CTACCACCGCCCAATGTAAA | 195 | TJo |
| EU889291 | L: GTAATGCGCDTADCCTTTGAGGG | 198 | TIX |

L, light chain; H, heavy chain.
 regional and local scale. Indeed, COI has been routinely used to successfully distinguish cryptic species in different “simple” organisms, e.g. Anisakid nematodes (Hu et al., 2001; Cross et al., 2006; Derycke et al., 2007).

Furthermore, the problems for non-specialists concerning the correct identification of minute mesopsammic organisms have always been a hindrance in the use of the meiofauna in ecological surveys and in assessing the actual extent of local biodiversity (Kennedy and Jacoby, 1999). Therefore, in the light of the present trend for reducing taxonomic expertise (the so-called taxonomic impediment, Boero, 2001), the finding of suitable primers for COI may be an invaluable contribution for future researches. Indeed, DNA barcoding (for a review, see Moritz and Cicero, 2004) by means of COI sequencing has been suggested as a promising tool to assess the actual level of marine biodiversity.

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