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Molecular data on two mitochondrial genes of a newly discovered crustacean species (*Lightiella magdalenina*, Cephalocarida)

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*Cephalocarida is a rare and poorly known class of small benthic crustaceans, consisting of only eleven species belonging to five genera. Thus far, only one species (*Hutchinsoniella macracantha*) has been studied at molecular level. We report the partial sequences of two phylogenetically important mitochondrial genes (Cytochrome c Oxidase I and Cytochrome b) from the newly discovered Mediterranean species,* Lightiella magdalenina. *The genetic relationships between the two cephalocarid species are discussed.*

Keywords: COI, Cyt-b, phylogenesis

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Cephalocarida is a class of small benthic crustaceans distributed from the intertidal zone to approximately 1550 m depth. To date, only eleven species belonging to five genera have been described. *Lightiella magdalenina* Carcupino, Floris, Addis, Castelli, Curini-Galletti, 2006, the most recently discovered species from La Maddalena Archipelago (Sardinia, Italy) (41°13′N 9°25′E), is characterized by a high degree of endemism. No other species have been reported in Europe, and the discovery of *L. magdalenina* in the Mediterranean Sea (Carcupino *et al.*, 2006) fills a gap in the worldwide distribution of the entire class. Its type locality is characterized by a muddy sand bottom very rich in organic matter with little seagrass beds. Since its first description (Sanders, 1955), Cephalocarida was considered the most primitive living crustacean class. However, it remains a poorly known taxon, and its phylogenetic position is controversial. Molecular data are only available for one species, *Hutchinsoniella macracantha* Sanders, 1955, and refer to a complete mitochondrial genome (Lavrov *et al.*, 2004), to two mitochondrial genes (Giribet *et al.*, 2001) and to six nuclear genes (Spears & Abele, 1997; Colgan *et al.*, 1998; Regier & Shultz, 1998; 2001; Shultz & Regier, 2000; Giribet *et al.*, 2001; Richter *et al.*, 2007). Nevertheless, the molecular analyses have not provided unequivocal results in terms of phylogenetic relationships: even when the survey was limited to mtDNA, Cephalocarida has been tentatively related with Remipedia (Giribet *et al.*, 2001), Maxillopoda and Pentastomida (Lavrov *et al.*, 2004).

The aim of this short note is to provide molecular data for this rare and poorly known crustacean class, based on the sequencing of two mitochondrial genes of *L. magdalenina*. These genes, selected for their value in phylogenetic analysis, are Cytochrome c Oxidase subunit I (COI) and Cytochrome b (Cyt-b). The former has been proposed by Hebert *et al.* (2003) as a sort of genetic 'barcode', which can serve as the core of a global bioidentification system for animals. The latter, widely used in vertebrate evolutionary studies, is particularly effective for the reconstruction of molecular phylogeny in invertebrates (Simmons & Weller, 2001).

For PCR amplifications of a partial region of both genes, we used primers designed by Folmer *et al.* (1994) for COI, and Boore & Brown (2000) for Cyt-b. Standard DNA procedures were used to extract from the whole body of a specimen about 2 mm long. The PCR amplification mix contained: 0.4 μM of each primer; 2.5 U of Taq DNA Polymerase; 2.5 mM of $MgCl_2$; 200 μM of dNTPs. The PCR profile consisted of 35 cycles (denaturation: 1′ at 94°C; annealing: 1′ at 52°C; extension: 1′30″ at 72°C).

The PCR amplifications yielded a product of 618 bp for the COI gene, and 345 bp for the Cyt-b gene (Genbank accession numbers: EU530536 and EU530537). Comparison of the two sequences with those of *H. macracantha* revealed 143 changes (66 transitions and 77 transversions) for COI and 126 changes (44 transitions, 79 transversions, and 3 deletions belonging to the same codon) for Cyt-b. These mutations led to 38 amino acid changes over 206 for the COI enzyme, and 50 amino acid changes and the deletion of one aspartate over 116 for the Cyt-b enzyme. For further analysis, the two genes were combined, excluding the third base of each codon, for a total of 644 bp.

A median joining network analysis was carried out with Network 4.5.0.0 software (http://www.fluxus-engineering.com) (Bandelt *et al.*, 1999), using the sequences of *L. magdalenina* and those of 8 representative species of Pancrustacea, whose complete genomes were reported in Lavrov *et al.* (2004) (Figure 1). As shown by the short length of the torso, the network analysis, based on a neighbour joining (NJ) approach, is not appropriate for deep level phylogeny. However, it allows discrimination of the phylogenetic status of specific residues.

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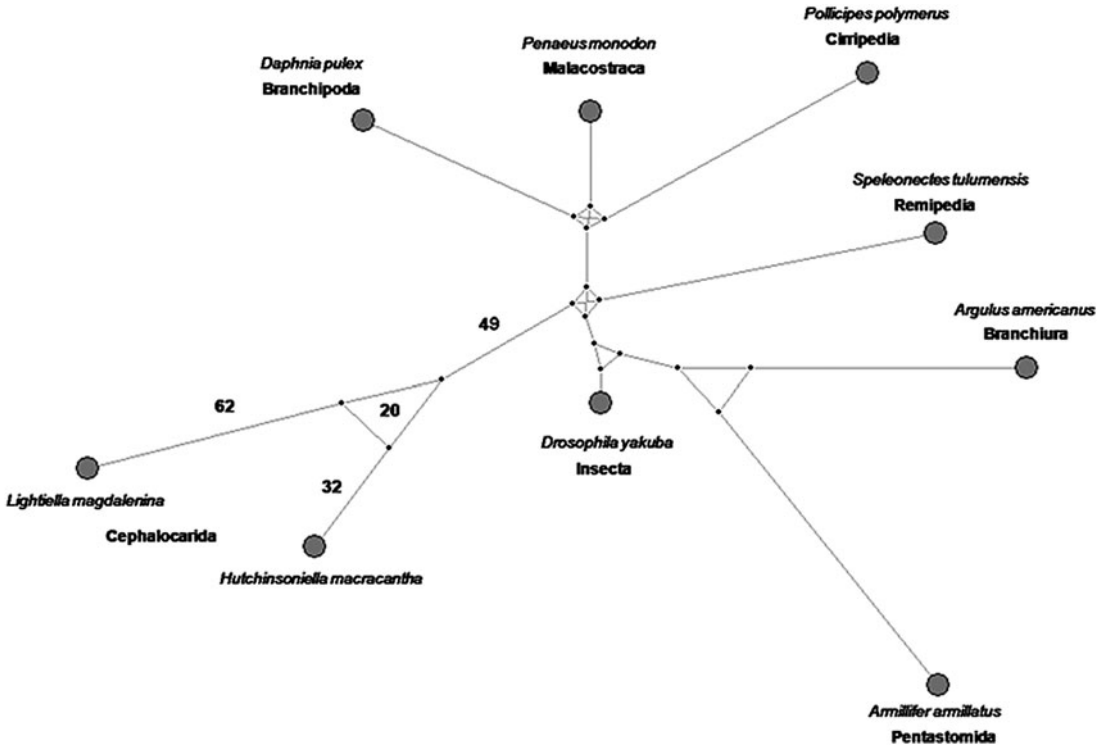

Fig. 1. Median joining network of the combined Cytochrome c Oxidase I and Cytochrome b partial genes from *Lightiella magdalenina* and 8 Pancrustacea species.

The cephalocarid clade is separated by the basal median vector by 49 changes, which represent the plesiomorphic status for the class. *Lightiella magdalenina* and *H. macracantha* are separated by 114 nucleotide differences: 62 of them are apomorphic for *L. magdalenina*, 32 for *H. macracantha*, while 20 cannot be univocally assigned (resulting in a triangular

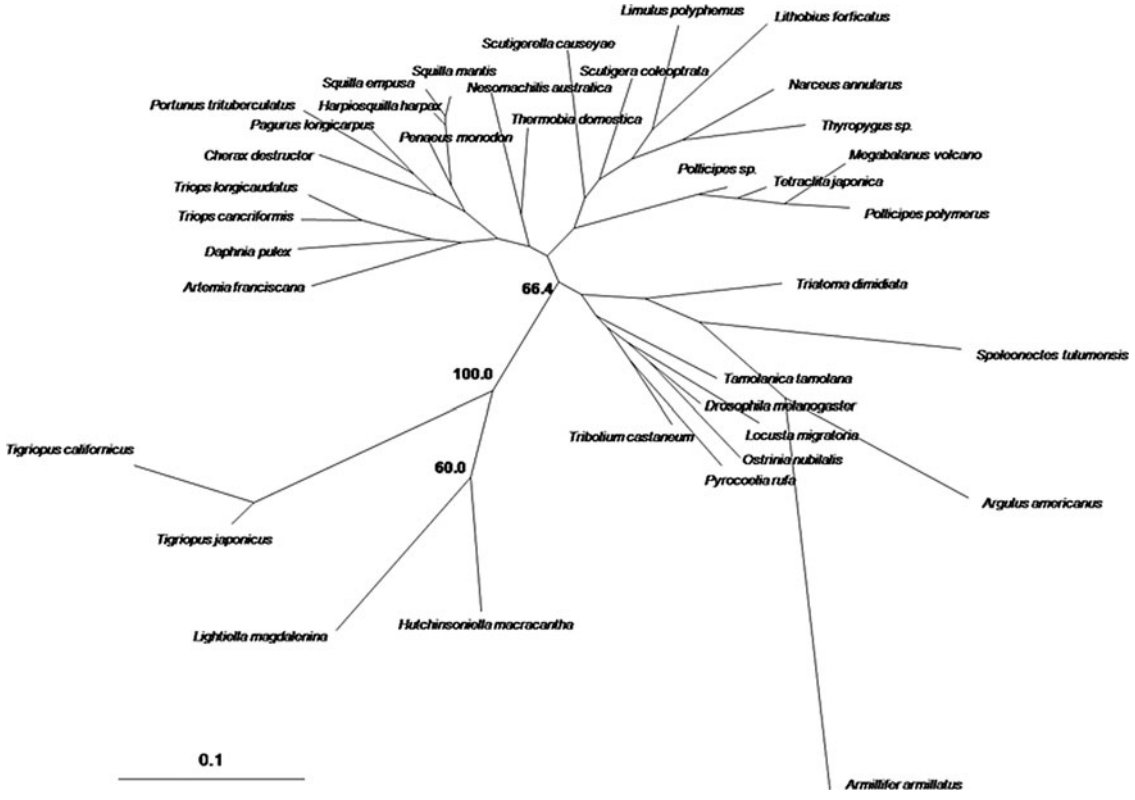

Fig. 2. Maximum likelihood unrooted tree of combined Cytochrome b and Cytochrome c Oxidase I partial genes from *Lightiella magdalenina* and 36 arthropod species (above). Numbers refer to the LR-ELW values (10,000 replicates) for the Cephalocarida branch.

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reticulation of the network). The prevalence of apomorphisms over plesiomorphisms suggests a very ancient separation of the two species from a common ancestor. Equality of the evolutionary rate of the two species was tested for both genes with the method proposed by Tajima (1993) using the MEGA4 software (Tamura *et al.*, 2007). In spite of the observed differences in the number of apomorphic nucleotides, the Tajima relative rate test of neutrality was non-significant for both genes, whatever the other sequence used as outgroup. A maximum likelihood (ML) analysis was carried out with Treefinder (http://www.treefinder.de) (Jobb, 2008), applying edge support (LR-ELW) (Strimmer & Rambaut, 2002) (Figure 2). *Lightiella magdalenina* was analysed together with another 30 Pancrustacea species, using as outgroup 5 Myriapoda and one Chelicerata species. The general topology of ML analyses appears to be consistent with the network structure, showing a deep divergence of the cephalocarid clade and an ancient separation of the two species. As previously observed by Hassanin (2006), the affinity between Cephalocarida and Copepoda suggested by ML analysis, could be interpreted as a consequence of a long branch attraction phenomenon due to reverse strand bias (Felsenstein, 1978).

A discussion of arthropod evolution is far beyond the scope of this short note. Nevertheless, our contribution of molecular data for a newly discovered species increases the knowledge on the genetic variation within Cephalocarida, which can be useful for future research in this strongly debated field.

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