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Phylogeny of Eutardigrada: New molecular data and their morphological support lead to the identification of new evolutionary lineages

Roberto Bertolani [a], Roberto Guidetti [b,*], Trevor Marchioro [b], Tiziana Altiero [a], Lorena Rebecchi [b], Michele Cesari [b]

[a] Department of Educational and Human Sciences, University of Modena and Reggio Emilia, Reggio Emilia, via Allegri 9, 42121 Reggio Emilia, Italy
[b] Department of Life Sciences, University of Modena and Reggio Emilia, Modena, via Campi 213/D, 41125 Modena, Italy

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ABSTRACT

An extensive study of the phylogeny of Eutardigrada, the largest class of Tardigrada, has been performed analyzing one hundred and forty sequences (eighty of which newly obtained) representative of one hundred and twenty-nine specimens belonging to all families (except Necopinatidae) of this class. The molecular (18S and 28S rRNA) results were compared with new and previous morphological data, allowing us to find new phylogenetic relationships, to identify new phylogenetic lineages, to erect new taxa for some lineages, and to find several morphological synapomorphies supporting the identified clusters. The class Eutardigrada has been confirmed and, within it, the orders Apochela and Parachela, the superfamilies Macrobiotoidea, Hypsibioidea, Isohypsibioidea, and Eohypsibioidea, and all the families and subfamilies considered, although with emended diagnoses in several cases. In addition, new taxa have been erected: the new subfamily Pilatobiinae (Hypsibiidae) with the new genus *Pilatobius*, as well as an upgrading of *Diphascon* and *Adropion* to genus level, previously considered subgenera of *Diphascon*. Our results demonstrate that while molecular analysis is an important tool for understanding phylogeny, an integrative and comparative approach using both molecular and morphological data is necessary to better elucidate evolutionary relationships.

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1. Introduction

With the application of DNA sequencing to tardigrades, new information has been obtained on the phylogenetic position of both the phylum and its main evolutionary lines. Tardigrades have been included in the clade Ecdysozoa by Aguinaldo et al. (1997), within the Panarthropoda, together with Onychophora and Arthropoda (Rota-Stabelli et al., 2010; Campbell et al., 2011), although the position of Tardigrada within this last group is still undetermined (Nielsen, 2012).

Molecular analyses within the phylum confirmed the subdivision of the higher taxa of Tardigrada, namely Heterotardigrada (Jørgensen and Kristensen, 2004; Jørgensen et al., 2010) and Eutardigrada (Guidetti et al., 2005; Nichols et al., 2006). In contrast, recent molecular studies at the genus and species levels (Guidetti et al., 2005, 2009; Kiehl et al., 2007; Møbjerg et al., 2007; Sands et al. 2008; Jørgensen et al., 2010, 2011; Guil and Giribet, 2012; Guil et al., 2013b) revealed some incongruence with the traditional morphological systematics of the phylum. In particular, the papers by Sands et al. (2008), and by Marley et al. (2011) revealed different evolutionary lines within Parachela that were only partially in agreement with those revealed by the previous morphological classical approaches. In particular, four main clusters were identified for which four superfamilies were proposed, which were partially confirmed in further studies (Jørgensen et al., 2010; Guil and Giribet, 2012). The superfamilies were initially without any morphological support, which was presented only later by Marley et al. (2011). The most surprising conclusion of Sands et al. (2008) was the attribution of *Hypsibius* and *Isohypsibius* to two different superfamilies, since these two genera were previously considered belonging to the same sub-family (Hypsibiinae).

Considering that several eutardigrade families and genera were not included in previous papers based on molecular analysis, we carried out a further phylogenetic study analyzing two molecular markers expanding the study to all eutardigrade families (except Necopinatidae) and increasing the number of analyzed genera and species. In addition, considering that Eutardigrada, the largest class of the phylum, is generally characterized by a low level of morphological diversity (especially compared with Heterotardigra-

* Corresponding author. Fax: +39 0592055548.
E-mail addresses: roberto.bertolani@unimore.it (R. Bertolani), roberto.guidetti@unimore.it (R. Guidetti), trevor.marchioro@gmail.com (T. Marchioro), tiziana.altiero@unimore.it (T. Altiero), lorena.rebecchi@unimore.it (L. Rebecchi), michele.cesari@unimore.it (M. Cesari).

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da; Fig. 1), and the identification of synapomorphies for each of the phylogenetic lineages has been a particularly difficult task, we analyzed morphological characters of the studied taxa more in depth, utilizing light and scanning electron microscopy, in order to acquire better knowledge of eutardigrade phylogeny and systematics. Therefore, for classifying organisms within a phylogenetic systematic framework, we used an integrative approach (as in Bertolani et al., 2011a), analyzing molecular phylogenies and compare them with morphological characters. With our integrative approach our goals were both to verify the current evolutionary lineages (or to identify new ones) and to provide further morphological support for those taxa, which to date have a taxonomic designation but are insufficiently described and thus not easily identifiable.

2. Materials and methods

2.1. Species sampling

Eighty eutardigrade specimens belonging to 26 genera were used. The full list of specimens, including collecting information, is provided in Table S1. Tardigrades were extracted from different substrates (Table S1) collected in Europe and in USA. Moss, grass and leaf litter were placed in water for about half an hour. Then, animals were isolated from all substrates, including freshwater sediments, using sieves. Finally, tardigrades were individually picked up using a glass pipette under a stereomicroscope and processed.

2.2. Molecular analyses

2.2.1. Species identification for molecular analysis

Before DNA extraction, each tardigrade specimen used in molecular analysis was observed *in vivo* by light microscopy (with a Leitz DM RB microscope, using differential interference contrast – DIC – and phase contrast – PhC, with 40× and 100× immersion oil objectives) and identified according to its morphological characters (Guidetti and Bertolani, 2005; Pilato and Binda, 2010). Before DNA extraction, pictures of the sclerified structures of *in vivo* specimens were also taken by using a Nikon DS-Fj1 photocamera, following the protocols described in Cesari et al. (2011) and Bertolani et al. (2011b).

2.2.2. DNA extraction, PCR amplification, and sequencing

Genomic DNA extraction was carried out from 80 single specimens (Table S1) through a rapid salt and ethanol precipitation (Cesari et al., 2009). Regions of the nuclear ribosomal subunit genes 18S and 28S rRNA were amplified using the following primer combinations: SSU F04 (5′-GCT TGT CTC AAA GAT TAA GCC-3′) and SSU R26 (3′-CAT TCT TGG CAA ATG CTT TCG-5′; Kiehl et al., 2007) for 18S, and 28S 1274 (5′-GAC CCG TCT TGA AAC ACG GA-3′) and 28S 689 (5′-ACA CAC TCC TTA GCG GA-3′) for 28S. For both genes, the polymerase chain reaction was carried out in 20 µl of reaction volume, which consisted of 2 µl reaction buffer (including 20 mM of $MgCl_2$), 2.5 mM of each dNTP, 10 pmol (final concentration) of each primer, 1 U of Dream*Taq* polymerase (Fermentas) and 2 µl of template DNA. A negative control lacking template DNA was carried out to test the possibility of contamination with foreign DNA. PCR was performed in a PCR Sprint Thermal Cycler (Hybaid). The protocol for 18S consisted of 35 cycles with 1 min at 94 °C, 35 s at 52 °C and 2 min at 72 °C, with a final elongation step at 72 °C for 10 min. The protocol for 28S consisted of 40 cycles with 45 s at 96 °C, 1 min at 48 °C and 1 min at 72 °C, with a final elongation step at 72 °C for 10 min. The amplified products were gel purified using the Wizard Gel and PCR cleaning kit (Promega). Sequencing reactions were performed using the ABIPRISM® BigDye™ Terminator Version 1.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on

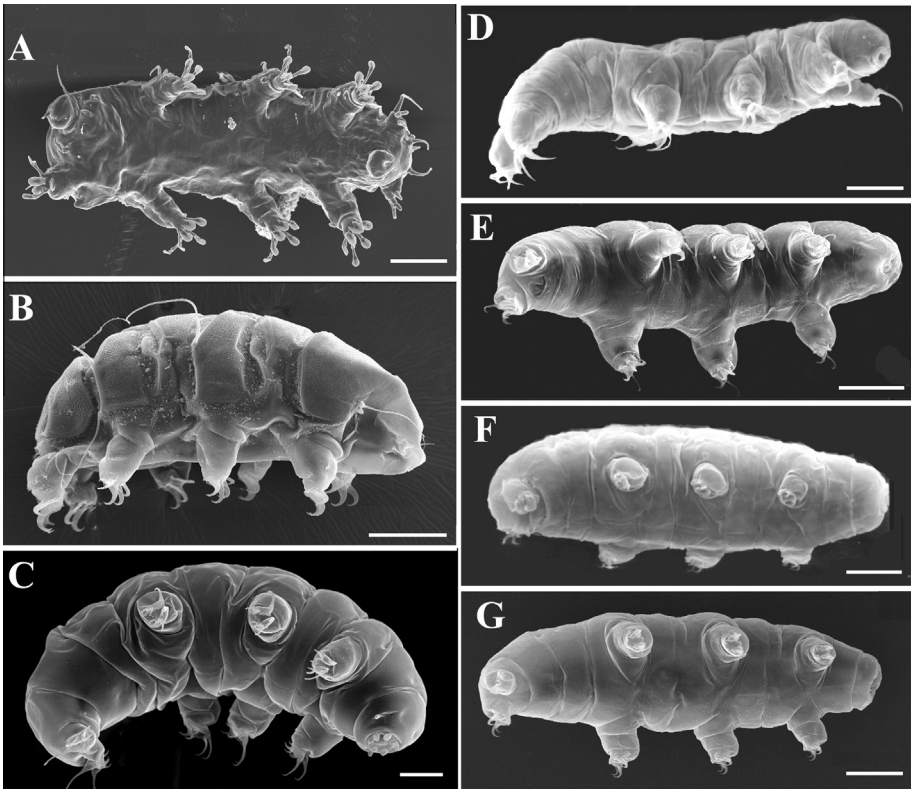

Fig. 1. Scanning electron micrographs of tardigrades. A: *Batillipes* (Arthrotardigrada) (picture kindly donated by C. Schulze and A. Schmidt-Rhaesa). B: *Echiniscus* (Echiniscoidea). C: *Milnesium* (Apochela). D: *Thulinius* (Parachela, Isohypsibioidea). E: *Ramazzottius* (Parachela, Hypsibioidea). F: *Bertolanius* (Parachela, Eohypsibioidea). G: *Paramacrobiotus* (Parachela, Macrobiotoidea). Scale bars: A = 20 µm; B–G = 100 µm.

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