

## The 18S rDNA sequences support polyphyly of the Hypsibiidae (Eutardigrada)

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### ABSTRACT

To extend data on 18S rDNA gene phylogeny within the Eutardigrada and to provide additional information on unclear taxonomic status of a glacier tardigrade *Hypsibius klebelsbergi*, gene sequences from seven tardigrade species of the family Hypsibiidae (*Hypsibius klebelsbergi*, *Hypsibius cf. convergens 1*, *Hypsibius cf. convergens 2*, *Hypsibius scabropygus*, *Hebesuncus conjungens*, *Isohypsibius cambrensis*, *Isohypsibius granulifer*) were analysed together with previously published sequences from ten further eutardigrade species or species groups. Three distinctly separated clades within the Hypsibiidae, 1) the Ramazzottius - Hebesuncus clade, 2) the Isohypsibius clade (*Isohypsibius*, *Halobiotus*, *Thulinus*), and 3) the Hypsibius clade (*Hypsibius spp.*) have been obtained in each of four phylogenetic trees recovered by Maximum Parsimony, Neighbour Joining, Minimum Evolution and UPGMA. *Hypsibius klebelsbergi* has been located always within the Hypsibius clade. The detailed sister group relationship was not resolved adequately, but there is robust support for a sister group relationship between the Hypsibius clade and the remaining clades. We cannot exclude that the Ramazzottius - Hebesuncus clade is a sister group of the Macrobiotus clade. Our findings suggest polyphyly of the Hypsibiidae, and thus multiple evolutions of some structures currently applied as diagnostic characters (e.g., claws, buccal apophyses).

Key words: Tardigrada, Hypsibiidae, polyphyly, 18S rRNA, morphological characters

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### 1. INTRODUCTION

Previous studies using 18S rDNA gene sequences have suggested that Tardigrada 1) belong to the Ecdysozoa and 2) are the sister group of the Arthropoda (Garey *et al.* 1996; Giribet *et al.* 1996; Moon & Kim 1996; Aguinaldo *et al.* 1997; Garey 2001). These suggestions are largely congruent with morphological data (e.g. arthropod relationships were discussed in detail already by Marcus 1929) and elaborated with additional details in numerous subsequent articles (e.g., Dewel & Dewel 1997; for review see Schmidt-Rhaesa *et al.* 1998). Within the Tardigrada the classes Heterotardigrada and Eutardigrada are well established by morphological characters (for review of the older literature see Marcus 1929) and the monophyly of both classes as well as the ancestral Apochela and the derived Parachela (see Schuster *et al.* 1980) among the Eutardigrada appears to be supported by molecular data (Garey *et al.* 1999; Jørgensen & Kristensen 2004; Nichols *et al.* 2006). Currently, relationships within and between the various tardigrade families, based mainly on morphological characters and cladistic analyses, exist for the Echiniscidae (Kristensen 1987; Jørgensen 2000) and Macrobiotidae (Guidetti & Bertolani 2001). More recently Nichols *et al.* (2006) studied morphological data and 18S rDNA to evaluate congruence of morphological and molecular data at the family level and Guidetti *et al.* (2005) within the Macrobiotidae.

In a study on the biology and diagnostic morphological characters of the unique glacier dwelling eutardigrade *Hypsibius klebelsbergi* Mihelčič, 1959, it was suggested that some structural traits in this species (e.g. slightly asymmetrical, concave and flattened apophyses of the mouth tube, claws in shape between *Isohypsibius*- and *Hypsibius*-type with shortened main branches and their flattened accessory spines) actually did not allow a clear assignment to the genus *Hypsibius* (see Dastych *et al.* 2003).

This prompted us to analyse 18S rDNA gene sequences of some Hypsibiidae including *H. klebelsbergi* to prove the above suggestion regarding relationships of this species within the family and the Hypsibiidae in general, and to extend the taxa sampling for improvements of future molecular analyses of Eutardigrada. In contrast to Jørgensen & Kristensen (2004), our molecular data indicate that the Hypsibiidae might be polyphyletic.

### 2. METHODS

Specimens of *Isohypsibius cambrensis* Morgan, 1976 (status nov.: in preparation), *Isohypsibius granulifer* Thulin, 1928, *Hebesuncus conjungens* (Thulin, 1911), *Hypsibius scabropygus* Cuénot, 1929, *Hypsibius cf. convergens 1*, *Hypsibius cf. convergens 2* and *H. klebelsbergi* were collected at several localities in Germany and Austria (see Tab. 1). The species colonizing each sample were firstly diagnosed on permanent mounted specimens at maximum magnification

**Tab. 1.** List of tardigrade species collected for the study and their origin.

Species	Substrate	Locality / date	m (a.s.l.)	Geogr. Coordinates
<i>Isohypsibius cambrensis</i>	Mosses on a low, concrete fence near the Zoological Museum	Germany, Hamburg / 17.2.2005	8	9°58'37" E, 53°34'02" N
<i>Isohypsibius granulifer</i>	Algae from pond in a garden, coll. M. Preuß	Germany, Henstedt-Ulzburg near Hamburg / 27.3.2005	37	9°59'21" E, 53°45'18" N
<i>Hypsibius scabropygus</i>	Bryophytes from silicate rocks	Austria, the Ötztal Alps, Mt. Festkogel / 20.6.2003	3020	11°03'03" E, 46° 51' 08" N
<i>Hypsibius cf. convergens 1</i>	Edge of oak forest, wet mosses on soil, near a brook	Germany, Siebeneichen / 21.2.2005	12	10°37'36" E, 53°30'36" N
<i>Hypsibius cf. convergens 2</i>	Mosses on a low, concrete wall at the Zoological Museum	Germany, Hamburg / 14.3.2006	8	9°58'37" E, 53°34'02" N
<i>Hypsibius klebelsbergi</i>	Cryoconite holes	Austria, the Ötztal Alps, glacier Langtalferner / 29.8.2005	2580	11°00'26" E, 46° 48'02" N
<i>Hebesuncus conjungens</i>	Bryophytes from silicate rocks	Austria, the Ötztal Alps, slope of Mt. Hangerer, NN ridge, alpine meadow / 31.8.2004	2620	11°00'57" E, 46°50'07" N

(1200×). For molecular analysis living specimens from another sub-sample have been identified individually at 400× magnification (up to 2-3 individuals on each slide, under cover glass) and transferred with micro-pipette into 100% ethanol.

Five to 10 specimens were used. DNA was prepared according to manufacturer's specifications with the DNeasy tissue kit (Quiagen) with an over night incubation step with proteinase K. 18S rDNA was amplified with the primer pairs SSU\_F\_04 (GCTTGTCTCAAAGATTAAGCC) and SSU\_R\_26 (CATTCTTGGCAAATGCTTTCG); the resulting DNA-pieces consisted of 920 to 953 bp. PCR was carried out for 15 min initial denaturing at 94 °C followed by 35 cycles with 30 sec denaturing at 94 °C, 60 sec annealing at 50 °C, and 180 sec extension at 72 °C. The program ended with 20 min incubation at 72 °C, the sample volume was 25 µl [sample concentrations of 0.5 mM each dNTP, 0.5 µM each primer, 6 mM Mg<sup>++</sup>, 1.25U Taq (Biomaster), 1 µl template-DNA in 1x PCR-buffer (Biomaster)]. Sequencing was carried out in both directions. PCR-products were sequenced by SeqLab (Göttingen).

The sequences of the 18S rDNA of the following species were taken from Genbank data. *Echiniscus viridissimus* Péterfi, 1956 (AF056024) (Garey et al. 1996), *Halobiotus stenostomus* (Richters, 1908) (AY582121) (Jørgensen & Kristensen 2004), *Macrobiotus hufelandi* group (X81442) (Giribet et al. 1996), *Macrobiotus areolatus* group (U32393) (Garey et al. 1996, named *Macrobiotus tonolli* Ramazzotti, 1956) *Macrobiotus echinogenitus* Richters, 1904 in the figures in Nichols et al. 2006), *Milnesium tardigradum* (Doyère, 1840)(AY582120) (Aguinaldo et al. 1997), *Ramazzotius oberhaeuseri* (Doyère, 1840), (AY582122) (Jørgensen & Kristensen 2004), *Richtersius coronifer* (Richters, 1903) (AY5821239) (Jørgensen & Kristensen 2004), and *Thulinus stephaniae* (Pilato, 1974) (AF056023) (Garey et al. 1996; *Thulinia* renamed *Thulinus* by Bertolani 2003).

Sequences were aligned with Clustalw (Thompson et al. 1994) and phylogenetic trees were calculated using Maximum Parsimony (MP), Neighbour Joining (NJ), Minimum Evolution (ME) and Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) analyses of corresponding regions (ranging from 755 to 849 bps) of the first half of the 18S rDNA were performed using MEGA2 (Kumar et al. 2001). Confidence levels were evaluated by bootstrap analysis with 500 and 1000 bootstrap replicates. The heterotardigrade *Echiniscus viridissimus* Péterfi, 1956 was used as outgroup.

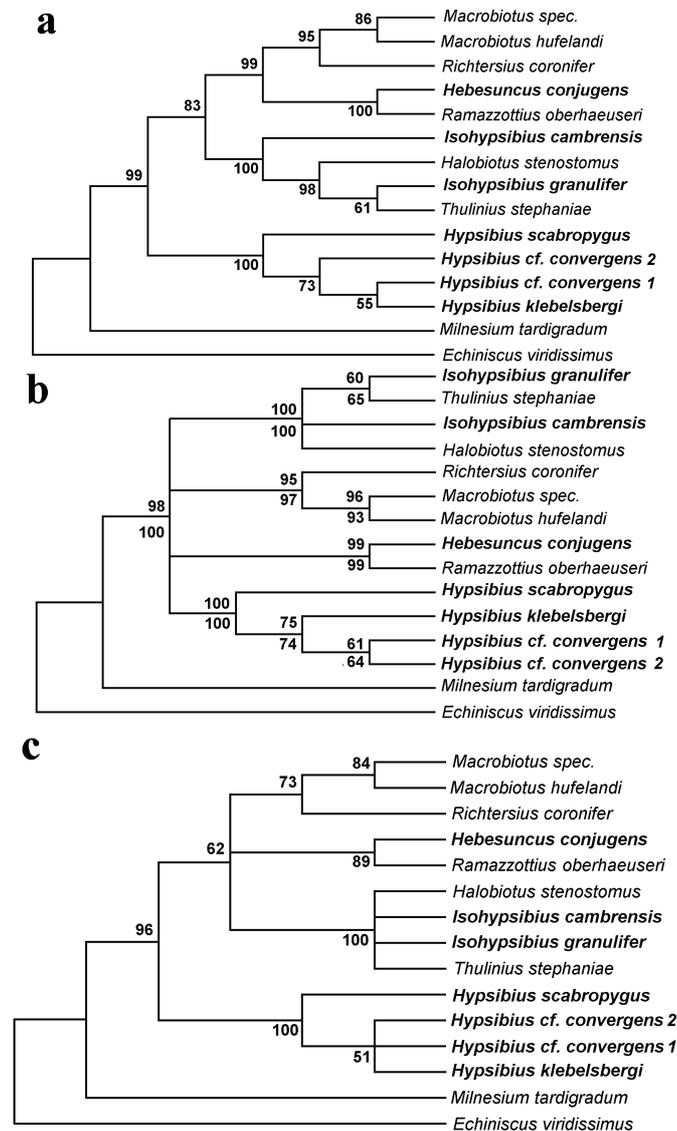
GenBank accession numbers of the partial 18S rDNA genes obtained in the present investigation are: *Hebesuncus conjungens* (AM 500646), *Hypsibius cf. convergens 1* (AM 500647), *Hypsibius klebelsbergi* (AM 500648), *Hypsibius scabropygus* (AM 500649), *Hypsibius cf. convergens 2* (AM 500650), *Isohypsibius granulifer* (AM 500651), *Isohypsibius cambrensis* (AM 500652).

### 3. RESULTS

Cladograms recovered from Maximum Parsimony, Neighbour Joining, Minimum Evolution and Unweighted Pair-Group Method using Arithmetic Averages revealed four clades among the Parachela identical in all trees, but partly with unresolved relationships (Figs 1a and 1c). Within the Eutardigrada monophyly of the Parachela is well supported (bootstrapping values 96-100%).

With the exception of the outgroup and the APOCHELA (*Milnesium tardigradum*), the four clades identified are 1) the *Isohypsibius* clade (*Isohypsibius*, *Halobiotus*, and *Thulinus*), 2) the *Macrobiotus* clade (*Macrobiotus*, *Richtersius*), 3) the *Ramazzotius* – *Hebesuncus* clade, and 4) the *Hypsibius* clade (all *Hypsibius* species examined). Bootstrap support of the four clades varied from 73% to 100% (Fig. 1).

All trees show a high bootstrap value (96-100%) supporting a sister group relationship between the *Hyp-*



**Fig. 1. a-c:** Phylogenetic trees of Eutardigrada based on the comparison of corresponding pieces of 18S rDNA gene sequences recovered for UPGMA (a), Neighbour Joining and Minimum Evolution (b), and Maximum Parsimony (c). In b are the numbers above each fork the bootstrap values for Minimum Evolution, below are the numbers for Neighbour Joining. Branches are not to scale. Numbers are bootstrap values. For further explanation see text.

*sibius* clade and the remaining clades identified in the Parachela. Relationships between the *Ramazzottius* - *Hebesuncus* clade, the *Macrobiotus* and the *Isohypsibius* clade were not resolved by Neighbour Joining (Fig. 1b), Maximum Parsimony (Fig. 1c) and Minimum Evolution (Fig. 1b). In the UPGMA-tree the *Isohypsibius* clade appears to be the sister group of a *Macrobiotus* - *Ramazzottius* - *Hebesuncus* clade (bootstrap value 83%) and the *Ramazzottius* - *Hebesuncus* clade a sister group of the *Macrobiotus* clade (bootstrap value 99%) (Fig. 1a).

#### 4. DISCUSSION

Analyses of the 18S rDNA gene sequences taken from Genbank data correspond (as expected) with those

presented by Garey *et al.* (1999), Jørgensen & Kristensen (2004) and Nichols *et al.* (2006), i.e. support of the monophyly of Hetero- and Eutardigrada and sister group relationships of Parachela and Apochela. Analysis of the broader taxa sampling, however, that covers various previously analysed Hypsibiidae (H. *stenostomus*, T. *stephaniae*, R. *oberhaeuseri*) and those examined newly herein (I. *cambrensis*, I. *granulifer*, H. *conjugens* and four *Hypsibius* species: H. *klebelsbergi*, H. cf. *convergens* 1, H. cf. *convergens* 2 and H. *scabropygus*) strongly suggests polyphyly of the Hypsibiidae. Jørgensen & Kristensen (2004) suggested monophyly of this eutardigrade family, based on the analysis of *Thulinus* and *Halobiotus* species only; an undetermined *Hypsibius* sp. also considered by these authors very

probably is *Thulinus* (see Garey *et al.* 1999). Further, they inserted *R. oberhaeuseri* in the Macrobiotidae without any comment (see *l.c.*, page 667, Tab. 1), where this species forms a clade with the Macrobiotidae with bootstrap values of 86 and 96% (see Jørgensen & Kristensen 2004, Fig. 2). Our results obtained by UPGMA analysis are in congruence with this finding. Independently from the procedure of analysis we always identified three distinctly separated clades currently included in the Hypsibiidae, i.e., the *Ramazzottius* - *Hebesuncus* clade (perhaps a sister group of the *Macrobiotus* clade: see above), the *Isohypsibius* clade and the *Hypsibius* clade, including *H. klebelsbergi*. The close relationship between *Macrobiotus* and *Ramazzottius* (*Hebesuncus*) is inconsistent from the morphological point of view, as some characters of the bucco-pharyngeal apparatus and claws suggest stronger affiliation between *Macrobiotus* and *Isohypsibius* (e.g. Pilato 1969).

Jørgensen & Kristensen (2004) analysed 18S rDNA of two Hypsibiidae (*Thulinus* and *Halobiotus* species; regarding *Ramazzottius* see the comments given above), which have been inserted in the *Isohypsibius* clade due to the new dataset presented herein. Interestingly, the close relationship of *Thulinus* and *Isohypsibius* is corroborated by similar analyses using the sequences of translational elongation factor EF2 and Pol II (RNA-Polymerase II) (Regier *et al.* 2004).

In a few articles the most important morphological characters of tardigrade taxa were mapped onto the gene tree (see Garey *et al.* 1999; Nichols *et al.* 2006) showing a considerable congruence of morphological and molecular data even at the family level (see Nichols *et al.* 2006). However, the Hypsibiidae were represented there (*l.c.*) only by *T. stephaniae* and *Hypsibius dujardini* (Doyère, 1840).

Claw structure and features of the buccal apparatus have been used for a long time to classify Eutardigrada (e.g., Thulin 1928; Marcus 1929; Ramazzotti 1972; Pilato 1969, 1982; Schuster *et al.* 1980; Bertolani & Kristensen 1987; Guidetti & Bertolani 2001; Guidetti *et al.* 2005). If the putative polyphyly of the Hypsibiidae should be confirmed by further molecular (and morphological) studies including further genera, e.g. *Doryphoribius*, the family would need to be re-diagnosed. We do not want to discuss this problem in detail herein, but, currently used key characters, including those at the generic level, show problems with clear application of these features. The difficulties are partly reflected in two recent general identification keys for tardigrades by Nelson (2001) and Nelson & McInnes (2002). The Macrobiotidae and Murrayidae recently separated from the Macrobiotidae (Guidetti *et al.* 2005) are clearly distinguished by their claws; the two double claws per leg are of similar size and shape and the sequence of the branches is secondary, primary, primary, secondary (2112). The Hypsibiidae share asymmetrical claws (sequence of the claw branches is alternate with the

sequence: secondary, primary, secondary, primary (2121)) with the Calohypsibiidae, Eohypsibiidae and Microhypsibiidae. However, current taxonomy and our gene trees indicate multiple evolutions of asymmetrical claws. Within the Hypsibiidae an *Isohypsibius*-claw (present in the members of our *Isohypsibius* clade), i.e., where the secondary branch of the claw forms a right angle (often difficult to define precisely) with its base and a *Hypsibius*-claw, where the secondary claw is continuous with its base forming (more or less distinctly) an arc, are distinguished. This type is present in our *Hypsibius* clade and the *Ramazzottius* - *Hebesuncus* clade. However, within the *Hypsibius*-claw, two subtypes are differentiated, (see e.g., Binda & Pilato 1986; Nelson 2001; Nelson & McInnes 2002). In view of our findings these subtypes deserve further attention. Other diagnostic features such as peribuccal lamellae and the buccal apparatus (symmetry and shape of anterior apophyses of the mouth tube) are currently not suitable to distinguish our clades. Interestingly, *Ramazzottius* - *Hebesuncus* (as well as the Macrobiotidae) lay ornamented eggs freely on the substrate, whereas "Hypsibiidae" (from the *Hypsibius* and *Isohypsibius* clade) deposit smooth eggs mostly in their exuviae (*H. klebelsbergi* seems to lay eggs freely or in the exuviae and *Acutuncus antarcticus* (Richters, 1904) lays free, partly ornamented eggs).

We have presented herein data derived from the 18S rDNA. However, the suitability of this genetic marker is limited to relatively ancient relationships (e.g. Abouheif *et al.* 1998). Therefore, more independent data from several molecules are required to ascertain phylogenetic trees. Studies on the Internal Transcribed Spacer 1 and 2 are in progress to include more rapidly evolving sequences.

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