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# A model study for tardigrade identification

Roberto Bertolani, Lorena Rebecchi, Michele Cesari

**Abstract** — Using tardigrades from a single moss sample as a case study, we propose a new method for tardigrade species identification, which is often problematic, due to the low number of morphological characters. Identification at generic level was carried out on adults, while morphological analyses were performed on animals (LM) and eggs (LM and SEM), including hologenophores, vouchers used also for molecular analysis of COI mtDNA. This multi-approach method revealed the presence of three species of the “*Macrobotus hufelandi* group” instead of the two species identified in a previous study. The validity of the method is shown, indicating that it could be applied to studies of problematic meiofauna taxa.

**Index Terms** — COI, DNA barcoding, morphology, Tardigrades, taxonomy.

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## 1 INTRODUCTION

Tardigrades consist of more than 1,000 described species [1], [2] colonizing marine, limnic and terrestrial environments, including “hostile to life” and unpredictable habitats. In the seventies, a new evaluation of the intraspecific variability and new morphological characters for species identification were proposed [3], [4], [5], which led the number of tardigrade species to increase from less than 500 species described to that date to the current number. An example of this improvement in identifying species can be found in *Macrobotus hufelandi*, the first described [6] and most commonly identified tardigrade species. What was considered a single species is currently represented by more than 25 species. Nonetheless, tardigrade identification at the species level is often problematic due to the low number of taxonomic characters. During our work it was not rare to find in the same moss sample more than one tardigrade species, not only in the same genus but also in the same species group, creating problems of species identification. For this reason we have begun to identify species by coupling a detailed evaluation of animal and egg shell morphology with DNA barcoding [7]. Using one moss sample as a case study, we propose a new method for tardigrade species identification and, in general, for identification of meiofaunal taxa whose morphological

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characters are often very limited.

## 2 MATERIAL AND METHODS

The moss sample was collected at Andalo (Central Alps; province of Trento, Italy, 46°N 10.133, 011°E 00.017, 1050 m) on a rock already examined by us (Fig. 1) [7], [8]. Several tardigrade species were present in the sample, belonging to different genera of eutardigrades (*Macrobotus*, *Minibiotus*, *Ramazzottius*, *Milnesium*) and heterotardigrades (*Echiniscus*) but only the specimens belonging to the so-called “*Macrobotus hufelandi* group” have been used in this study. Two species in this group, *Macrobotus macrocalix* Bertolani & Rebecchi, 1983 (amphimictic) and *M. cf. terminalis* (parthenogenetic), were already found in the previous collections [7], [8].



Fig. 1 – The rock located in Andalo (Italy), with the moss patch that was used in the study.

Morphological analyses of paragenophore voucher specimens (*sensu* Pleijel *et al.* [9]) were carried out by mounting animals and eggs (that have species-specific shell processes) in Faure-Berlese fluid for light microscopy (LM) observations. Other eggs were fixed and dehydrated for scanning electron microscopy (SEM) analysis. Additional animals were stained with acetic lactic orcein for gender identification.

For molecular analysis, DNA was extracted from single entire animals. Some of these specimens were newborns hatched from isolated eggs, whose shells were mounted in Faure-Berlese fluid, obtaining hologenophore voucher specimens (*sensu* Pleijel *et al.* [9]). Amplification and sequencing of 684 bp of the COI mtDNA gene were carried out, following the procedures described in Cesari *et al.* [7]. Kimura 2-parameters distances between haplotypes were scored by using MEGA4 [10], while neighbor joining and maximum parsimony dendrograms were computed using PAUP\* 4.01b10 [11], also using sequences retrieved from GenBank (EU244599; FJ435804-7; AY598773-5; FJ176203-17). For possible further investigations, a fragment of the moss sample was stored at -80°C.

### 3 RESULTS

Observations of animals stained with acetic lactic orcein confirmed the presence of males and females morphologically attributable to *M. macrocalix* by the presence of a strong buccal armature, with thick crests and large bands of evident teeth and with a relatively wide buccal tube, also observed in mounted specimens (Fig. 2a). In addition, males were also found among the specimens characterized by a weaker buccal armature and narrower buccal tube (Fig. 2b).



Fig. 2 – Buccal-pharyngeal apparatuses (Faure-Berlese fluid, phase contrast). a: Strong buccal armature and wide buccal tube. b: Weak buccal armature and narrow buccal tube. Scale bars = 10  $\mu$ m.

Molecular analysis of specimens and eggs belonging to the “*Macrobotus hufelandi* group” revealed three clearly distinct haplogroups (a-c, Fig. 3), with very high genetic distances among them (Tab. 1).

		1	2	3	d
1	Haplogroup a				0.005
2	Haplogroup b	0.193			0.000
3	Haplogroup c	0.169	0.181		0.001

Tab. 1 – Kimura 2-parameters distances computed among (under the diagonal) and inside (column d) haplogroups.

A detailed analysis of egg shell morphology both of the hologenophores and of other voucher specimens (paragenophores) showed the presence of three types of eggs, all bearing processes as inverted goblets on the shell (Fig. 4). One type of egg exhibited high (9.6-10.7  $\mu$ m) and wide (7.9-8.6  $\mu$ m) processes with large (7.0-8.0  $\mu$ m) smooth distal discs and very large pits located only around the process bases (typical of *M. macrocalix*) (Fig. 4a, d). The second type of egg was characterized by clearly smaller processes (7.4-8.4  $\mu$ m) than

those of *M. macrocalix* and having an irregularly edged distal disc (6.3-7.0 µm in diameter) and a non-uniform reticulated egg shell with a thick meshwork (Fig. 4b, e). The third type of egg had small processes (5.0-5.3 µm in height) with a slightly irregular edge on the distal disc (4.7-5.2 µm in diameter), and a very uniform reticulated egg shell with a very thin meshwork (Fig. 4c, f).

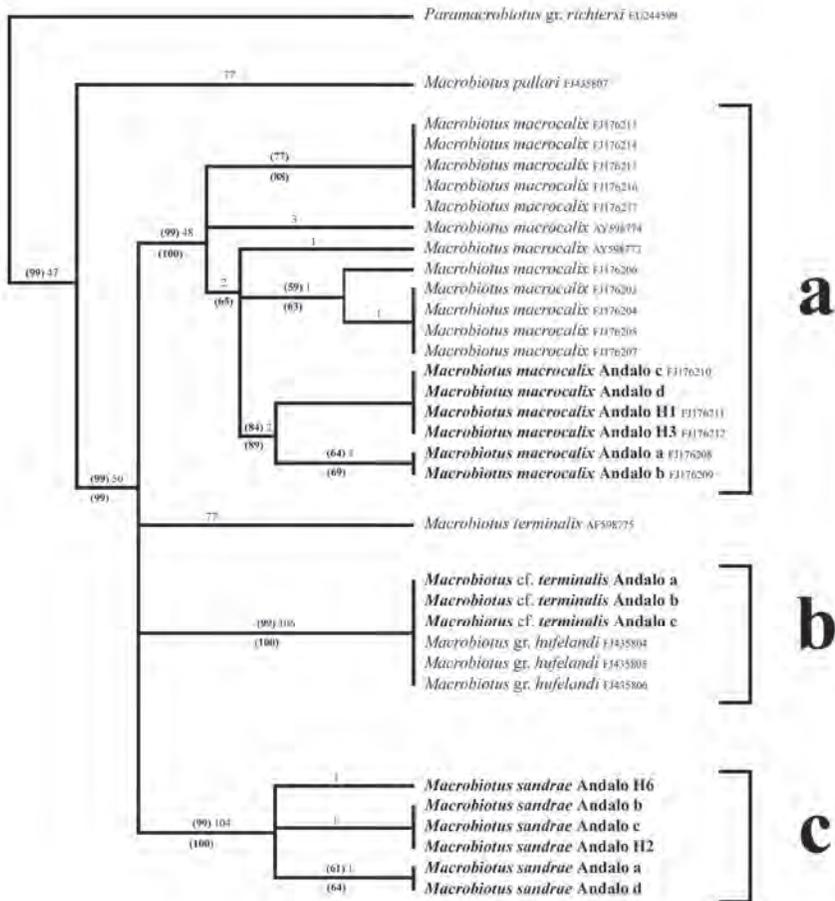


Fig. 3 – Dendrogram combining neighbor joining (NJ, ME score: 0.731) and maximum parsimony (MP, consistency index: 0.743; retention index: 0.920; rescaled consistency index: 0.684) analyses. Numbers above branches indicate mutational steps, while numbers in parentheses show bootstrap values computed after 2000 replicates (above branches: MP; below branches: NJ). **a-c** denote different haplogroups, while H denotes individuals for which hologenophore voucher specimens are available. Names in bold indicate specimens pertaining to the studied moss.

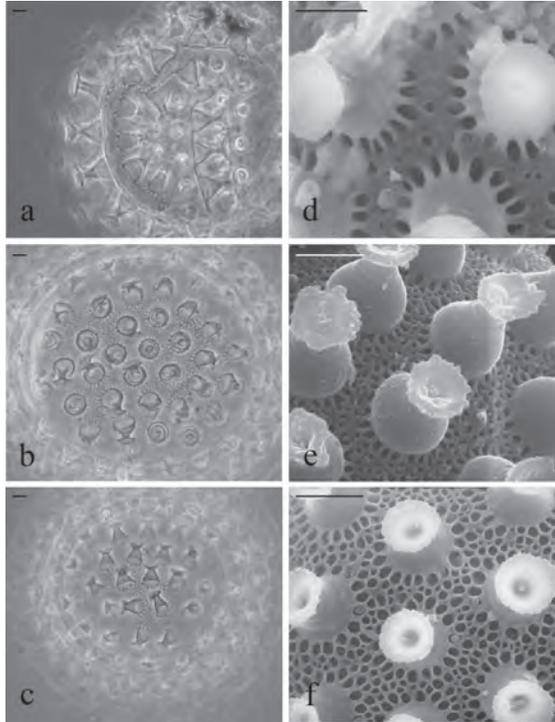


Fig. 4 – Voucher specimens consisting of egg shells. **a-c**: Faure-Berlese fluid (LM, phase contrast). **a**: *Macrobiotus macrocalix*, haplogroup a (hologenophore H1). **b**: *M. cf. terminalis*, haplogroup b (paragenophore). **c**: *M. sandrae*, haplogroup c (paragenophore). **d-f**: SEM (paragenophores). **d**: *M. macrocalix*. **e**: *M. cf. terminalis*. **f**: *M. sandrae*. Scale bars = 5 µm.

## 4 DISCUSSION

The sex ratio analysis of the tardigrades belonging to the “*Macrobiotus hufelandi* group” in the moss sample revealed a much more complicated situation than that known from the literature [7], [8]. Nevertheless, by comparing the results of a detailed morphological analysis with those obtained by DNA barcoding, and in particular by sequencing the newborns’ DNA and linking their sequences to the related egg shell shapes (hologenophores), the problem can finally be solved.

The distance values among the three different haplogroups are very high, far exceeding the 3% threshold and the 10x rule proposed by Hebert *et al.* [12], [13], [14], thus supporting the specific rank of the three haplogroups. Two species, *M. macrocalix* and *M. cf. terminalis* (currently being described as a new species), morphologically correspond to what was previously found on the same rock at Andalo [7], [8]. With regards to the third species, the animals look similar to the specimens of *M. cf. terminalis* (even through probably smaller), but the eggs are quite distinguishable and allow us to attribute them to *Macrobiotus sandrae* Bertolani & Rebecchi, 1983. This species is known to be amphimictic

[8], a situation consistent with the presence of males among the animals with a weaker buccal armature and narrower buccal tube.

## 5 CONCLUSIONS

The methods described here allow us to solve intricate tardigrade identification problems, validating our new approach based on linking morphological and molecular data. The use of voucher specimens, and in particular of the hologenophores, is critical for obtaining a correct species diagnosis. A hologenophore can also be obtained by culturing an isolated female until oviposition, mounting it as voucher and using its developing eggs either for molecular analysis and/or as further vouchers. Further information important for identification can also be obtained from other tardigrades, which can be photographed *in vivo* up to maximum magnification (100x objective) before being used in molecular investigations.

In our opinion, our multi-approach method for tardigrade identification can be easily applied to other meiofaunal taxa, whose few morphological characters can generate problems in species identification.

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