https://www.biodiversityjournal.com - Edizioni Danaus
Biodiversity Journal, 2024, 15 (4): 935–955 - **Article**
https://doi.org/10.31396/Biodiv.Jour.2024.15.4.935.955

Biodiversity Journal
ISSN 2039-0394 (Print Edition)
ISSN 2039-0408 (Online Edition)

Discussion on the morphological and molecular data used in the study of tardigrade systematics

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ABSTRACT The tardigrade taxonomy is recently based on morphological and molecular data. Some cases are here discussed starting from the reasons that lead to consider the family Hexapodibiidae a valid taxon together with an evaluation on its position within the superfamily Isohypsibioidea. The matters that support the institution of the family Hexapodibiidae also offer the occasion to discuss not only this particular case but, in general, the use of molecular and morphological data in systematic and phylogenetic studies of Tardigrada. In particular, I try to warn against errors of diagnosis of the material used to obtain the molecular sequences, or the use of a low number of data, which could lead to even considerable errors in the construction of the phylogenetic trees.

KEY WORDS Phylogeny; characters weighting; discussion.

Received 22.11.2024; accepted 21.12.2024; published online 30.12.2024

INTRODUCTION

Much molecular research has been carried out during the last two decades regarding limno-terrestrial tardigrades (e.g. Garey et al., 1999; Jørgensen & Kristensen, 2004; Regier et al., 2004; Nichols et al., 2006; Jørgensen et al., 2010, 2011; Guidetti et al., 2005, 2009, 2016, 2019; Kiehl et al., 2007; Sands et al., 2008a,b; Cesari et al., 2009, 2016 a,b; Marley et al., 2011; Guil & Giribet, 2012; Guil et al., 2013a,b, 2018; Bertolani et al., 2014, 2022; Morek et al. 2019, 2020; Gąsiorek et al., 2019 a,b,c; Morek & Michalczyk, 2020, Topstad et al., 2021;Tumanov & Tsvetkova, 2023; Vecchi et al., 2023; Dey et al., 2024; Pust et al., 2024; Gąsiorek et al., 2024; Morek et al., 2024; Vincenzi et al., 2024). Those studies, integrated with the available morphological data, according to the authors, allowed a better reconstruction of the phylogeny of Phylum Tardigrada. However, as will be highlighted later, some changes to taxonomy and phylogeny predominantly based on molecular data, in some cases lead to unlikely conclusions compared to morphological analyses.

In the present paper some cases will be discussed starting with the reasons that support the validity of the family Hexapodibiidae together with an evaluation of its position within the superfamily Isohypsibioidea.

The family Calohypsibiidae Pilato, 1969b had been instituted for Eutardigrada having claws of the *Calohypsibius* Thulin, 1928 type, and in the family description some morphological differences had been stressed between the genus *Calohypsibius* on one hand and the other genera of the family on the other hand (Fig. 1, from Pilato 1969b). These differences (Figs. 1, 2) were confirmed in subsequent papers regarding the same family Calohypsibiidae (Pilato 1982b, pp. 223–224; Pilato & Beasley (1987, p. 69); Pilato, 1989).

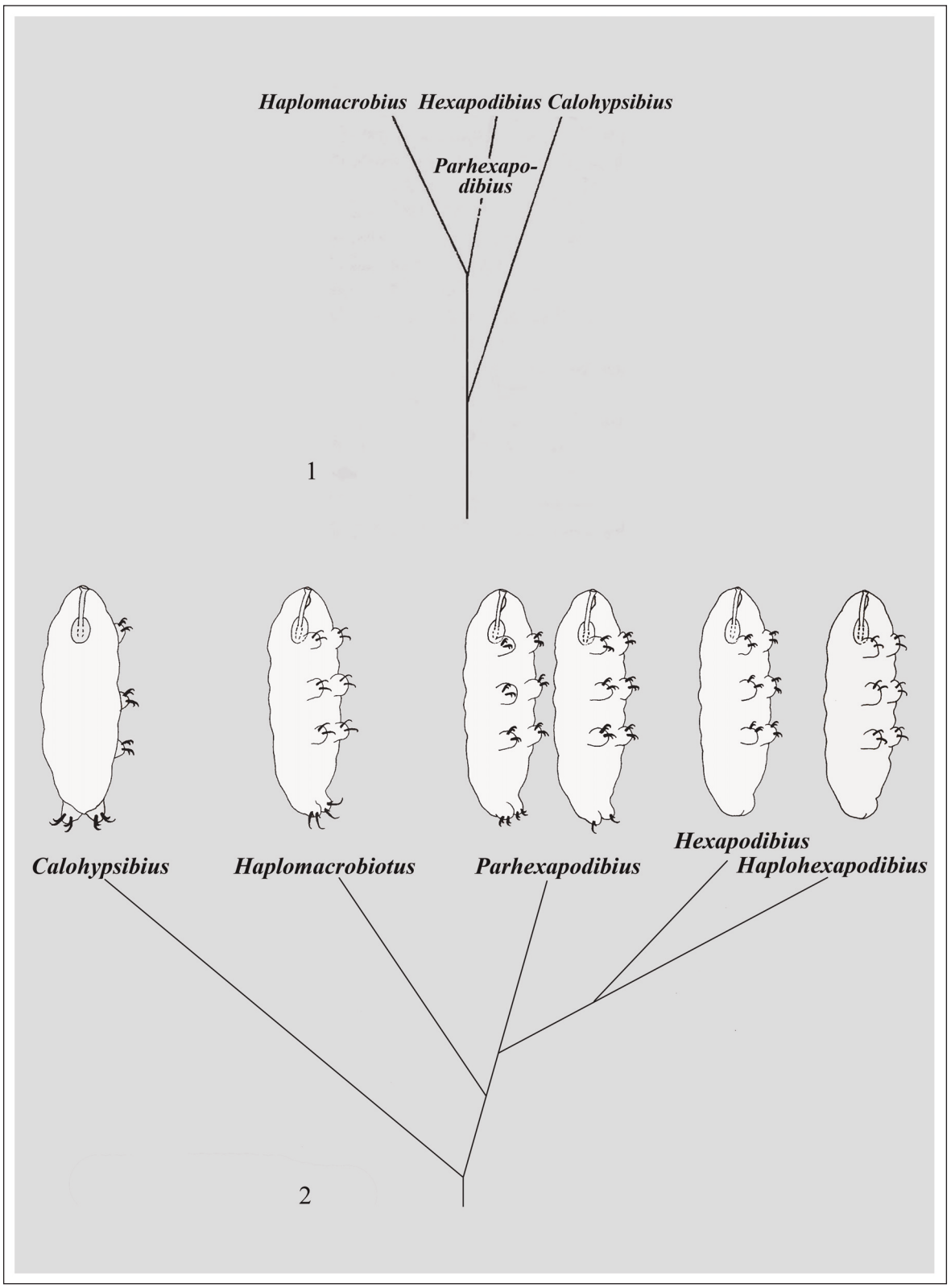
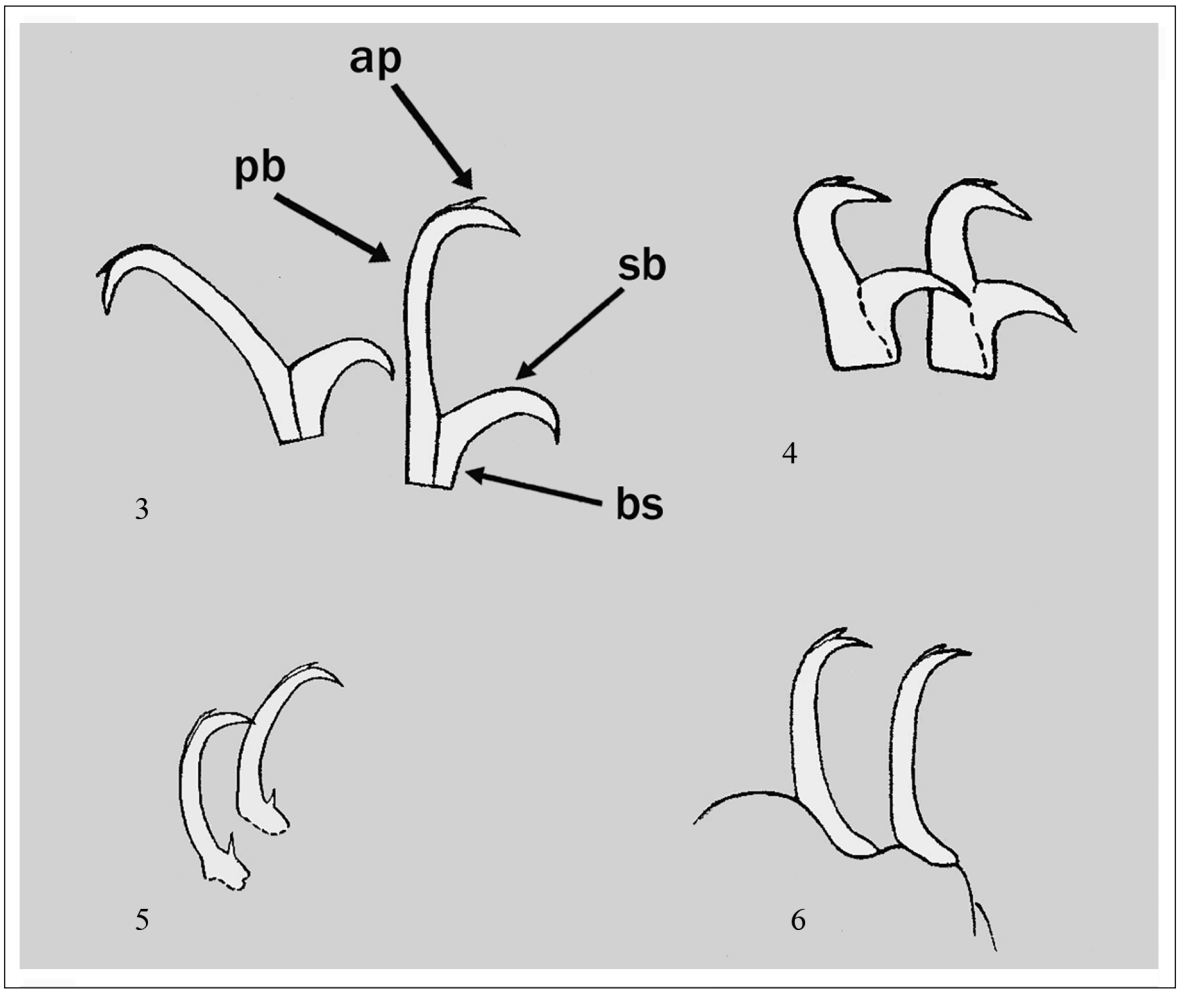


Figure 1. Phylogenesis of Calohypsibiidae according to Pilato (1969b) (only four genera were known at that time). Figure 2. Phylogenesis of the Calohypsibiidae confirmed by Pilato (1989) when all the five genera attributed to that family were known. A degree of separation of the genus *Calohypsibius* from the other genera of the family is evident.

Recent combined studies of the nucleotide sequences coding for 18S rRNA and 28S rRNA, and new morphological observations about Calohypsibiidae, led Bertolani et al. (2014) to leave only the genus *Calohypsibius* ascribed to the family Calohypsibiidae, confirming this family's position in the superfamily Hypsibioidea. At the same time, mainly on the basis of molecular affinities of *Hexapodibius* Pilato, 1969, accompanied by a difference in the claw structure and notable differences in the buccopharyngeal apparatus compared to *Calohypsibius*, they transferred the genus *Hexapodibius* and the other three genera previously ascribed to the family Calohypsibiidae (*Haplomacrobiotus* May, 1948; *Hexapodibius*; *Parhexapodibius* Pilato, 1969 and *Haplohexapodibius* Pilato et Beasley, 1987) to the superfamily Isohypsibioidea. All four of these genera have similar claws, or clearly derived claws with a much shorter or absent secondary branch (Figs. 3–6), and similar bucco-pharyngeal apparatuses.

In 2014, only the family Isohypsibiidae was attributed to this superfamily and, as a consequence, Bertolani et al. (2014) added the four above mentioned genera into the family Isohypsibiidae, stressing that that family appeared clearly polyphyletic. Afterwards, Cesari et al. (2016b), adding molecular information on *Haplomacrobiotus,* noted that both



Figures 3–6. Claws of the *Hexpodibius* type of the various genera of Hexapodibiidae. Fig. 3: claws with the secondary branch normally developed (genus *Parhexapodibius* Pilato, 1969b). Fig. 4: claws with the secondary branches reduced (genus *Hexapodibius* Pilato, 1960a). Fig. 5: hind legs of *Haplomacrobiotus* May, 1948 where the secondary branches are reduced to a simple spur. Fig. 6: claws of *Haplohexapodibius* Pilato et Beasley, 1987 in which the secondary branches are absent; ap = accessory points; bs = basal section; pb = primary branch; sb = secondary branch (from Pilato & Binda, 2010).

molecular and morphological data led to the same conclusion, namely that those four genera belong to a homogeneous phyletic lineage clearly different from the other lineages of the superfamily Isohypsibioidea. For this reason they instituted for those four genera the new family Hexapodibiidae Cesari, Vecchi, Palmer, Bertolani, Pilato, Rebecchi et Guidetti, 2016.

The position of Hexapodibiidae as taxa of the Isohypsibioidea is accepted without discussion by the various authors who have taken them into consideration, even though the evaluation of the position of the Hexapodibiidae within the Isohypsibioidea is not uniform. In fact, even though in the phylogenetic tree of figure 4 of Bertolani et al. (2014) and figure 3 of Cesari et al. (2016b) *Hexapodibius* and the Hexapodibiidae, respectively, seem to be not basal among the Isohypsibioidea (at that time composed by only one family), due to the low number of data the authors are careful not to emphasize this fact in their discussions, aware that a much greater number of data would have been needed to confirm this situation. Bertolani et al. (2014) state "*Within the phyletic lineage of Isohypsibioidea (Fig. 4), species attributed to seven genera are present (....), whose relationships are not always well-resolved.*" Prudence seems to characterize Gąsiorek et al. (2019a), who represent a comb-like phylogenetic tree of the Isohypsibioidea, i.e. without identification of a basal branch. However, in the same year Gąsiorek et al. (2019c) state that the Isohypsibiidae (from which numerous species and genera have been removed to erect new families), and therefore not the Hexapodibiidae, represent the basal branch of the Isohypsibioidea and that (pp. 1 and 41) "*the Isohypsibioidea are most likely the most basally branching evolutionary lineage of eutardigrades*". It should be emphasized that this statement is based on the same low number of sequences used by previous authors. It also should be noted that there is already evidence here that different conclusions are drawn with the same molecular data. In my opinion, as I will discuss later, the morphological data do not lead to this conclusion.

In this paper it seems opportune to stress the reasons which led me (as co-author) to consider the new family Hexapodibiidae as valid and to express an opinion about the importance of attributing the proper role of molecular and morphological data in attempting to construct the phylogeny of Eutardigrada. For this reason I also examined other eutardigrade species and genera whose purported identification or phylogenetic position were perplexing, considering both preparations and descriptions in various publications. It is evident that any errors in identifying the species compromise the validity of the molecular data and the identification of their phylogenetic position, and it is clear that molecular data, especially those lacking strong support, can lead to risks of misinterpretation of phylogeny.

MATERIAL AND METHODS

Morphological observations were conducted on species of almost all the genera attributed to the superfamily Isohypsibioidea; many examined specimens were mounted in polyvinyl-lactophenol and deposited in the Binda and Pilato collection (Museum of the Department of Biological, Geological and Environmental Sciences, University of Catania, Italy). Three specimens of *Isohypsibius papillifer* (Murray, 1905) were also examined, kindly loaned by Sandra McInnes (British Antarctic Survey, Cambridge). Photomicrographs were made under x100 oil immersion, using a Leica Phase Contrast Microscope equipped with "Canon S40" digital camera and using Adobe Photoshop Elements 2.0 digital imaging software. For this paper no molecular study has been carried out, and all molecular data are from Sands et al. (2008b), Bertolani et al. (2014, 2022), Cesari et al. (2016b), Gąsiorek et al. (2019c) and Guidetti et al. (2019).

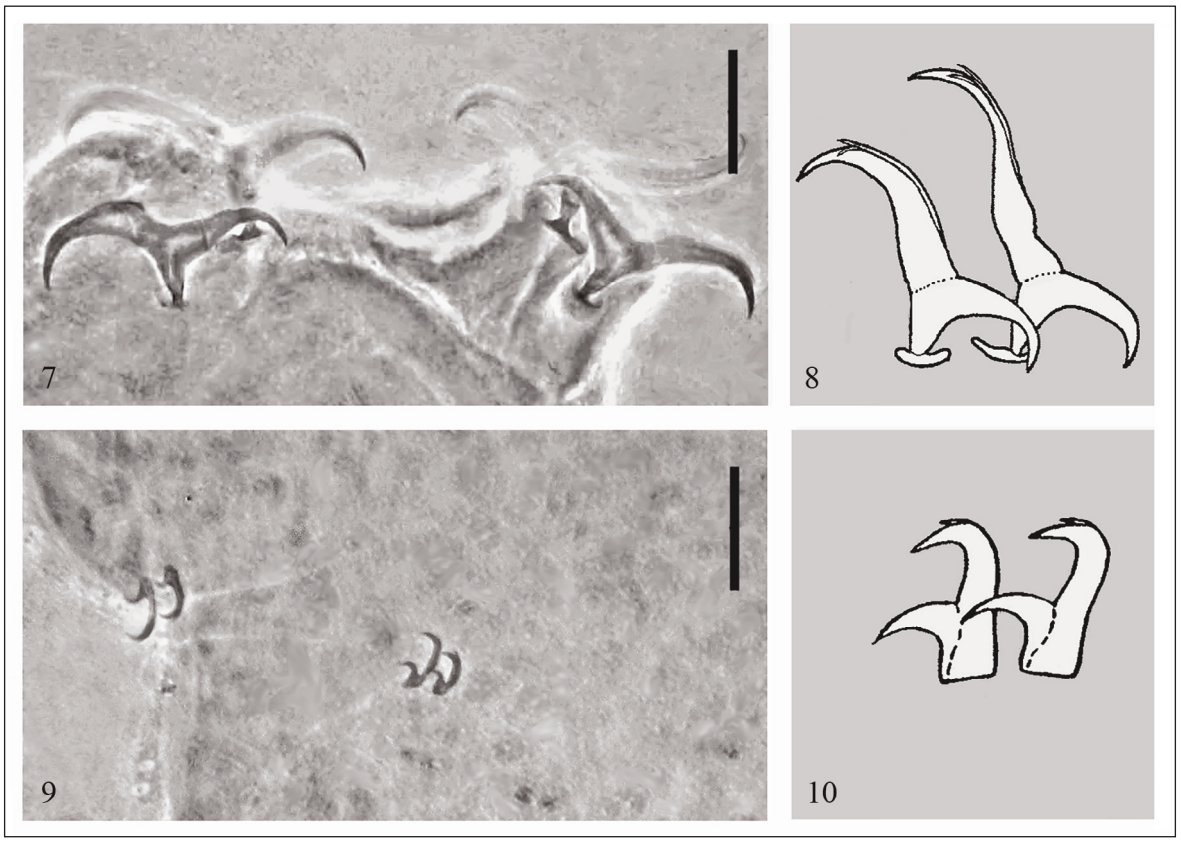
RESULTS AND DISCUSSION

A. Evaluation of the family Hexapodibiidae, highlighting of some cases of forced and insufficiently supported phylogenetic interpretations based on a molecular evidence

An in-depth review of the cuticular structures observing the available slides before me of the known genera of Hexapodibiidae (*Hexapodibius*, *Parhexapodibius*, *Haplomacrobiotus*, *Haplohexapodibius*) confirmed the morphological uniformity and peculiarity, and therefore the validity, of that family, which I had already approved as co-author

in Cesari et al. (2016b). Nonetheless, I here take occasion for resolutely disagreeing, from the morphological point of view, with the attribution of those four genera to the family Isohypsibiidae by Bertolani et al. (2014) and with the recent proposal of a basal position of this family within the Isohypsibioidea and the eutardigrades (Gąsiorek et al. 2019c). In the first case, placing claws with different structure in the same family, there would be an asymmetry in the evaluation of the same character, which does not occur within the other superfamilies of eutardigrades. Bertolani et al. (2014) transferred those four genera into the family Isohypsibiidae on the basis of combined studies of two DNA sequences (18S and 28S genes). They correctly noticed the differences between the claws, of the *Hexapodibius* type of those genera, and the claws of the *Isohypsibius* type of the Isohypsibiidae, but they deemed to solve the problem by considering the claws of the *Hexapodibius* type as derived from claws of the *Isohypsibius* Thulin, 1928 type due only to a reduction of the basal tract.

Also Gąsiorek et al. (2019c, Page 19), defined the claws of *Hexapodibius* type as "*a morphotype of the claws of the* Isohypsibius *type*"; i.e. admitting, like Bertolani et al. (2014), that the claws of the *Hexapodibius* type derived from claws of the *Isohypsibius* type by reduction of the basal tract. However, it is difficult to think that the claws of the *Hexapodibius* type derived from claws of the *Isohypsibius* type, differing not only in the shape but also in the structure (obviously the simple dimensions are not important). The claws of the *Isohypsibius* type (Figs. 7, 8) have a basal portion that continues with the secondary branch, and the main branch is joined to the secondary branch obliquely, whereas in the claws of the *Hexapodibius* type (Figs. 3–6 and 9, 10 of this paper, and 7B of Bertolani et al., 2014) main and secondary branches are joined to each other as far as the very base, and



Figures 7–10. Claws of the *Isohypsibius* type (Figs. 7, 8)**,** and of the *Hexapodibius* type (Figs. 9, 10). The two types of claw differ from one another in both structure and shape. The claws in Fig. 9 refer to the second and third pairs of legs of the holotype of *Hexapodibius chirstenberryae* Pilato et Binda, 2003**.** Scale bars = 10 μm.

the suture, well visible, has an orientation clearly different from that of the claws of the *Isohypsibius* type. If a reduction of the basal portion is imagined in the claws of the *Isohypsibius* type, since the suture has a different orientation, it (Figs. 7–10) would remain different, and this indicates a very probable distinct origin of those two types of claws. It is very difficult to imagine a separation of the branches and then a re-joining according to a different position in order to produce claws of the *Hexapodibius* type. It seems more likely to hypothesize that from a common ancestor, with four separate claws per leg, the Apochela and ancestor of the Parachela arose; in the Apochela the four claws of each leg did not join; from the descendant who had been ancestor of the Parachela, some lineages derived whose claws, independently one from the others, joined two by two forming double claws. From one of these descendants that acquired double claws, it is possible that two lineages evolved whose molecular sequences have not diversified like the structure of the claws. In these two lineages the four claws per leg joined two by two in different manners giving double claws of the *Isohypsibius* type in a descendent lineage (the Isohypsibiidae), and of the *Hexapodibius* type in the other (the Hexapodibiidae). Starting about fifty years ago (Pilato 1969b), but also see Tumanov (2021), the importance of distinguishing the shape from the structure of the claws was stressed. Tumanov (2021) wrote: "*Modern family-level system of the Eutardigrada is mainly based on the works of Pilato..... who reconstructed the phylogeny of this group using the organization of the claws as a primary character and that of the buccal-pharyngeal apparatus as a secondary character*". In some cases it seems that distinction between shape and structure is today neglected, and the result is to avoid dealing with the problem of the discordance between morphological and molecular data.

With regard to the use of molecular data in eutardigrade phylogeny, on one hand, considering the origin of the claws of *Macrobiotus* Schultze, 1834 type, Bertolani et al. (2014) wrote (Page 113): "*The main parachelan clade is formed by four well supported evolutionary lines that can be identified with the four superfamilies: Isohypsibioidea, Hypsibioidea, Eohypsibioidea, and Macrobiotoidea (Figs. 2 and 3). Only the tree obtained with the BI, under GTR model, shows the Isohypsibioidea basal to the other superfamilies and the Hypsibioidea as sister-group of the clade with the other two superfamilies (Eohypsibioidea, Macrobiotoidea) being closely related (Page 114, Fig. 2). The BI (under CAT model) and ML analyses are not able to solve the relationships among superfamilies)*". On the other hand, Gąsiorek et al. (2019c) seem to have less perplexities, and, exceeding the caution of Bertolani et al. (2014), wrote on page 41: "*Isohypsibioidea are most likely the most basal lineage in the order Eutardigrada* (Sands et al., 2008; Bertolani et al., 2014a)". And in the same page 41: "*Given the phylogenetic relationships between the orders* (Bertolani et al., 2014a)*, asymmetrical claws are most likely a plesiomorphy of the Eutardigrada whereas claw symmetry should be considered as a macrobiotid autapomorphy*".

Those who consider "*the Isohypsibioidea the most basal lineage on the order Eutardigrada*", should explain in what way asymmetrical claws of the *Isohypsibius* type became symmetrical claws of the *Macrobiotus* type, considering the differences in claw structure between those two groups, describing a reasonable process in this transition and not simply speak of "*macrobiotid autapomorphy*". It is difficult to imagine that from an ancestor having claws of the *Isohypsibius* type, i.e. with branches asymmetrically joined (and with main and secondary branches clearly different in length from one another) a descendant derived in which the claws, of *Isohypsibius* type, changed so deeply, including their symmetry, structure and shape. One can also hypothesize that the branches of the claws of *Isohypsibius* parted and subsequently joined in a different way giving origin to the symmetrical claws of the *Macrobiotus* type, but both these hypotheses appear very improbable.

Moreover, Gąsiorek et al. (2019c), discussing the phylogeny of Eutardigrada, about the buccopharyngeal apparatus state: "*In other words, the presence of the ventral lamina should be treated as an example of parallel evolution within Eutardigrada being at the same time the autapomorphy of Macrobiotoidea as well of Hexapodibiidae and some genera of Doryphorybiidae fam. nov.*" It seems strange to admit the same autapomorphy in the Macrobiotoidea, in the Hexapodibiidae and in some genera of Doryphoribiidae, but it is even stranger that: A) Gąsiorek et al. (2019c, p. 18) write that "*The lack of ventral lamina in Hetero-*

tardigrada Marcus, 1927, Apotardigrada, Hypsibioidea, and many Isohypsibioidea suggests that it is a derived trait" without hypothesizing what the ancestor provided with that lamina would have been like; and B) they hypothesize that after the reduction of the lamina in an ancestor of the Macrobiotoidea, in this taxon the same structure reappeared as an autapomorphy. In these cases requiring massive and complicated morphological changes, I believe that too much importance has been given to molecular data that is still very weak.

Even when only or mainly molecular data are considered, problems frequently occur in Eutardigrade phylogenetic analysis. Eutardigrada are traditionally considered a class subdivided into two orders: Apochela and Parachela. Recently, Guil et al. (2018), mostly on a molecular basis proposed to consider those two orders as classes named, respectively, Apotardigrada and Eutardigrada; but Morek et al. (2020), and also Fleming & Arakawa (2021), considered the proposal by Guil et al. (2018) unjustified and re-established the traditional taxonomy.

B. Examples of misdiagnosis of taxa and consequent incorrect coupling of morphological and molecular data

Other no less important problems arise when there are errors in identifying the taxa that are used for sequencing. The possibility of mistakes in specific or generic diagnoses of the material used for molecular research (so that molecular and morphological evaluation may appear discrepant, and phylogenetic evaluation at least dubious) is a demonstrable fact. According to the molecular analysis of Sands et al. (2008b), *Isohypsibius papillifer* (Murray, 1905) appeared clearly different from all the other studied species of the genus (as intended in 2008). For many years I had the suspicion that this species did not belong to the genus *Isohypsibius* (see Plate III, Fig.15C in Murray, 1905). In order to be sure, I asked for a loan from Sandra McInnes of the specimens of *Isohypsibius papillifer* tested by Sands et al. (2008b) and thanks to her kindness I was able to examine three specimens attributed to *Isohypsibius papillifer* by those authors. In the label of only one of those slides it was written "*Isohypsibius* cf. *papillifer*", i.e a doubt was expressed about the specific diagnosis, but no doubt about the genus. In the paper by Sands et al. (2008b) no doubt is expressed about the specific diagnosis. Authors who referred to the paper of Sands et al. (2008b) (as examples Bertolani et al., 2014; Guil et al., 2018; Gąsiorek et al., 2019c) recorded *Isohypsibius papillifer* without reference to eventual doubts on the specific diagnosis. In any case, as regards the considerations expressed in this paper, if the specimen used by Sands et al. (2008b) is identical to the three specimens I examined, the doubt about the specific diagnosis is less important than the fact that the species studied by those authors, and named *Isohypsibius papillifer,* does not belong to the genus *Isohypsibius* but to a different genus: *Mixibius* Pilato, 1992 or to a genus morphologically extremely similar to *Mixibius*. This, in my opinion, is demonstrated by the internal claw shape (Figs. 11, 12) compared with a claw (Fig. 13) of *Mixibius fueginus* Pilato et Binda, 1996, and by the morphology of the apophyses for the insertion of the stylet muscles (Figs. 14 and, better, Fig. 15) relative to a specimen in lateral view).

In Fig. 16, by comparison, a photo relative to *Mixibius parvus* Lisi, Sabella et Pilato, 2014 is presented; it is evident that each apophysis of the specimen named *Isohypsibius papillifer* by Sands et al. (2008b) (Figs. 14, 15) is a hook followed by a longitudinal thickening of the buccal tube wall as in *Mixibius parvus* of Fig. 16 and not a ridge thickening as it is in the species of *Isohypsibius* (Fig. 16 and Figs. 17, 18).

Many authors (Guil & Giribet, 2012; Bertolani et al., 2014; Guil et al., 2018) did not examine that material and therefore did not notice the mistake regarding the genus and continued to ascribe that species to the genus *Isohypsibius.* Also Gąsiorek et al. (2019c) did not notice the mistake but, based on the presence of cuticular pointed gibbosities covered with reticulum, for that species, and for *Isohypsibius sattleri* (Richters, 1902), instituted the new genus *Dianea* that, if the mistake of Sands et al. (2008b) will be confirmed, should be revised. One can also suspect that the specimen whose sequence has been studied by Sands et al. (2008b) was different from those sent to me.

This case highlights the fact that an unrecognized incorrect diagnosis may be used in phylogenetic studies by subsequent researchers with consequent incorrect evaluations. Particularly serious consequences may occur if, as an example, a species of genus 'A' is erroneously ascribed to

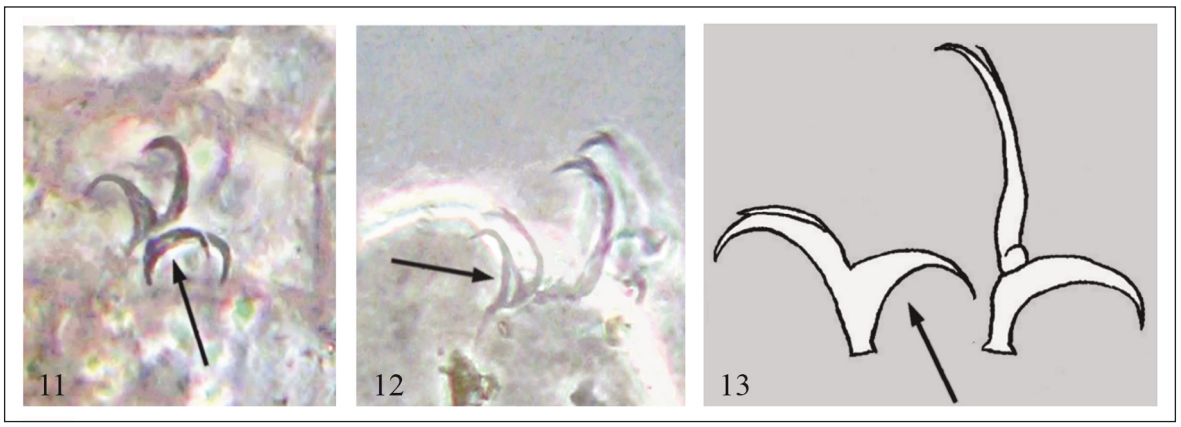
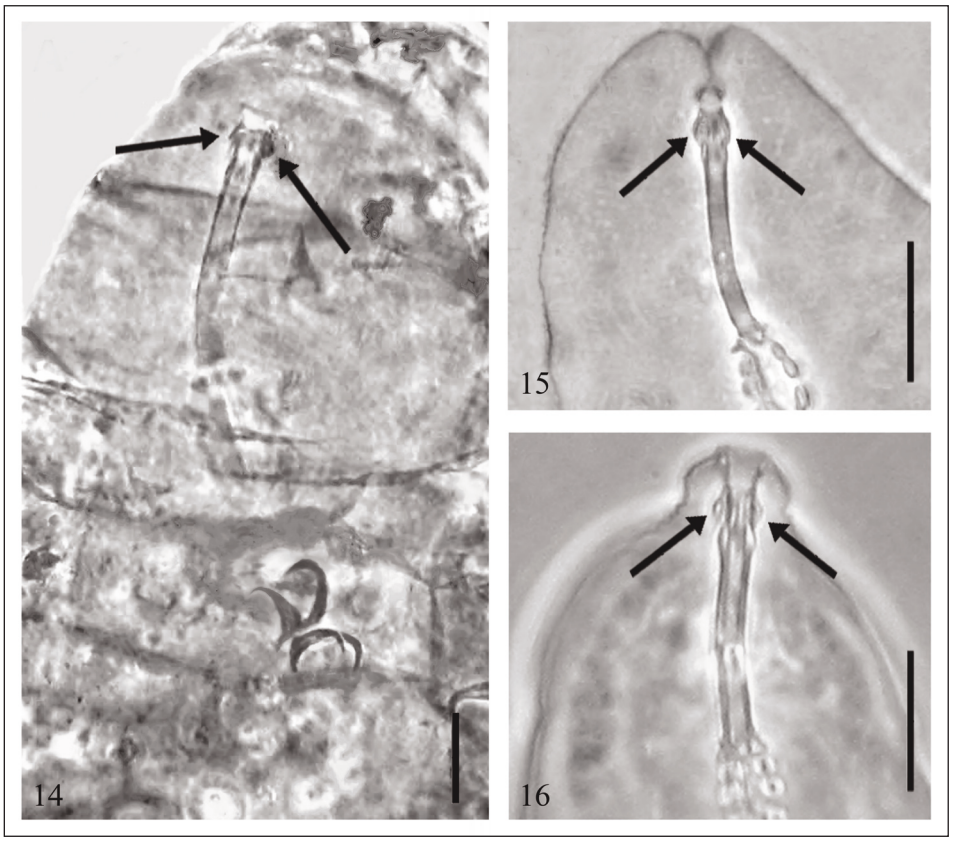
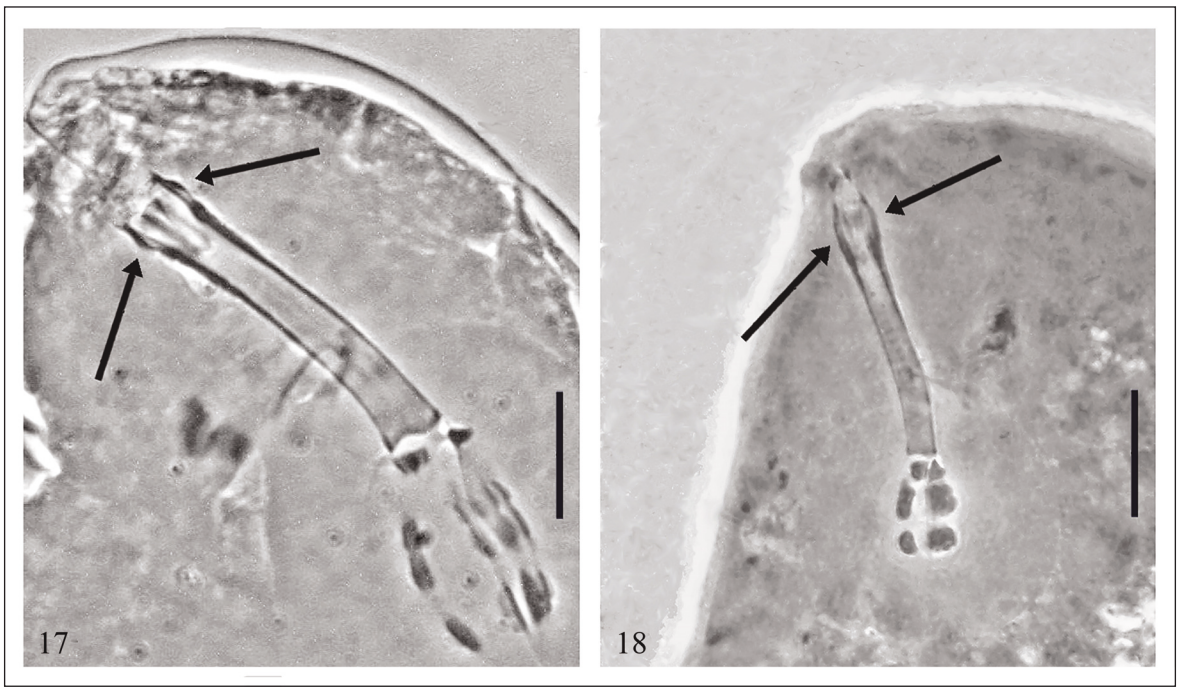


Figure 11. Claws of the second pair of legs of a specimen, attributed to *Isohypsibius papillifer* (Murray, 1905) of the population whose 18S rDNA sequence had been studied by Sands et al. (2008). Figure 12. Claws of the hind legs of the same specimen. Figure 13. Claws of *Mixibius fueginus* Pilato & Binda, 1996. The arrows indicate the angle larger than 90°, between the basal portion and the secondary branch of the internal claws.



Figures 14, 15. Bucco-pharyngeal apparatus of specimens attributed to *Isohypsibius papillifer* (Murray, 1905), of the population whose 18S rDNA sequence had been studied by Sands et al. (2008). The apparatus of Fig. 14 is of the same specimen showed in Figs. 11 and 12. Figure 15 shows the bucco-pharyngeal apparatus, in lateral view, of a second, identical, specimen studied by the same authors. Figure 16. Bucco-pharyngeal apparatus of *Mixibius parvus* Lisi, Sabella et Pilato, 2014. The arrows indicate the apophyses for the insertion of the stylet muscles. In the specimens studied by Sands et al. (2008) each apophysis is not a ridge as in the species of the genus *Isohypsibius* (Figs. 17, 18) but rather a hook as in *Mixibius* (Fig. 16), and a thickening of the buccal tube wall is present caudally to the apophyses. Scale bars = 10 μm.



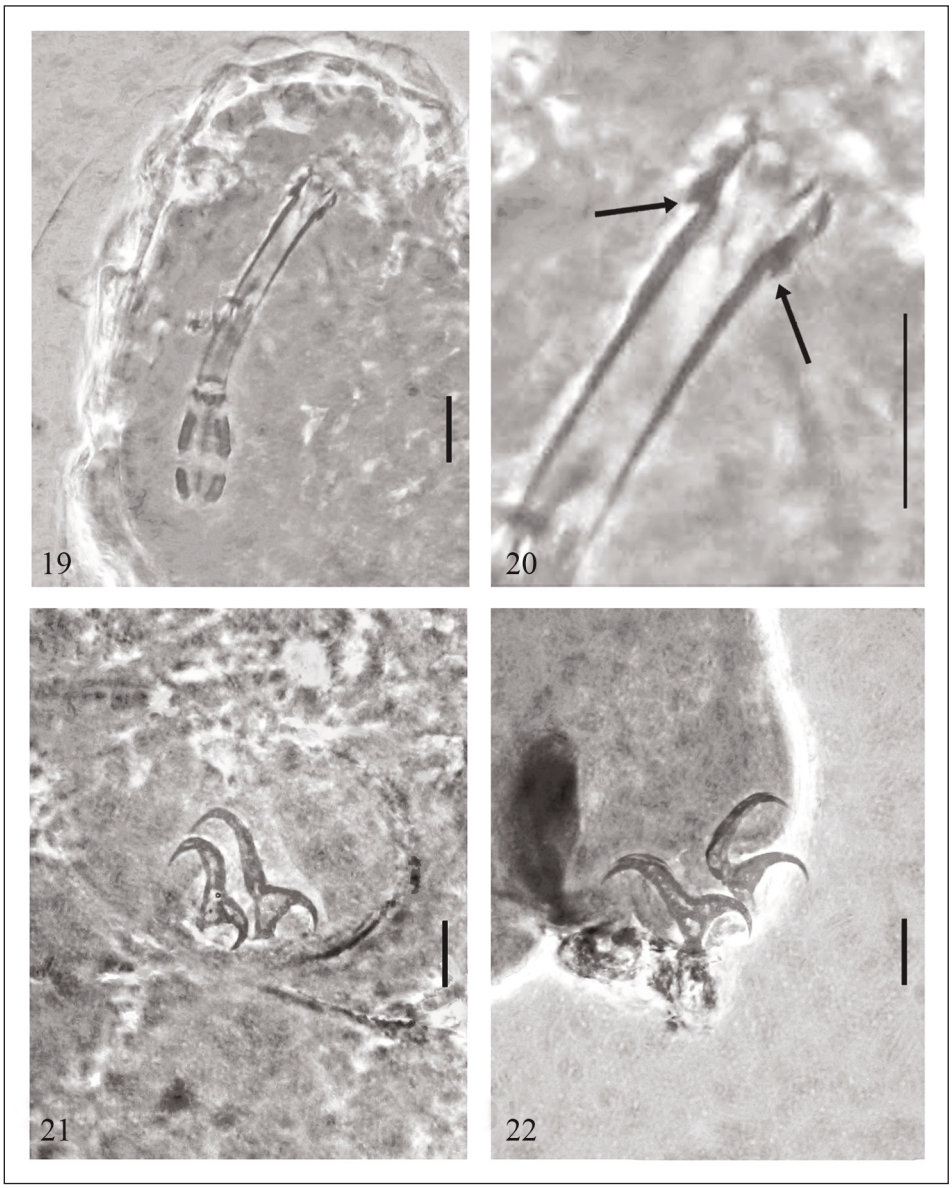
Figures 17, 18. Buccal tube of the *Isohypsibius* type. Fig. 17: *Isohypsibius verae* Pilato et Catanzaro, 1989. Fig. 18: *Isohypsibius barbarae* Pilato et Binda, 2002. Each apophysis for the insertion of the stylet muscles (arrows) is a continuous ridge. Scale bars = 10 μm.

genus 'B' lacking sequences from other species; the phylogenetic position attributed to the genus 'B' may be absolutely erroneous, with possible consequences regarding the higher taxa to which that genus is ascribed.

Another alarm has to be stressed: the tendency of several authors to subordinate the morphological data to the molecular data without a careful, critical evaluation. As an example, Gąsiorek et al. (2019c) instituted the new genera *Dianea* and *Ursulinius* stressing as a morphological difference the different shape of the cuticular gibbosities. *Ursulinius* has: "*large, mammillose and round gibbosities*", whereas in *Dianea* the gibbosity is "*less regular and clearly narrows toward the apex*". However, gibbosities similar to those of *Ursulinius* are present also in some species of *Doryphoribius* Pilato 1969b, and gibbosities similar to those of *Dianea* are present in the specimen attributed by Sands et al. (2008b) to *Isohypsibius papillifer* that, as mentioned above, does not belong to that species and probably not even to that genus. Gasiorek et al. (2019c) stated (page 19) that according to Binda & Pilato (1971), Kristensen & Hallas (1980) and Pilato (1982a) the use of dorsal gibbosities as a generic trait was a subject of criticism, but they solved the problem with a hypothesis by writing: "*However, recent data show that in a single sample, numerous, potentially closely related or pseudocryptic species can be found* (e.g. see Faurby et al., 2011; Morek et al., 2019)". The possibility to find cryptospecies cannot be denied, but it does not seem correct to institute two new genera without a precise discussion of the characters taken into consideration, but only on the basis of a hypothesis. It seems also strange that Gąsiorek et al. (2019c) consider important at the generic level the shape of the cuticular gibbosities and not also the number of rows of gibbosities and the presence or absence of a median line of gibbosities. In addition, according to the suggestion of Gąsiorek et al. (2019c), it should be necessary to transfer to the genus *Dianea* also *Isohypsibius papillifer*, but, as above mentioned, first it should be ascertained whether Sands et al. (2008b) examined the molecular sequences of a true *Isohypsibius* instead of a *Mixibius* (or another Isohypsibioidea).

There is an additional problem concerning the affinity between *Acutuncus* Pilato et Binda, 1997

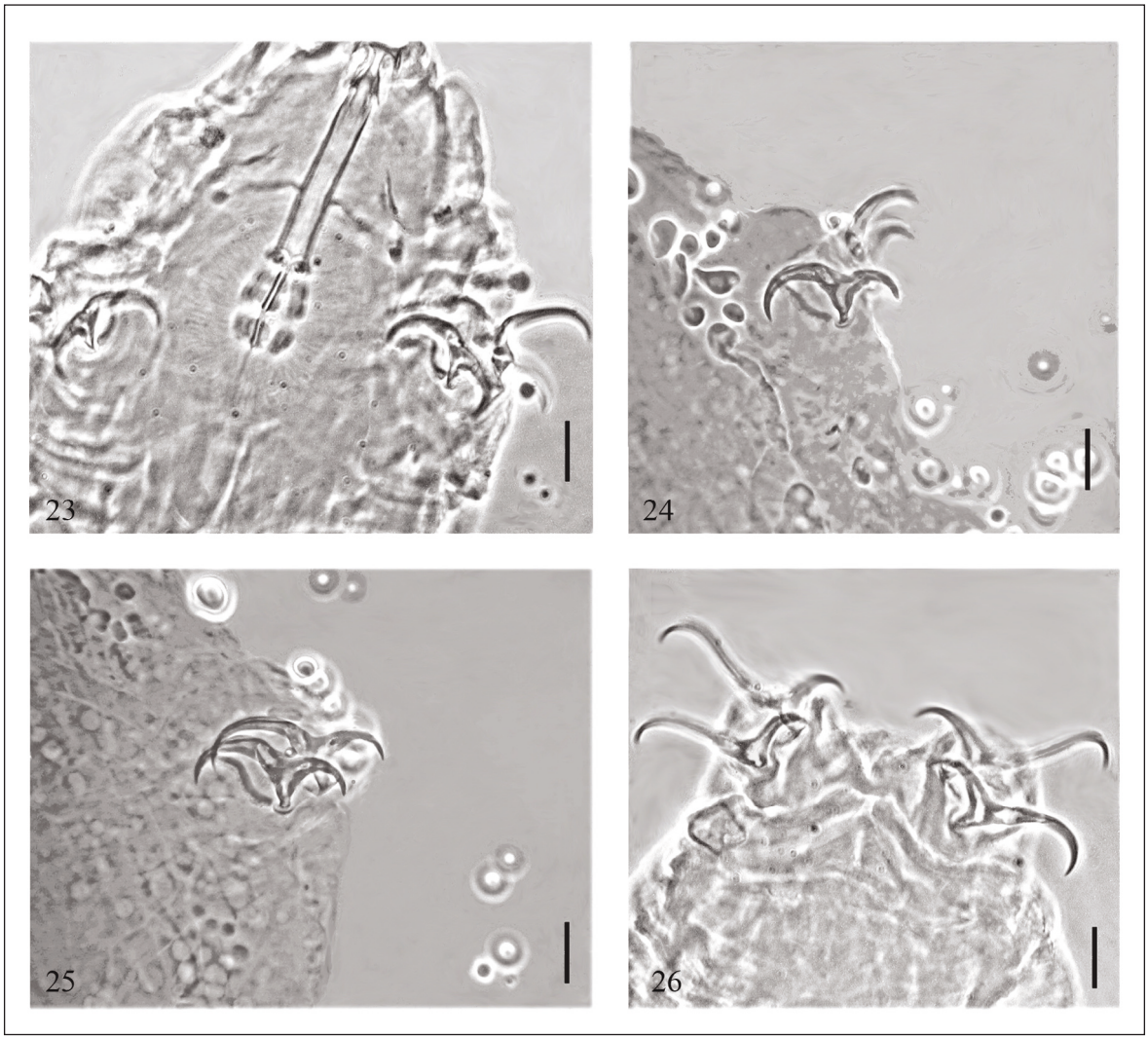
and *Mixibius*, that cannot be discussed at length here. This problem is not solved because a mistake in the literature may be noticed about the genus *Acutuncus.* The possibility of mistakes about this genus is suggested by the paper of Cesari et al. (2016a) where the authors studied populations of supposed *Acutuncus antarcticus* Richters, 1904 from seven localities of Victoria Land. In Figure 3 of page 640 they furnished 6 photos of claws all attributed to *Acutuncus antarcticus*; but only the claws of the photos 3A and 3D should be attributed to *Acutuncus antarcticus*, while the others have to be attributed to a species of a different genus (at least the claws of Figs. 3C and 3F correspond to claws of *Mixibius*). In order to notice the difference from *Acutuncus*, the readers may carefully compare in those photos the shape of the basal portion of the secondary branch of the internal claws of the figures 3A and 3D of Cesari et al. (2016a) with those of Figs. 21 and 22 of this paper.



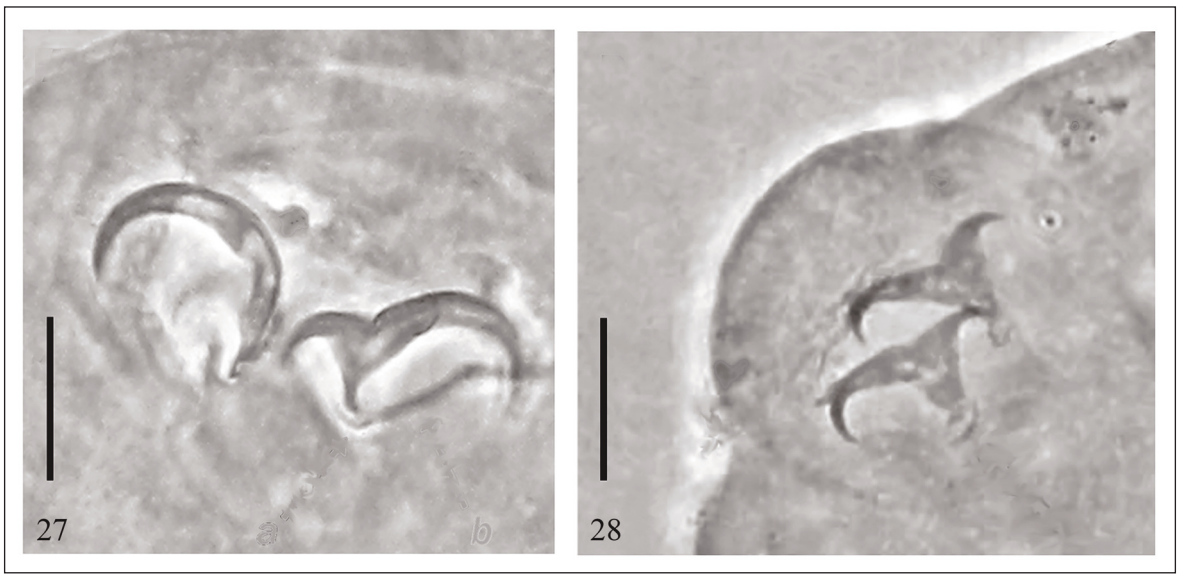
Figures 19–22. *Acutuncus antarcticus* (Richters, 1904) from Victoria Land. Fig. 19: bucco-pharyngeal apparatus. Fig. 20: apophyses for the insertion of the stylet muscles (arrows). Fig. 21: claws of the third pair of legs. Fig. 22: claws of the hind legs. Photos from Pilato et al. (2017). Scale bars = 10 μm.

Another mistake can be found in the paper of Gąsiorek et al. (2019c). They wrote on page 21: "*The* I. dastychi *group exhibit claws with branches forking at a very wide, approaching a 180° angle, present also in* Eremobiotus *(fig. 8E). Interestingly, the topology of the tree indicates the affinity of these two groups as* I. dastychi *and* Eremobiotus *sp. nov. are in a single polytomous clade (that includes also* Ursulinius *gen. nov.)*". However, the claws of Fig. 8E in Gąsiorek et al. (2019c) are not of *Isohypsibius dastychi* Pilato, Bertolani et Binda, 1982, as demonstrated by Figs. 23–26 of the present paper where claws of the holotype and of a paratype of *Isohypsibius dastychi* are shown.

It is evident (Figs. 27, 28) that in the species of *Eremobiotus* Biserov, 1992 the main branches of the internal claws, particularly those of the hind legs, are rigidly joined and that the shape of the claws does not appear very different as a consequence of their position; in addition it is true that the branches of those claws fork at very wide angle. On the contrary, in *Isohypsibius dastychi* the junction of the main branch is flexible and this allows both claws to assume different positions and, apparently, different shapes; only in some positions



Figures 23–26. *Isohypsibius dastychi* Pilato, Bertolani et Binda, 1982. Fig. 23: claws of the first pair of legs of the holotype. Fig. 24: claws of the first pair of legs of a paratype. Fig. 25: claws of the third pair of legs of the same paratype of Fig. 24. Fig. 26: claws of the hind legs of the holotype. Scale bars = 10 μm



Figures 27, 28. *Eremobiotus ginevrae* Lisi, Binda et Pilato, 2016. Fig. 27: claws of the third pair of legs. Fig. 28: claws of the hind legs. Scale bars = 10 μm.

the branches fork at an angle wider than 90°, while in other positions it is not so. In Figs. 23, 24 the claws of the first pair of legs of the holotype and a paratype of *Isohypsibius dastychi* are shown, and it is evident that they seem of different shape as a consequence of the different positions. In Fig. 26 the claws of the hind legs of the holotype of *Isohypsibius dastychi* are shown, and it is evident that the branches do not fork at a very wide angle, and they are very different from the claws of *Eremobiotus*.

Yet another problem regards the genus *Fractonotus* Pilato, 1998. This genus included only one species, *Fractonotus caelatus* (Marcus, 1928), having claws whose main branch is continuous with the thin basal portion, and with the secondary branch rigidly joined to the main branch. This claw structure is different from that of the families Isohypsibiidae and Hypsibiidae. Based on this, the genus *Fractonotus*, together with the genus *Microhypsibius* Thulin, 1928, had been ascribed to the family Microhypsibiidae Pilato, 1998 that I considered related to the Hypsibiidae. In agreement with this opinion, Marley et al. (2011) and Bertolani et al. (2014), ascribed the family Microhypsibiidae to the superfamily Hypsibioidea.

However, recently Gąsiorek et al. (2019a) studied *Fractonots caelatus, Isohypsibius gilvus* Biserov, 1986 and specimens attributed to *Calohypsibius verrucosus* (Richters, 1900), and (Page 72) they ascribed these three species to the genus *Fractonotus* transferring this genus into the family Isohypsibiidae. However, only the latter species was analyzed also for 18S, 28S and ITS2 genes, and of this species they studied neither the type material, nor specimen collected in the locus typicus.

In their new definition of the genus *Fractonotus* (Page 76) Gąsiorek et al. (2019a) wrote: "*Claws of the modified Isohypsibius type, with triangular bases and strongly curved branches (Figs. 12A, 12B)*". As a difference between the claws of *Fractonotus* and *Calohypsibius* and *Isohypsibius,* Gąsiorek et al. (2019a) referred only to the claw shape but not also to the claw structure. It is unacceptable not to distinguish the shape from the structure of the claws, to change the definition of the genus *Fractonotus*, and to transfer this genus from a superfamily to another one completely overlooking the structure of the claws that is exactly the main character for which the genus, and a new family, where erected. Anyhow, it must be stressed here that, in addition to what is specified above about the structure, also the shape of the claws of Figs. 11A–D and 12A–B in the paper of Gąsiorek et al. (2019a), and also Figs. 7G and 9A of the paper of Gąsiorek et al. (2019c), do not appear to be of a species of the family Isohypsibiidae, since the secondary branch of the internal claws forms an angle much wider than 90° with the basal portion.

As above mentioned, Gąsiorek et al. (2019a) analyzed from the molecular point of view specimens that they considered belonging to *Calohypsibius verrucosus,* and the data obtaianed on the 18S, 28S and ITS2 genes correctly induced to attribute that species to the Isohypsibioidea. As a consequence of their opinion about the claws, and of those molecular data, Gasiorek et al. (2019a), as above mentioned, attributed the three studied species to the genus *Fractonotus* but they also wrote (page 76): "*given the differences on claw morphology, there is a possibility that* F. verrucosus *n. comb. and* F. gilvus *n. comb. belong to a neo isohypsibioid genus, and are only delusively similar to* Fractonotus*".* I think probable that Gasiorek et al. (2019a) studied the molecular data of a species of Isohypsibiidae and arbitrarily attributed the same molecular data to *Fractonotus*, but *Fractonotus caelatus* remains today the unique known species of this genus, and such genus has to be attributed to the family Microhypsibiidae (Hypsibioidea). It seems evident that the problem has to be studied again.

C. Underestimate of the morphological data in the descriptions of new species and of apomorphies in phylogenetic evaluations

An example of incorrect evaluation of morphological characters that appears due to the subordination of the morphology to nucleotide sequences can be noted. Gąsiorek et al. (2019c) instituted, in the framework of Isohypsibioidea, the new family Doryphoribiidae and ascribed to it the genera *Grevenius* Gąsiorek, Stec, Morek et Michalczyk, 2019c*; Thulinius* Bertolani, 2003; *Pseudobiotus* Nelson, 1980; *Doryphoribius* Pilato, 1969 and *Apodibius* Dastych, 1983. According to Gąsiorek et al. (2019c) the definition of the family Isohypsibiidae (Page 37) is: "*Terrestrial eutardigrades with six peribuccal lobes or with a continuous peribuccal ring, and peribuccal lamina. Lacking peribuccal lamellae and ventral lamina on the buccal tube. AISM ridgelike and asymmetrical with respect to the frontal plane (only* Fractonotus*) or symmetrical (remaining five genera). Stylet furcae of the* Hypsibius *type. Claws with secondary branches clearly shorter than primary branches (br_≤ 0.70)*". On page 39 Gąsiorek et al. (2019c) gave the definition of the new family Doryphoribiidae where they wrote: "*Freshwater (limnic) or terrestrial eutardigrades with six peribuccal lobes, or with continuous peribuccal ring. Mouth opening surrounded by peribuccal lamellae, often partially or almost completely fused (*Paradiphascon*,* Pseudobiotus*,* Thulinius*) or by a peribuccal lamina (*Apodibius*;* Doryphoribius*;* Grevenius *gen. nov.). Ventral lamina on the buccal tube present (*Apodibius*;* Doryphoribius*) or absent (*Grevenius *gen. nov.,* Paradiphascon*;* Pseudobiotus*;* Thulinius*). AISM ridge-like, well-developed and asymmetrical in genera with no ventral lamina or greatly reduced and asymmetrical in genera exhibiting the ventral lamina. Flexible pharyngeal tube present (*Paradiphascon*) or absent (all remaining genera). Two claw types: the dominant type, with secondary branches being similar on height to the primary branches (all genera with the exception of some* Doryphoribius *spp.); and the second, with secondary branches being clearly shorter than the primary branches (only in some* Doryphoribius *spp.)*".

An immediate question arises: what is a clear, constant, morphological difference to distinguish those two families? To Doryphoribiidae are ascribed both terrestrial and fresh-water species while to the Isohypsibiidae are ascribed only terrestrial species, but on Page 52 Gąsiorek et al. (2019c) ascribed to the genus *Isohypsibius* also the species *Isohypsibius marcellinoi* Binda et Pilato, 1971 and *Isohypsibius reticulatus* Pilato, 1973 i.e. two species found in freshwater (Pilato 1973, 1974). On the same page 52 are ascribed to the genus *Ursulinius* also the species *Isohypsibius elegans* Binda et Pilato, 1971 and *Isohypsibius lunulatus* (Iharos 1966), the former found by Pilato (1973), and the latter found by Pilato & Catanzaro (1989) in Sicilian rivers. Therefore, either Gąsiorek et al. (2019c) should change the definition of the family Isohypsibiidae, or they need to ascribe to a different family the four above mentioned species. Peribuccal lamellae offer an apparent clear difference, absent in the family Isohypsibiidae and present in some Doryphoribiidae where they may be partially or almost completely fused. However, peribuccal lamina and peribuccal lamellae seem to be homologous structures. Fused peribuccal lamellae can form a peribuccal lamina, or, on the contrary, incisions of the peribuccal lamina can create peribuccal lamellae. From this possibility it seems consequential to affirm that to have a peribuccal continuous lamina or a peribuccal lamina with incisions forming

peribuccal lamellae is not a difference for which it is correct to distinguish two families. If a terrestrial species is found having continuous peribuccal lamina and a buccal tube provided with ventral lamina will it be ascribed to Isohypsibiidae or to Doryphoribiidae? And if an aquatic species will be found having peribuccal lamellae and a buccal tube without ventral lamina will it be ascribed to Isohypsibiidae or to Doryphoribiidae?

The unique difference between the families just discussed is based on very limited molecular data regarding the 18S rRNa and the 28S rRNA sequences (with some data drawn from GenBank), so this is another clear example of morphology subordinated to the nucleotide data with consequent confusion about taxonomy and phylogeny.

Another, less important problem can be indicated: Gąsiorek et al. (2019c) transferred into the new genus *Grevenius* the species *Isohypsibius tubereticulatus* Pilato et Catanzaro, 1989; *Isohypsibius verae* Pilato et Catanzaro, 1989 and *Isohypsibius kristenseni* Pilato, Catanzaro et Binda, 1989. In the legend of Fig. 1 (Page 8) of their paper, where they present the schemes of the possible oral armature in Isohypsibioidea, at point A those authors wrote: "*a continuous peribuccal lamina, two bands of teeth (*Apodibius*,* Grevenius *gen nov.,* Halobiotus*,* Hexapodibius*)*". From this, it appears that all the species of *Grevenius* have in the mouth two bands of teeth, but in the above indicated three species of which I am coauthor, and attributed by Gąsiorek et al. (2019c) to the genus *Grevenius*, I have not been able to see an anterior band of teeth, and it is pointed out, dubiously, the presence of a posterior band of teeth only in *Isohypsibius verae*. Gąsiorek et al. (2019c) did not specify whether they examined the three above mentioned species; if they did, perhaps my specimens are not good because their teeth are not visible, but if those authors did not examine the three species, it is necessary to better examine them before making any generalizations.

In other cases it seems sometimes COI and other sequences less conservative than 18S and 28S sequences, are given a value higher than they should deserve, and new species are described only based on these molecular data.

As an example, Schill et al. (2010) instituted, exclusively on the basis of molecular differences (ITS2), three new species of *Paramacrobiotus* Guidetti, Schill, Bertolani, Dandekar et Wolf, 2009 morphologically undistinguishable from one another and from *Paramacrobiotus richtersi* (Murray, 1911). The aim of the present paper is not to discuss the choice of Schill et al. (2010) of instituting those new species, even though some perplexities are justified, as also expressed by Bertolani et al. (2014) and Guidetti et al. (2019), but it is very important to stress that Schill et al.'s research clearly showed that differentiation regarding at least the ITS2 sequences (but not necessarily resulting in complete speciation) may start before morphological differentiation. On the other hand, it is well known that the Eutardigrada are conservative also as regards to morphological characters (Pilato 1975, 1979), and therefore it is possible that within a phyletic lineage, a descending lineage acquired a difference regarding some conservative nucleotide sequences, without at first also developing morphological differences from the sisterlines. But, on the contrary, in my opinion it seems also possible that, at least in some cases, one of the diverging lineages from a common ancestor acquired clear differences regarding the nucleotidae sequences more slowly than the morphological differences. If this is possible, it is easy to explain the case of the Hexapodibiidae that are similar to the Isohypsibiidae as regards the nucleotide 18S and 28S sequences but already very different as regards to claw structure.

The above mentioned possibility is hypothesizable in other cases. For example the phylogenetic analysis based on 18S+COI sequences (Stec et al. 2021) confirmed by data in Bertolani et al. (2022), shows one highly supported monophyletic lineage grouping all the species of the *Macrobiotus hufelandi* group, including *Macrobiotus pallarii* Maucci, 1954 and related species that produce eggs completely different from these of that group. According to Massa et al. (2021) and Bertolani *et al*. (2022), the *Macrobiotus hufelandi* group also includes the genus *Xerobiotus* Bertolani et Biserov, 1996 that, due to the synapomorphy of its greatly modified claws, should be considered valid. Stec et al. (2022) confirmed that the species of *Xerobiotus* belong to the *Macrobiotus hufelandi* group, but subordinating the claw morphology to the molecular data, proposed the abolition of that genus, disagreeing with the opinion of Massa et al. (2021), and Bertolani et al. (2022). I wish to stress here that morphological differences, if marked and regarding structures

surely significant for survival and evolution, cannot be ignored and subordinated to molecular data.

CONCLUSIONS

All the statements and evaluations expressed above do not mean that molecular data should not have a major role in phylogenetic studies. On the contrary, it is evident that they are absolutely useful because sometimes they reveal mistakes relative to the interpretation of morphological data, sometimes they reveal previously missed morphological details, and therefore they can add to our understanding of phylogenetic relationships. In one of the cases discussed here, as an example, the molecular data provided clarification of the systematic position of the group of genera previously ascribed to the family Calohypsibiidae and now distributed in two families (Calohypsibiidae and Hexapodibiidae).

In studies about phylogeny, the best situation is when molecular and morphological data are consistent. But, when there is no concordance, in order to propose a taxonomy and a hypothesis of phylogeny, it is often necessary to consider prioritising the former or the latter data, and in this case considerable experience is needed to evaluate the relevance of morphological characters at the considered taxonomic level and to evaluate what is the most reasonable hypothesis.

Bertolani et al. (2014) correctly wrote on pages 117-118: "*The erection of new taxa (at any level) only on the basis of molecular data should be avoided, in agreement with a recommendation of the International Commission of Zoological Nomenclature (1999). Without morphological support, the risk of a mistake is high and the information for identifying the taxa is substantially lacking, especially when micrometazoans, such as tardigrades, are considered*". That is perfect, and Guidetti et al. (2019) and Bertolani et al. (2022) agree, but the concept is valid not only when new taxa are erected but also when genera are transferred from one family to another, or when a family is transferred from one superfamily to another.

As regards the institution of new species, the Commission of Zoological Nomenclature is clear, but unfortunately, according to some authors, it will soon be time to no longer follow its indications. For example, Morek et al. (2019) wrote (page 16) "*It may become inevitable that in the near future new tardigrade species, in which phenotypic characters are insufficient for delimitation, will be differentiated mostly or solely by genetic traits*". It is desirable, in agreement with Bertolani et al. (2014) and with Guidetti et al. (2019), that the future will be different.

It is becoming more and more common, to use nucleotide sequences to describe new species or genera, to change the taxonomy at genus or family level, and Guil et al. (2018) used them also to elevate orders to class level. Recently Morek & Michalczyk (2020) revealed within the genus *Milnesium* Doyère,1840 "*no congruence between genetic markers and morphological traits*", and on Page 690 they wrote: "*Genetic data alone, although may provide valuable insights into the phylogeny, have limited power in explaining evolution and are taxonomically useless if they are not tightly associated with phenotypic data*". But those authors also wrote, (same paper, Page 684) that "*to estimate the number of species utilized in this study, we used a molecular species delimitation method*". This second sentence seems to exclude the necessity of a deeper study of the morphology of the species of *Milnesium*, but this appears as a questionable conclusion, and, overall, it must be remarked that even if this may be valid for *Milnesium,* it is necessary to be prudent as regards the other taxa.

Similar problems may concern other taxa very different from Tardigrada, and it is important to note here the perplexities and recommendations expressed by various authors such as Will et al. (2004), Rubinoff (2006), DeSalle (2006), Löbl (2014), Páll-Gergely (2017). In particular, just as an example, DeSalle (2006), in distinguishing between "species delimitation" and "species identification", wrote (Page 1545): "*In a taxonomic context DNA sequence information in the absence of other corroborating evidence can never be used by itself as an indicator of species delimitation*".

Experience suggests that it seems possible to imagine a sort of independent evolution of morphological characters and some nucleotide sequences that are little, or not at all, bound to morphology. This hypothesis may explain the fact that in many cases we find a concordance between molecular and morphological data but in some others this concordance is not recognizable. On the

other hand, as above mentioned, it cannot be excluded that in some cases there may be a discrepancy due to mistakes of the researchers who, uninformed of the mistake, or on the basis of insufficient data, propose incorrect taxonomic and phylogenetic evaluations.

When there is concordance between the morphological and molecular data, it is possible to draw a very probable correct phylogenetic hypothesis, but when a discordance results, great caution is needed in drawing conclusions.

In conclusion, the importance of molecular data is evident for taxonomy and phylogeny and some proponents of molecular tools frequently note that errors can occur when they are not included in analyses. But my point is that errors (diagnostic mistakes, insufficient data, voucher specimens neglected) can also be introduced with molecular studies. These wrong or limited data, if not pointed out and corrected, may be considered correct by the other researchers and may lead to underestimating the indications deriving from morphology (sometimes also misinterpreting their indications) often to overcome contradictions between the molecular and morphological data. If regardless of these possibilities one draws phylogenetic conclusions, morphological evaluations become subordinated to the study of some (and often very few) nucleotide sequences, not always verified. It must also be stressed that the morphological characters too are determined by nucleotide sequences (in my opinion usually overlooked in the molecular studies). A clear sign of this tendency may be the fact that in the literature it is already possible to read of "COI species", "molecular species", "genetic species", "molecular phylogeny", and "morphological phylogeny" whereas there is only one phylogeny, which should be reconstructed on the basis of all the data from all sources we possess, and of the evaluations and hypotheses that appear more reasonable on the basis of the available knowledge of that moment. However, hypotheses must not be trusted solely from the probabilistic, cold, calculations of a computer but also from experienced eyes and the human brain.

Unfortunately, the enthusiasm for carrying out molecular research seems to lead some authors to the above indicated errors; only in this way the above mentioned opinion of Morek et al. (2019) about the possible future necessity of differentiation of the species "*solely by genetic traits*" can be explained. Similarly, Guil et al. (2013b, Page 1, lines 1-2) wrote: *"Much of what is known about the phylogenetic relationships of the neglected phylum Tardigrada comes from molecular data rather than morphology-based phylogenetic studies*" and, (Page 2): "*morphological phylogenies have only been proposed by four studies*". It is possible to provide here a list of papers, starting from Thulin (1928), both regarding the morphology and the phylogeny of tardigrades, preceding the onset of research on nucleotide sequences, but the literature is available and it is superfluous to insist on presenting it here. But it is also correct to not ignore that the molecular data often simply confirm the lineages of traditional systematics only sometimes requiring a change the rank of various taxa already well identified, and to establish new taxa for lineages that were already well recognized even if not defined as officially named (see as an example Figs. 1 and 2, the two lineages recognizable within the family Calohypsibiidae since 1969 (Pilato 1969b) recently distinguished by Bertolani et al. (2014).

By disregarding the literature many data can be put forward as new, and today, unfortunately, one can notice some signs of this trend. It seems opportune to ask morphologists to turn their attention to some recent proposals of systematics and phylogeny, and to the method adopted; in particular, caution should be adopted with molecular indications that may prevent correct evaluation of the indications of morphology. It is also opportune that all readers do not think that my alarm is only a personal opinion; as a matter of fact, other researchers gave analogous alarms regarding different taxa than Tardigrada, and also non-animal organisms, and for this reason it seems opportune to conclude this paper with some sentences of Páll-Gergely (2017) (who is not a tardigradologist): (pages 594–595) "*For instance, incorrect identifications of specimens used for molecular studies* (Nilsson et al. 2006; Groenenberg et al. 2011), *taxonomic inflation* (Isaac et al. 2004; Harris & Froufe 2005)*, and the increasing gap between phylogeny and classification* (Franz 2005) *are much more serious problems*"; and (page 595) "*On the other hand, since the world's biodiversity is largely unknown [....] and the number of this decreasing* (Bebber et al. 2014; Wheeler 2014) *[...]. Especially, given that if someone wants to describe*

something, he/she can find a way to do so in local, small, non peer-reviewed journals or self-published books"; and to conclude (Page 595): "*One-sided critiques emphasising only the taxonomic value of molecular assessment could well result in the weakening trust of taxonomists (mostly the ones not dependent on impact factor) in peer-reviewed journals, which is already a major problem in today's taxonomy. Morphology is still what makes the organism a tangible entity beyond its DNA*".

ACKNOWLEDGMENTS

I thank the colleagues Sandra McInnes (British Antarctic Survey, Cambridge) who kindly sent me three specimens of *Isohypsibius papillifer* (with the permission to publish photos of them); the geneticist colleague Salvatore Saccone and the colleagues Oscar Lisi, Venera Ferrito and Anna Maria Pappalardo (University of Catania, Italy) with whom all I discussed genetic and phylogenetic issues. I also thank the anonymous reviewers who spent their time to improve this paper.

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