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Two new species of Macrobiotidae (Tardigrada: Eutardigrada) from the United States of America, and some taxonomic considerations of the genus *Murrayon*

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Abstract.—Two new species of eutardigrades, *Macrobiotus nelsonae* and *Murrayon stellatus,* collected in Tennessee, U.S.A., are described. *Macrobiotus hibernicus* and *Macrobiotus dianeae,* related to *M. stellatus,* are re-examined and ascribed to the genus *Murrayon* on the basis of the claw type and the presence of a ventral hook on the strengthening bar. A dichotomous key to the genus *Murrayon* is proposed.

Thulin (1928), Marcus (1936) and Ramazzotti (1962), in their systematic monographs on tardigrades, included all eutardigrades (except *Milnesium*) in the family Macrobiotidae and all those with so-called 'symmetric' claws, i.e., with respect the median plane of the leg, in the genus *Macrobiotus.* Pilato (1969a, 1969b) proposed to separate the eutardigrades in different families on the basis of the claw structure, ascribing to Macrobiotidae only the genera characterized by 'symmetric' claws, i.e., *Macrobiotus* and *Pseudodiphascon* (the latter erected as a subgenus by Ramazzotti in 1965). The status of this family remained almost unchanged until the 1980s, when new taxonomic characters, related to the bucco-pharyngeal apparatus and to the claws, were considered for the identification of the genera (Schuster et al. 1980, Bertolani 1981, Pilato 1981, Bertolani & Kristensen 1987). Today 11 genera are placed in the Macrobiotidae. Moreover, the number of known species has been increased remarkably due to a more in-depth analysis and the use of a greater number of characters. Within the genus *Macrobiotus,* for example, is the so-called '*hufelandi*' group studied by light microscopy and scanning electron microscopy (SEM) by Biserov (1990a, 1990b) and by Bertolani & Rebecchi (1993), who identified several new species within this group with so-called high intra-specific variability. Within the genus *Macrobiotus,* natural groups of species sharing similar morphology of the animals and/or of spermatozoa and eggs were identified, which led to the hypothesis of further possibilities of subdivision at the genus or subgenus level (Guidi & Rebecchi 1996).

Tardigrades from new material collected in Tennessee (U.S.A.) and comparisons with known related species have expanded our knowledge of the systematics of Macrobiotidae, one of the larger and more complex families of eutardigrades, leading to the erection of two new species and the redesignation of two other species.

Methods

Specimens from 44 samples of beech leaf litter (*Fagus grandifolia* Ehrb.) collected in fall 1995, winter 1995–1996, and spring 1996 on Roan Mountain (Southern Appalachians, Carter County, Tennessee, U.S.A.) at different altitudes (1200 m, 1500 m and 1650 m asl) were examined. Tardigrades and their eggs were preserved for SEM examinations or directly mounted in polyvinyl-lactophenol or in Hoyer's medium, or stained with acetic carmine and subsequent-

ly mounted in Faure-Berlese's mounting medium for the light microscopy (LM) observations. Light microscopy observations were done with phase contrast and differential interference contrast (DIC) under oil immersion (100×). The specimens prepared for SEM were fixed in absolute ethanol, critical point dried in liquid CO_2 and sputter-coated with gold-palladium. All the specimens were examined using a Philips SEM 500 at the 'Centro Interdipartimentale Grandi Strumenti' of the University of Modena.

As comparison material, several specimens were used: *Murrayon pullari* (Murray 1907) from Monte Rondinaio, Emilia Romagna, Italy (from Bertolani's collection); *Murrayon hastatus* (Murray 1907) from Angerfjrden, Sweden (from Kristensen's collection); *Macrobiotus hibernicus* Murray 1911 from Valtellina, Lombardia, Italy (from Pilato's collection), from Valico of Gran San Bernardo, Valle d'Aosta, Italy, from Val Piantonetto, Piemonte, Italy (both from Maucci's collection; the latter presented to him by C. Robotti) and from Godhavn, Greenland (from Kristensen's collection); *Macrobiotus dianeae* Kristensen 1982 from Unartog, Greenland (paratypes from Kristensen's collection), from Monte Rondinaio, Emilia Romagna, Italy (from Bertolani's collection); a paratype and an egg of *Macrobiotus aviglianae* Robotti 1970, and the holotype and an egg of *Macrobiotus pallarii* Maucci 1954 (both from Maucci's collection).

Macrobiotus nelsonae, new species (Figs. 1–5, Table 1)

Etymology.—The species is dedicated to Dr. Diane R. Nelson, professor at East Tennessee State University, whose hospitality and help allowed this work.

Paratypes.—566 paratypes and 322 eggs mounted in polyvinyl-lactophenol, Hoyer's medium and Faure-Berlese (permanent slides), 3 paratypes and 3 eggs on stubs for SEM observation.

Holotype.—Adult male 786.1 μm in length, collected in January 1996, mounted in polyvinyl-lactophenol, slide labeled as 5N05a-S10 (Fig. 1A).

Type locality.—Roan Mountain (Carter County, Tennessee) on the north-facing slope at elevations between 1200 and 1650 m asl, in leaf litter from beech trees.

Diagnosis.—Animals of large size. Smooth cuticle with pores. Eye-spots anterior. Wide buccal tube; evident buccal armature, with two consistent bands of teeth and with transverse crests. Two rod-shaped macroplacoids and microplacoid. Claws of the '*hufelandi*' type, with lunulae. Eggs laid freely, with big conical reticulated processes inserted onto an areolate surface.

Description.—Length from 196.0 up to 925.4 μm. Colorless, although the largest animals may have a pink coloration. Eyespots in anterior position. Smooth cuticle, with oval cuticular pores not uniform in size and irregularly distributed, much more visible in the anterior and posterior part of the animal. In some animals a uniform and fine punctuation on the dorsal cuticle is visible, much more identifiable in recently prepared specimens (Fig. 2). Ten evident peribuccal lamellae. Buccal armature characterized by an anterior band of small teeth (mucrones), followed by a large posterior band of larger teeth, whose dimensions increase caudally (Figs. 1C, D). Three transverse crests, the dorsal ones longer than the ventral; in some animals the median ventral crest is subdivided in two or seldom into more segments of variable length. Wide buccal tube. Rounded pharyngeal bulb (ratio length/width 1.1–1.3:1) with large apophyses, two rod-shaped macroplacoids and an evident microplacoid. First macroplacoid the longest, with an evident narrowed midportion, the second with a subterminal constriction (Fig. 1B). Massive claws, of '*hufelandi*' type (Y-shaped; Figs. 1E, F), the claws of the front legs the smallest and those of the hind legs the largest. Main branch of the outer claw slightly longer than that of the inner one, both bearing

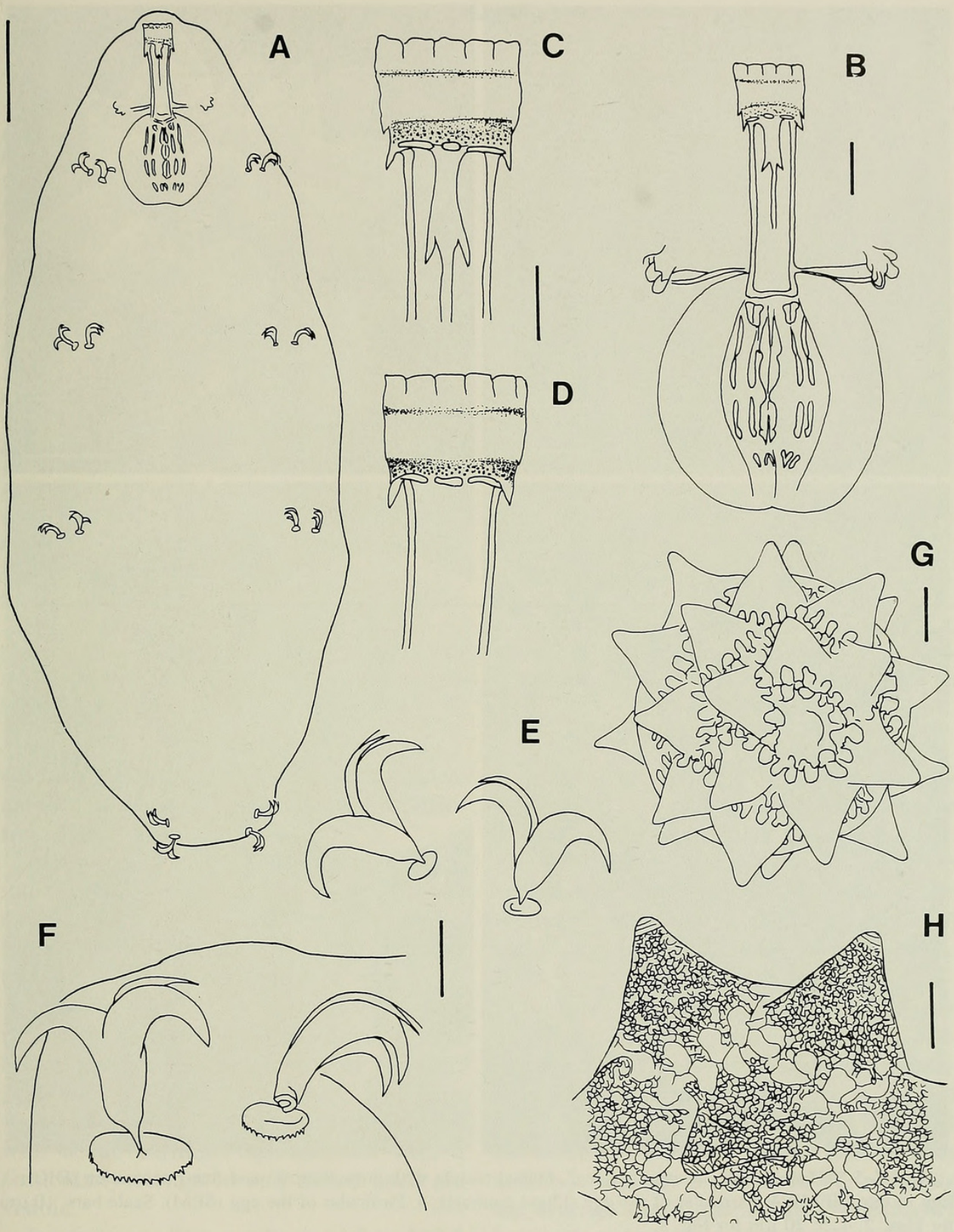
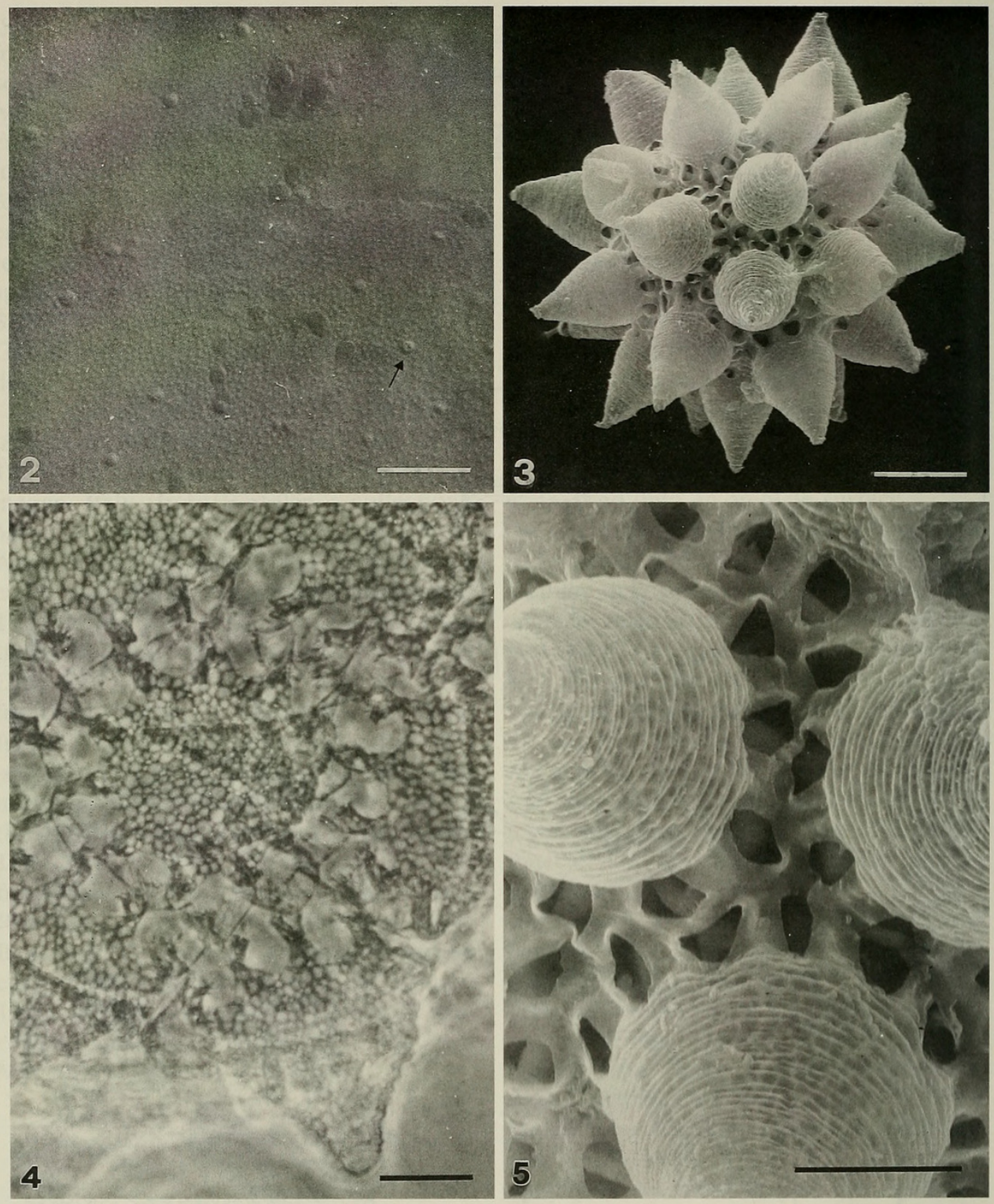


Fig. 1. Holotype of *Macrobiotus nelsonae* sp. n. A, Habitus (ventral view); B, Bucco-pharyngeal apparatus (frontal view); C, Buccal armature (ventral view); D, Buccal armature (dorsal view); E, Claws of the front legs; F, Claws of the hind legs; G, Egg. H, Particular of the egg. Scale bars, 100 μm for A; 20 μm for B, G; 10 μm for C, D, E, F, H.



Figs. 2–5. *Macrobiotus nelsonae* sp. n. 2, Dorsal cuticle with pores (arrow) and fine punctuation (DIC); 3, Egg in toto (SEM); 4, Particular of the egg (Phase contrast); 5, Particular of the egg (SEM). Scale bars, 10 μm for Figs. 2, 4, 5; 20 μm for Fig. 3.

evident accessory points. Small lunulae at the base of the claws of the first three pairs of legs. Lunulae surrounded by fine teeth in the largest animals. Lunulae of the hind claws large, even larger on the posterior claws, and with teeth of irregular dimensions in the largest animals. Three animals with modified claws on only one side of the hind legs. Two of them show a supernumerary spur (on the anterior claw in one

Table 1.—Measurements and *pt* index of the holotype* (786.1 μm in length) and nine paratypes of *M. nelsonae* sp. n.

	al	btl	btd	ssi	prl	1°pl	2°pl	ecl1°	icl1°	ecl3°	icl3°	pcl4°	acl4°
μm	embryo	42.6	5.9	34.7	16.8	9.9	5.0						
pt			13.8	81.5	39.4	23.2	13.8						
μm	230.0	33.7	5.0	27.7	14.9	7.9	5.9	7.9	6.9	8.9	7.9	9.9	8.9
pt			14.8	82.2	44.2	23.4	17.5	23.4	20.5	26.4	23.4	29.4	26.4
μm	258.7	34.0	4.0	27.0	16.8	8.9	5.9	8.9	7.9	8.9	7.9	9.9	8.9
pt			11.8	79.4	49.4	26.2	17.4	26.2	23.2	26.2	23.2	29.1	26.2
μm	437.8	46.5	6.9	37.6	26.7	14.9	10.9	10.9	9.9	11.9	9.9	12.9	11.9
pt			14.8	80.9	57.4	32.0	23.4	23.4	21.3	25.6	21.3	27.7	25.6
μm	587.1	61.4	10.9	51.5	38.6	21.8	13.9	12.9	11.9	14.9	11.9	16.8	14.9
pt			17.8	83.9	62.9	35.5	22.6	21.0	19.4	24.3	19.4	27.4	24.3
μm	626.9	57.4	10.9	48.5	36.6	21.8	12.9	12.9	11.9	15.8	12.9	17.8	16.8
pt			19.0	84.5	63.8	38.0	22.5	22.5	20.7	27.5	22.5	31.0	29.3
μm	786.1*	69.3	13.9	58.4	49.5	29.7	16.8	14.9	13.9	17.8	15.8	20.8	16.8
pt			20.1	84.3	71.4	42.9	24.2	21.5	20.1	25.7	22.8	30.0	24.2
μm	786.6	69.3	15.8	57.4	50.5	29.7	18.8	17.8	15.8	18.8	17.8	21.8	18.8
pt			22.8	82.8	69.3	42.9	27.1	25.7	22.8	27.1	25.7	31.5	27.1
μm	815.9	76.2	15.8	63.4	53.5	32.7	18.8	16.8	15.8	16.8	15.8	19.8	18.8
pt			20.7	83.2	70.2	42.9	24.7	22	20.7	22	20.7	26	24.7
μm	925.4	77.2	14.9	64.4	54.5	31.7	20.8	17.8	15.8	18.8	16.8	19.8	19.8
pt			19.3	83.4	70.6	41.1	26.9	23.1	20.5	24.6	21.8	25.6	25.6

al = animal length; btl = buccal tube length; btd = inner diameter of the buccal tube; ssi = stylet supports insertion level on the buccal tube; prl = macroplacoids row length; 1°pl = first macroplacoid length; 2°pl = second macroplacoid length; ecl = external claw length; icl = internal claw length (1° = front leg, 3° = second or third pair of legs); pcl = posterior claw length; acl = anterior claw length (4°= hind legs).

case, in the posterior one in another one); another one has a third branch on a posterior claw.

Measurements and *pt* indices (percentage ratio between the length of the considered structure and the length of the buccal tube; Pilato 1981) of the holotype and nine paratypes are reported in Table 1.

Midgut of the largest animals containing mastax of rotifers and sclerified parts of the bucco-pharyngeal apparatus of eutardigrades.

In the testis of male specimens stained with acetic carmine, spermatozoa with a particularly elongated and coiled head are visible.

Large eggs (diameter from 84.7 to 129.5 μm without processes; Figs. 1G, 3). Conical processes (height from 20.8 to 31.7 μm, diameter of the base from 20.0 to 34.7 μm) showing a reticular pattern with meshes of very irregular, variable shape and size (Figs. 1H, 4, 5). By SEM their surfaces appear annulated (Fig. 5). Top of the processes rounded and usually without reticulation but with a fine annulation, sometimes lightly notched. Each process is separated from the others by a double series of 11–12 polygonal areolations (5–6 μm in diameter) inside which no sculpture is visible (Figs. 4, 5).

Remarks.—Comparisons with the type material of *Macrobiotus pallarii* and *Macrobiotus aviglianae* confirmed the synonymy between these two species as pointed out by Pilato & Binda (1977), and indicated the close relationship between *Macrobiotus nelsonae* and *M. pallarii.* In *M. nelsonae* the average size of the animals is decidedly larger, there is a more evident microplacoid, a more complex buccal armature with a larger posterior band of teeth, a wider buccal tube, larger claws and a more posterior insertion of the stylet supports on the buccal tube. The most evident difference between *M. nelsonae* and *M. pallarii* is in the eggs.

The processes are more than twenty per hemisphere in *M. pallarii* (checked on type material) and always less than fifteen in *M. nelsonae*; they are also much longer. The areolations at the base of the processes are fewer in number in *M. pallarii* (8–9 instead of 11–12 in *M. nelsonae*) and never in a double series. In *M. nelsonae* the *pt* index seems to change with the size of the animal. Comparative studies on other species may provide further informations.

Repositories.—Holotype, 88 paratypes and 54 eggs are in R. Bertolani's collection in the Department of Animal Biology at the University of Modena; 473 paratypes and about 260 eggs in D. R. Nelson's collection in the Department of Biological Sciences of East Tennessee State University; 5 paratypes and 6 eggs are in the National Museum of Natural History, Smithsonian Institution of Washington, D.C.

Murrayon stellatus, new species
(Figs. 6–8, Table 2)

Etymology.—From *stellatus* (Latin), meaning "starry," for the star-like dots on the cuticle of the animals.

Paratypes.—6 paratypes mounted in polyvinyl-lactophenol and Hoyer's medium and 1 egg mounted in polyvinyl-lactophenol.

Holotype.—Length 234.1 μm, collected in January 1996 and mounted in Hoyer's medium, slide labelled as 5N08b-S12.

Type locality.—Roan Mountain (Carter County, Tennessee, U.S.A.), on the north-facing slope at elevations of 1500 m asl in leaf litter from beech trees.

Diagnosis.—Cuticle punctated, with evident dorsolateral bands of relatively large star-like dots. Three rod-shaped macroplacoids, microplacoid absent. Eggs laid freely, with small rod-shaped processes.

Description.—From 162.1 to 234.1 μm in length, colorless, without eye-spots. Entire cuticle has a fine punctuation; moreover, two dorsolateral bands (width about 10 μm and visible at low magnification, 5× objective) of star-like dots are also very visible (Figs. 6A, 7). They run through each side of the body and join anteriorly at the front of the animal and posteriorly just in front of the hind legs. The stars seem finer and closer together on the front of the animal and in sections above each leg, where they cover the basal portion of the dorsal surface (Fig. 7). Peribuccal lamellae evident. In these specimens the components of the buccal armature are not identifiable. Stylet supports inserted on the buccal tube at about two-thirds of its length. Shape of the strengthening bar characteristic of the genus, with a showy ventral hook. Buccal tube relatively large. Pharyngeal bulb round (ratio length/width 1.1–1.2:1), containing large apophyses and three slender rod-shaped macroplacoids (Fig. 6B). The first is the longest; the second, very close to the first, the shortest; the third shows an evident subterminal constriction. Legs long. Claws small, of '*pullari*' type (V-shaped and with a peduncle, quadrate in lateral view; Figs. 6C, D), larger on the hind legs. Main branch of the claws with evident accessory points. Small smooth lunulae at the base of each claw.

Measurements and *pt* indices of the holotype and two paratypes, compared with two paratypes of *Macrobiotus dianeae* and two specimens of *Macrobiotus hibernicus* (coll. Kristensen), are reported in Table 2.

A small broken egg was found at the type locality and attributed to this species because of its similarity with that of the related species *M. dianeae* and *M. hibernicus.* The egg has rod-shaped processes of 3–4 μm in height with a large base and a smaller head shaped like that of a nail (Fig. 6E). The processes look often curved. Some processes have a short thin spine on their heads. At some points a hyaline matrix covering the processes is visible. The processes are aligned to include wide polygonal areas, sometimes incomplete. The surfaces of these areas seem smooth.

Remarks.—*Murrayon stellatus* is similar to *Macrobiotus dianeae* and to *Macrobiotus*

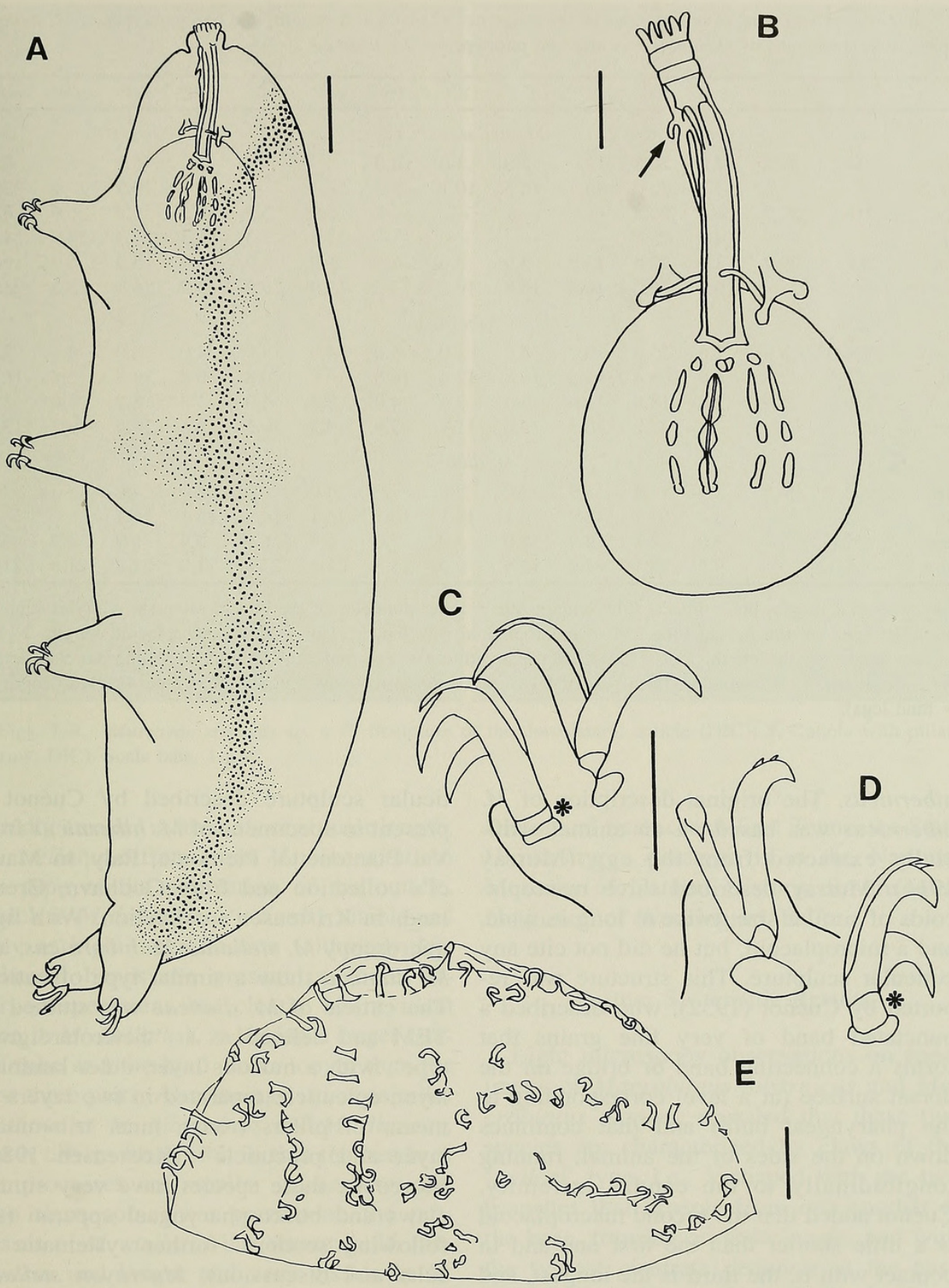


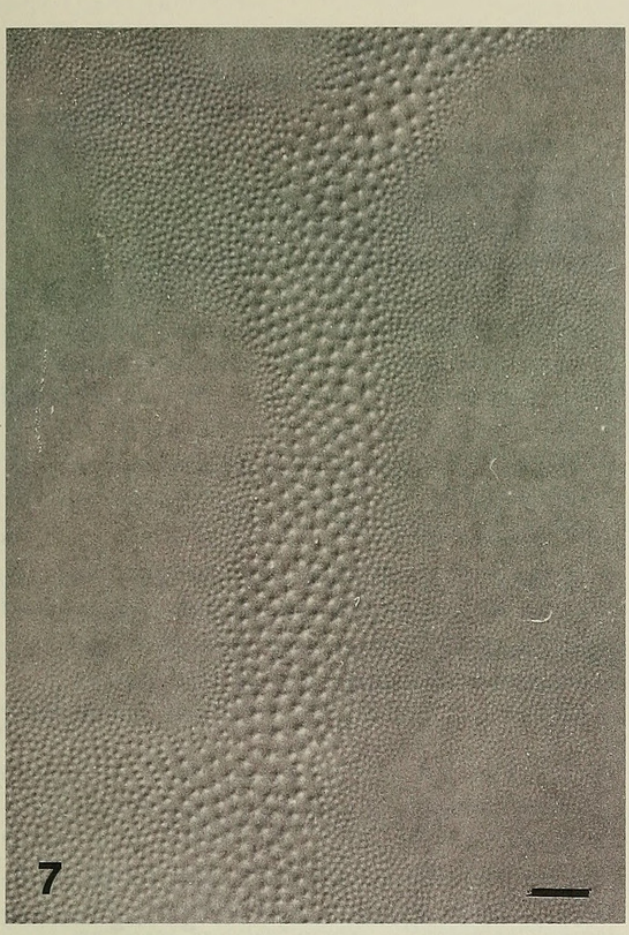
Fig. 6. Holotype of *Murrayon stellatus* sp. n. A, Habitus (lateral view); B, Bucco-pharyngeal apparatus (lateral view); C, Claws of the third pair of legs; D, Claws of the hind legs; E, Egg detail. Note in B the hook on the ventral tube (arrow) and in C and D the quadrate peduncle at the bases of the claws (asterisks). Scale bars, 20 μm for A; 10 μm for B, E; 5 μm for C, D.

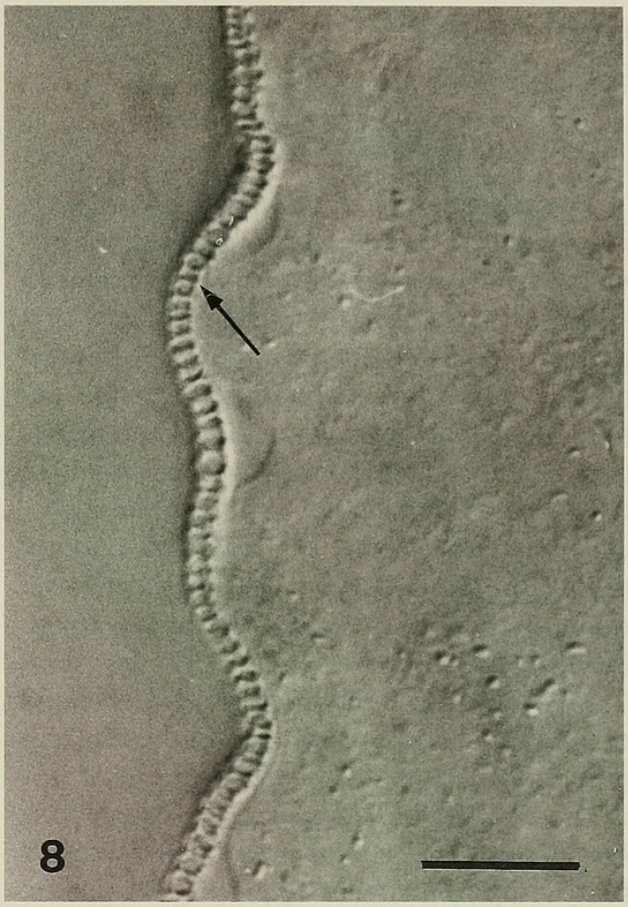
Table 2.—Measurements and *pt* index of the holotype* (234.1 μm in length) and two paratypes of *M. stellatus* sp. n., two specimens of *M. hibernicus* and two paratypes of *M. dianeae.*

	al	btl	btd	ssi	prl	1°pl	2°pl	3°pl	ecl1°	icl1°	ecl3°	icl3°	pcl4°	acl4°
						M. stellatus sp. n.								
μm	214.1	29.7	4.0	20.8	12.9	5.0	3.0	4.0	—	—	5.9	5.9	6.9	6.9
pt			13.5	70.0	43.4	16.8	10.1	13.5	—	—	19.9	19.9	23.2	23.2
μm	219.1	27.7	4.0	20.8	—	—	—	—	5.9	5.9	5.9	5.9	6.9	6.9
pt			14.4	75.1	—	—	—	—	21.3	21.3	21.3	21.3	24.9	24.9
μm	234.1*	29.7	3.0	20.8	13.9	5.0	3.0	4.0	5.9	5.9	5.9	5.9	6.9	6.9
pt			10.1	70.0	46.8	16.8	10.1	13.5	19.9	19.9	19.9	19.9	23.2	23.2
						M. hibernicus								
μm	204.0	23.8	2.0	15.8	10.9	4.0	3.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
pt			8.4	66.4	45.8	16.8	12.6	16.8	16.8	16.8	16.8	16.8	16.8	16.8
μm	239.0	25.7	2.0	18.8	11.9	4.0	3.0	4.0	5.0	5.0	5.0	5.0	5.0	5.0
pt			7.8	73.2	46.3	15.6	11.7	15.6	19.5	19.5	19.5	19.5	19.5	19.5
						M. dianeae								
μm	204.0	24.8	2.0	16.8	11.9	4.0	3.0	4.0	4.0	4.0	4.0	4.0	—	—
pt			8.1	67.7	48.0	16.1	12.1	16.1	16.1	16.1	16.1	16.1	—	—
μm	259.0	31.7	3.0	23.8	14.9	5.0	3.0	5.0	6.9	6.9	6.9	6.9	6.9	6.9
pt			9.5	75.1	47.0	15.8	9.5	15.8	21.8	21.8	21.8	21.8	21.8	21.8

al = animal length; btl = buccal tube length; btd = inner diameter of the buccal tube; ssi = stylet supports insertion level on the buccal tube; prl = macroplacoid row length; 1°pl = first macroplacoid length; 2°pl = second macroplacoid length; 3°pl = third macroplacoid; ecl = external claw length; icl = internal claw length (1° = front leg, 3° = second or third pair of legs); pcl = posterior claw length; acl = anterior claw length (4° = hind legs).

hibernicus. The original description of *M. hibernicus* was based on an animal artificially extracted from the egg (Murray 1911). Murray described three macroplacoids of similar size, twice as long as wide, and a microplacoid, but he did not cite any cuticular sculpture. This structure was reported by Cuénot (1932), who described a punctated band of very fine grains that forms a connecting band or bridge on the dorsal surface (at a level corresponding to the pharyngeal bulb) and that continues down on the sides of the animal, running longitudinally to the caudal extremity. Cuénot added that the second macroplacoid is a little shorter than the first one and in contact with it, the third is the longest, and the microplacoid is present, but can also be absent. The attribution of the Cuénot's material to *M. hibernicus* is not certain, but very probable because of the considerable similarity of the eggs with that described by Murray (1911). The same aspect of the cuticular sculpture described by Cuénot is present in specimens of *M. hibernicus* from Val Piantonetto, Piemonte, Italy, in Maucci's collection and from Godhavn, Greenland, in Kristensen's collection. With light microscopy *M. stellatus, M. hibernicus,* and *M. dianeae* show a similar type of cuticle. The cuticle of *M. dianeae* was studied by TEM and defined as a "'heterotardigrade type', with a mucous layer, outer laminary layer, epicuticle separated in two layers by means of pillars (rods), inner trilaminary layer and procuticle" (Kristensen 1982). Moreover, these species have very similar claws and bucco-pharyngeal apparati (see following sections: further systematic results and discussion). *Murrayon stellatus* differs from *M. dianeae* in having a more evident cuticular sculpture, very visible even with low magnification, in having a clearly rounded bulb and in having the first macroplacoid longer than the other two. Moreover the claws of *M. dianeae* are de-





Figs. 7–8. *Murrayon stellatus* sp. n. 7, Sculpture of the dorsolateral cuticle (DIC); 8, Cuticle with pillars (arrow; DIC). Scale bars, 1 μm.

cidedly smaller in animals of similar length. *M. stellatus* differs from *M. hibernicus* in the type of the punctation of the cuticle, which in *M. hibernicus* is hardly visible and only at high magnification (although dorsolateral bands are present in some observed specimens but clearly less evident than in *M. stellatus*), in having a larger buccal tube and the hind claws longer than the first three pairs. Also the egg is very similar to those of *M. hibernicus* and *M. dianeae,* but it differs from both in that the heads of the processes are smaller in diameter and also short spines are present on the processes of *M. stellatus* but absent in *M. hibernicus* and longer and very visible in *M. dianeae.*

Repositories.—Holotype, 4 paratypes and an egg are in R. Bertolani's collection in the Department of Animal Biology at the University of Modena, 2 paratypes are in D. R. Nelson's collection in the Department of Biological Sciences at East Tennessee State University; a paratype is in the National Museum of Natural History, Smithsonian Institution of Washington, D.C.

Further Systematic Results

Light microscopy observations on specimens of *Macrobiotus hibernicus* and *Macrobiotus dianeae* revealed that these two species are characterized by claws of the '*pullari*' type, i.e., V-shaped with the two branches that diverge from one another at the base, forming an acute angle, and with the typical quadrate peduncle at the base (Bertolani & Pilato 1988). Both also have an evident hook on the ventral margin of the strengthening bar of the buccal tube.

A personal communication from R. M. Kristensen on the presence of pillars in the cuticle of *Murrayon hastatus* suggests the

need for further investigations on the cuticle of the genus.

Discussion

The morphological observations on *Murrayon pullari, Murrayon hastatus, Macrobiotus hibernicus* and *Macrobiotus dianeae* allow reanalysis of the systematic position of some of these species and the opportunity to define the characteristics of the genus *Murrayon.* Based on the observations of abundant material of *M. dianeae* (where claws were very visible in profile), this species had claws of the '*pullari*' type, and not of the '*hufelandi*' type with a short basal tract, as reported by Bertolani & Pilato (1988).

The presence of '*pullari*' type claws and of a ventral hook on the strengthening bar are evidence that *M. hibernicus* and *M. dianeae* should be transferred to the genus *Murrayon.* The genus *Murrayon* is therefore composed of the following 7 species: *M. pullari* (type species), *M. hastatus, Murrayon hibernicus* comb. n., *Murrayon nocentiniae* (Ramazzotti 1961), *Murrayon dianeae* comb. n., *Murrayon ovoglabellus* (Biserov 1988) and *Murrayon stellatus.*

The discovery of *M. stellatus* and the transfer of *M. hibernicus* comb. n. and *M. dianeae* comb. n. indicate that the genus *Murrayon* is not exclusively limnic, as it was previously considered.

Systematic Key to the Genus *Murrayon*

1. Cuticle superficially sculptured with more or less evident punctation (dots) 2
– Cuticle superficially smooth, without punctation 4
2. Cuticle uniformly and finely punctated, egg processes immersed in a hyaline matrix, bearing spines and delimiting polygonal areas ..... *M. dianeae*
– Cuticle with two dorsolateral bands of more evident dots 3
3. Dorsolateral bands of dots very evident, egg processes with small heads, some bearing short spines on the top ..... *M. stellatus*
– Dorsolateral bands of dots barely evident, egg processes without any spine ..... *M. hibernicus*
4. Long thin macroplacoids, the first with a deep constriction, the second with an evident subterminal narrowing, egg with large processes covered by a hyaline matrix ..... *M. hastatus*
– Short wide macroplacoids 5
5. First macroplacoid with a slight median constriction, smooth egg .. *M. ovoglabellus*
– First macroplacoid very constricted in the middle; eggs with processes separated from one to another and inserted on a smooth shell 6
6. Eggs with small, rigid, conical tubercles, with surface of the egg visible *M. pullari*
– Eggs with long conical and bent processes with aspect of large aculeus .... *M. nocentiniae*

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