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Diversity of free-living flatworms (Platyhelminthes) in Cuba

YANDER L. DIEZ[1,2,*], CLAUDIA SANJUAN[2,3], CAMILA BOSCH[2,3], ALEJANDRO CATALÁ[3], MARLIES MONNENS[2], MARCO CURINI-GALLETTI[4] and TOM ARTOIS[2]

[1]*Museum of Nature Hamburg –Zoology, Leibniz Institute for the Analysis of Biodiversity Change (LIB), Martin-Luther-King-Platz 3, 20146, Hamburg, Germany*
[2]*Research Group Zoology: Biodiversity and Toxicology, Centre for Environmental Sciences, Hasselt University, Universitaire Campus Gebouw D, B-3590 Diepenbeek, Belgium*
[3]*Biology & Geography Department, Universidad de Oriente, Ave. Patricio Lumumba s/n, CP 90500, Santiago de Cuba, Cuba*
[4]*Dipartimento di Medicina Veterinaria, Università di Sassari, Sassari, Italy*

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Cuban biodiversity is characterized by high species richness and endemism; however, free-living flatworms have been neglected in studies of the fauna of the archipelago. These animals constitute an essential component of marine and freshwater ecosystems as top predators and secondary producers. In this contribution, we provide the first comprehensive analysis of turbellarian diversity in Cuba based on a long-term sampling effort in marine, brackish, freshwater and terrestrial environments. We used observed and estimated species richness as indicators of alpha diversity. As a result, we have collected, for the first time, 279 species in Cuba, including 189 species of rhabdocoels, 33 species of polyclads, 21 species of macrostomorphs, 14 species of proseriates, 12 species of prolecithophorans, seven species of triclads and one representative each of Prorhynchida, Gnosonesimida and Bothrioplanida. At least 184 species (67%) are new to science. Fifty of these species have been recorded in published journal contributions. The remainder are pending formal identification and/or description. We demonstrate the turbellarian fauna of Cuba to be one of high diversity and endemism. Estimated species richness is much higher than that observed, exemplifying the taxonomic impediments and stressing the need for more intense sampling campaigns in the archipelago.

ADDITIONAL KEYWORDS: biodiversity – Caribbean – endemism – habitat – invertebrates – Turbellaria.

RESUMEN

La biodiversidad cubana está caracterizada por una alta riqueza de especies y endemismo, no obstante los platelmintos de vida libre han sido excluidos en los estudios de la fauna del archipiélago. Estos animales constituyen un componente esencial en ecosistemas marinos y dulceacuícolas como depredadores y productores secundarios. En esta contribución se presenta el primer análisis de la diversidad de turbelarios en Cuba basado en el muestreo a largo plazo en ambientes marinos, salobres, dulceacuícolas y terrestres. Se utilizó la riqueza de especies observada y estimada como indicadores de diversidad alfa. Como resultado, se recolectaron por primera vez 279 especies en Cuba, incluyendo 189 rabdocelos, 33 policládidos, 21 macrostomorfos, 14 proseriatas, 12 prolecitóforos, 7 tricládidos, y 1 representante de Prorhynchida, Gnosonesimida y Bothrioplanida respectivamente. Al menos 184 especies (67%) son nuevas para la ciencia. Cincuenta de estas especies han sido registradas en artículos publicados en revistas. Las restantes están pendientes de identificación y/o descripción. Se ha demostrado que la fauna de turbelarios de Cuba es de alta diversidad y endemismo. La riqueza estimada de especies es muy superior a la observada, ejemplificando el impedimento taxonómico y la apremiante necesidad de intensificar las campañas de muestreo en el archipiélago.

PALABRAS CLAVE ADICIONALES: biodiversidad – Caribe – endemismo – hábitat – invertebrados – Turbellaria.

*Corresponding author. E-mail: yanderluis87@gmail.com

INTRODUCTION

The Cuban Archipelago is located in the Caribbean biodiversity hotspot (Myers *et al.*, 2000; Roberts *et al.*, 2002; Zachos & Habel, 2011). The Caribbean is characterized by high numbers of species in terrestrial, marine and freshwater habitats. More than 7000 plant species have been recorded in Cuba, 51% of which are endemic (Herrera, 2007). Terrestrial and freshwater habitats host a very rich fauna of invertebrates, including > 1400 gastropods (95% endemic) (Espinosa & Ortea, 2009), some of which are considered to be the most beautiful land molluscs in the world; *Polymita* spp. (Espinosa & Larramendi, 2013). Cuba also hosts ~1600 species of charismatic butterflies, 20% of which are endemic (Barro & Núñez, 2011).

The marine biota of the Caribbean Sea is represented by > 12 000 species (Costello *et al.*, 2010). This vast species richness results from phylogeographical processes derived from the complex palaeogeographical history of the Caribbean (Rocha *et al.*, 2008; Bowen *et al.*, 2013). This semi-enclosed system, surrounded by the continental American coasts and the Antilles arc, shows characteristics of a mediterranean sea, with specific climatic and oceanic current patterns (Iturralde-Vinent, 2006). The marine biota of the region was particularly affected by the rising of the Isthmus of Panama ~3 Mya (see Hurtado *et al.*, 2016). The isolation of the Caribbean biota from the Eastern Pacific led to large-scale speciation processes by vicariance (see Thacker, 2017; Lima *et al.*, 2020). In addition, there is a high diversity of ecosystems, dominated by coral reefs, mangroves and seagrasses, constituting niches for thousands of species (Espinosa & Ortea, 2007). Most Caribbean marine richness is recorded in the Greater Antilles, with 2781 species of the prevalent animals (sponges, corals, molluscs, amphipods and echinoderms) (Miloslavich *et al.*, 2010).

The Cuban archipelago is a reservoir for the largest number of marine species in the Caribbean (Miloslavich *et al.*, 2010). Reported numbers of species increase each year with the publication of dozens of articles on systematics, ecology and biogeography of the island. This is well illustrated for molluscs, for which Espinosa & Ortea (2021) recorded 1920 species. Other major invertebrate groups include 280 species of sponges (Alcolado, 2002), 152 stony corals (González, 2004), 428 nematodes (Pérez-García *et al.*, 2020), 1320 crustaceans (Lalana & Ortiz, 2000; Lalana *et al.*, 2005, 2014; Diez, 2014) and 385 echinoderms (del Valle & Abreu, 2007). Ascidians are represented by 35 species (Hernández, 1990). Fish have received preferential attention owing to their ecological and commercial importance, and 1226 species are recorded so far (Claro, 1994).

However, this picture is incomplete, because many marine phyla remain critically understudied in Cuba. This mainly includes the major meiofaunal groups, e.g. Acoelomorpha, Nemertea and Tardigrada. All these groups are characterized by a high global diversity, and the paucity of records is likely to reflect a lack of research effort, rather than absence from the Cuban archipelago (Zhang, 2013).

Platyhelminthes, in particular, have mainly received attention as marine parasitic species of vertebrates in Cuba. Apart from the parasitic neodermatans, only a few free-living polyclads (Hidalgo, 2007) and few triclads have been reported, i.e. *Girardia cubana* (Condreanu & Balcesco, 1973) and *Bipalium kewense* Moseley, 1878 (Condreanu & Balcesco, 1973; Morffe *et al.*, 2016). To fill this knowledge gap, we undertook a large-scale collection effort targeting turbellarians. This included numerous sampling expeditions over a period of several years, starting in January 2016, focusing mainly on Rhabdocoela and Polycladida. This has resulted in seven published papers (Catalá *et al.*, 2016; Diez *et al.*, 2018a, b, 2019, 2021; Gobert *et al.*, 2021, 2022), recording 50 species (39 species of rhabdocoels and 11 species of polyclads) from Cuba, including 23 new species. More recently, we also started to study other marine (Proseriata) and freshwater (Macrostomorpha and Rhabdocoela) flatworms.

Free-living flatworms, traditionally known as turbellarians, are a heterogeneous group. Their classification has been controversial; however, many of the traditionally recognized turbellarian taxa are themselves monophyletic (Egger *et al.*, 2015; Laumer *et al.*, 2015) . The taxonomical organization of the flatworm clades is based mainly on the previously mentioned phylogenetic studies and, therefore, the traditional Linnaean classification is not used over the family rank. However, WoRMS (2023) is still recognizing the traditional classification levels.

The aim of this study is to summarize our findings so far, after 7 years of collecting campaigns, and to report the general diversity patterns of Cuban turbellarian fauna, including marine, freshwater and terrestrial species.

MATERIAL AND METHODS

Study area

An intensive collecting campaign was conducted in eastern Cuba (from January 2016 to May 2022), with a few samples taken in western Cuba (Fig. 1). Seventy-two samplings were conducted at 42 localities (including one terrestrial, 17 freshwater, two brackish and 22 marine; Supporting Information, Table S1).

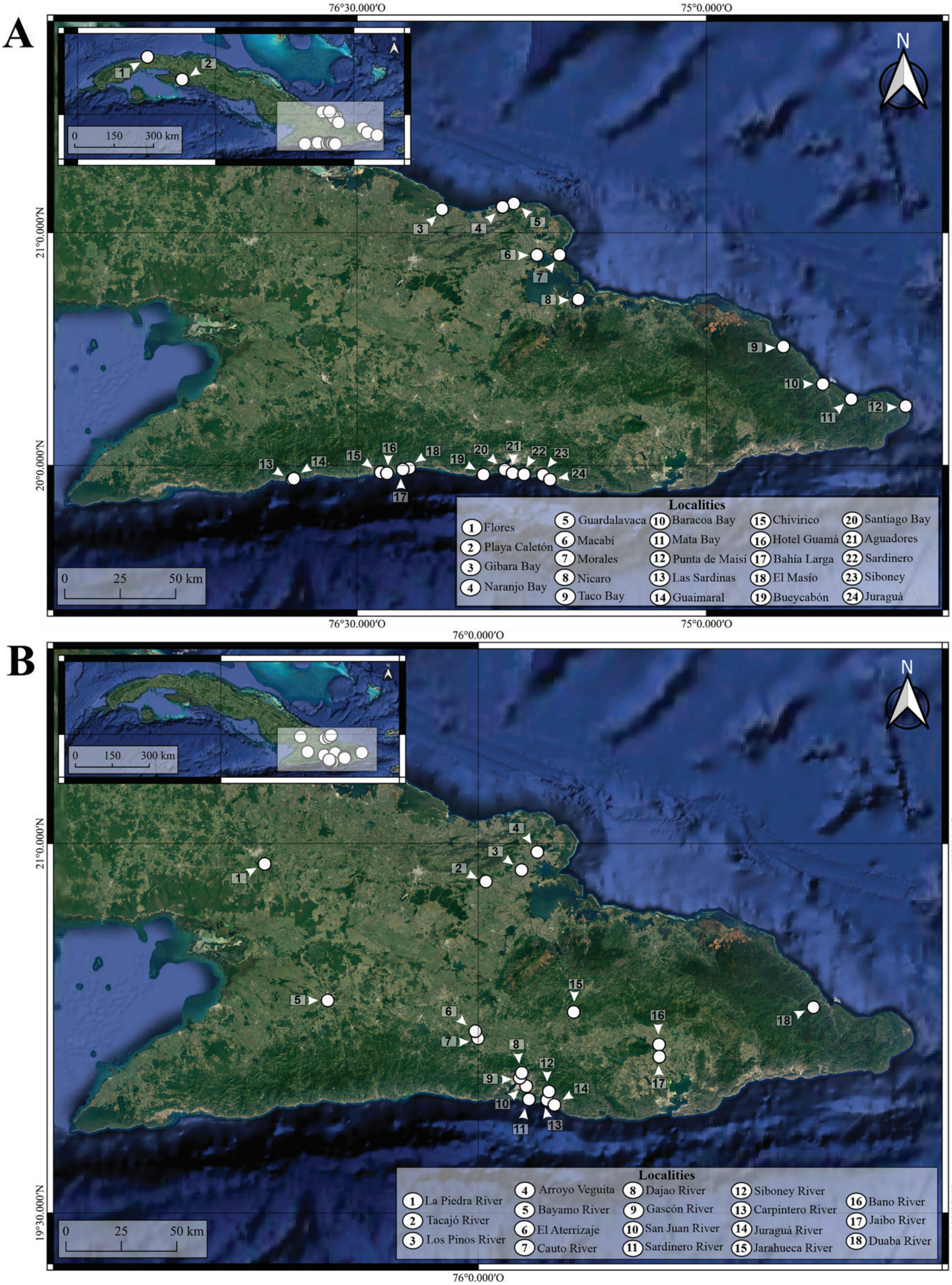

Figure 1. Maps of the sampled localities. A, marine and brackish. B, freshwater and terrestrial environments.

Collection, identification and preservation of specimens

Marine and brackish microturbellarians (Macrostomorpha, Rhabdocoela, Proseriata, Tricladida, Gnosonesimida and Prolecithophora) and small polyclads were collected from algae, seagrass and sediments (sandy and muddy sand) in the intertidal zone to a depth of 3 m. Specimens were extracted using the $MgCl_2$ method (Schockaert, 1996). Some specimens from marine samples very rich in organic matter (i.e. samples from mangroves) were extracted with the oxygen-depletion method (Schockaert, 1996). The marine Polycladida were mostly collected directly under rocks and shells and in oyster beds [*Crassostrea virginica* Gmelin, 1791] intertidally to 1 m deep. Freshwater species (Macrostomorpha, Rhabdocoela, Tricladida, Prorhynchida and Bothrioplanida) were extracted from vegetation, rotten vegetation and muddy sediment by oxygen depletion. Terrestrial Tricladida were collected under leaf litter.

Microturbellarians were studied alive and whole mounted with lactophenol on a Nikon Eclipse 80i microscope, using Nomarski interference contrast. For histological observation, freshwater triclads and microturbellarians were fixed in hot (50 °C) Bouin's fixative, embedded in paraffin and serially sectioned (3 µm thickness). After sectioning, they were stained with Heidenhain's Haematoxylin, using Erythrosine as the counterstain. Specimens for further molecular studies were preserved in ethanol (99%) and stored at −20 °C.

Polyclads were studied alive and anaesthetized in a solution of hexahydrate $MgCl_2$ and seawater (1:1). Larger specimens (> 0.8 cm in length) were fixed in 10% formaldehyde for 24 h (Newman & Cannon, 1995). Specimens < 0.8 cm in length were fixed in hot (50 °C) Bouin's fixative. After fixation, specimens were stored in 70% ethanol. For histological studies, the posterior region of the body, containing the genitalia, was sectioned at 7 µm thickness and stained as described for microturbellarians, following the protocol described by Bolaños *et al*. (2016). The remaining body of the specimens was whole mounted with Canada balsam.

Most of the material collected is stored in the collection of the Research Group Zoology: Biodiversity and Toxicology (Hasselt University, Belgium). The material of Polycladida and Tricladida is deposited in the Biology & Geography Department of Universidad de Oriente (Cuba). Holotypes of the species already described are deposited in the Swedish Museum of Natural History (Sweden) and the Finnish Museum of Natural History (Finland). The voucher codes can be found in the original descriptions (see Diez *et al*., 2018a, b, 2019, 2021; Gobert *et al*., 2021, 2022).

Data analyses

Species richness (*S*) was determined as the number of species identified in the samplings. From these values, estimated species richness was obtained by means of the non-parametric bootstrap method (Colwell & Coddington, 1994) for the more sampled taxa (marine Rhabdocoela and Polycladida, and freshwater Rhabdocoela + *Macrostomum* spp.). The estimates were calculated with the packages 'vegan' (Oksanen *et al*., 2019) and 'BiodiversityR' (Kindt & Kindt, 2019) for R (Basualdo, 2011).

RESULTS

Species richness and composition

Our collecting efforts in Cuba yielded 279 species of free-living flatworms that have been collected in Cuba for the first time (Supporting Information, Tables S2 and S3). The number of species included 189 rhabdocoels, 33 polyclads, 21 macrostomorphs, 14 proseriates, 12 prolecithophorans, seven triclads and one each of Prorhynchida, Gnosonesimida and Bothrioplanida (Table 1). In Table 1, we considered

Table 1. Number of free-living flatworms collected in Cuba (2016–2022), including new records, new species and species of doubtful classification

Taxa	Number of species	New records	New species	Doubtful
Macrostomorpha	21	5	12	4
Polycladida	33	27	2	4
Prorhynchida	1	–	–	1
Gnosonesimida	1	–	1	–
Rhabdocoela	189	26	154	9
Proseriata	14	1	13	–
Tricladida	7	–	2	5
Prolecithophora	12	–	–	12
Bothrioplanida	1	–	–	1
Total	279	59	184	36

'Doubtful' species to be those where we are uncertain whether they are new species or already known. We also collected the known freshwater triclad *Girardia cubana*. The most species-rich genera of marine microturbellarians were the rhabdocoels *Cheliplana*, *Gyratrix*, *Paulodora*, *Schizochilus*, *Reinhardorhynchus* and *Carcharodorhynchus*, with 17, 13, 11, 9, 8 and 8 species, respectively. Among marine rhabdocoels, 75 species belonged to Eukalyptorhynchia, 40 to Schizorhynchia, 33 to Thalassotyphloplanida, 15 to Neodalyellida and three to Mariplanellida. Freshwater rhabdocoels were represented by four eukalyptorhynchs and 19 limnotyphloplanids. Five of these species belonged to *Gieysztoria*, four to *Gyratrix* and three to *Phaenocora*.

These results have been published, in part, by Catalá *et al*. (2016), Diez *et al*. (2018a, b, 2019, 2021) and Gobert *et al*. (2021, 2022). These papers recorded 50 species from Cuba (39 rhabdocoels and 11 polyclads), including 23 new species of rhabdocoels. Therefore, 229 species are pending formal identification and/or description, including all freshwater and terrestrial species. Considering the recently described new species and those not yet described, 184 species (66%) are new to science, a number even higher within Rhabdocoela, with 154 new species (81%), and Proseriata, with 13 new species (93%) (Table 1). Most marine microturbellarians have been classified to genus level; however, 26 species are still unclassified. Thirteen freshwater species have not been assigned to a known genus (seven species of rhabdocoels, four species of triclads, one species of prorhynchid and one species of bothrioplanid), nor has the terrestrial specimen.

Based on the results of statistical analysis of our sampling effort, the estimated species richness is 200, 37 and 40 species for marine rhabdocoels, freshwater rhabdocoel + *Macrostomum* spp. and polyclads, respectively. According to the sampling effort, we have collected 83%, 81% and 83% of the estimated species richness of each taxon, respectively. In the three analyses, the species richness and the observed species richness curves do not reach the asymptote (Fig. 2).

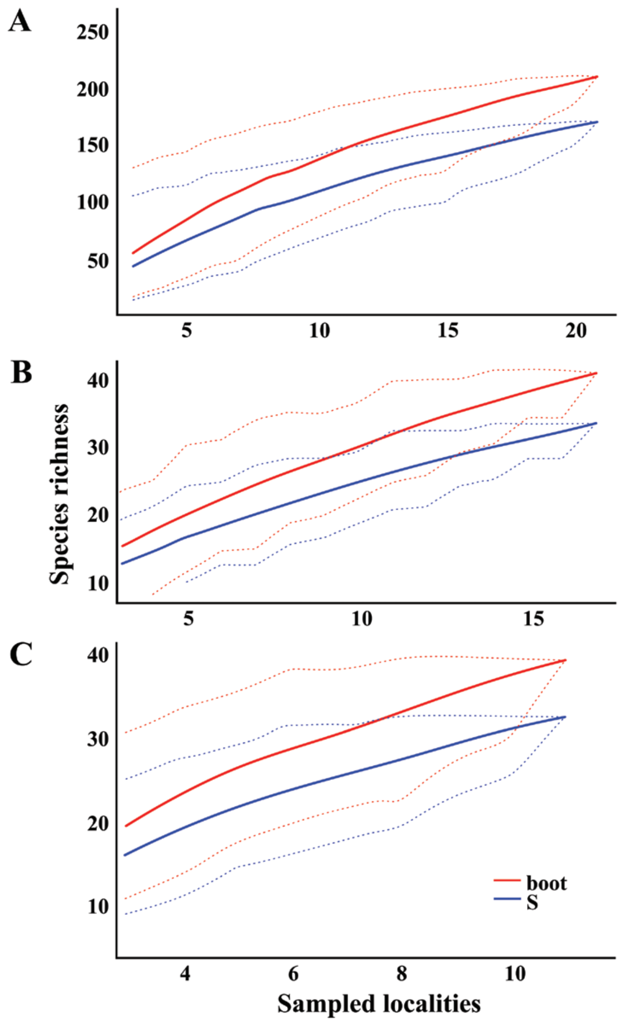

Figure 2. Curves of species accumulation of the observed (*S*) and estimated species richness for the non-parametric estimator bootstrap (boot) for the free-living flatworms of Cuba. A, marine Rhabdocoela. B, freshwater Rhabdocoela + *Macrostomum* spp. C, marine Polycladida.

Habitat

Collected species are mostly from marine (240) and freshwater (37) habitats, whereas two species are from brackish water and one is terrestrial (Table 2; Supporting Information, Tables S2 and S3). An unidentified species of *Paulodora* (Rhabdocoela; *Paulodora* sp. 6 in Supporting Information, Table S2) is the only species recorded from both marine and brackish habitats. Marine and brackish water rhabdocoels were mostly found in sediments (144 species); 33 species inhabited vegetation (algae and seagrass), and three were collected on rotten leaves of mangroves. Only 11 species (7%) were collected on both sediments and vegetation. Schizorhynchs were almost always inhabitants of sediments (except *Carcharodorhynchus spiniferus* Diez, Reygel & Artois, 2019, which was also found on algae). Eukalyptorhynchs, neodalyellids and thalassotyphloplanids were well represented in both sediments and on vegetation. Macrostomorphs were mostly found in sediments, whereas freshwater species were also found on vegetation. Proseriates were strictly found in marine sediments. Polyclads were found interstitially in sediments, under rocks and in oyster beds.

Table 2. Distribution in habitats of the species richness of the free-living flatworms collected in Cuba (2016–2022)

Taxa	Marine	Brackish	Freshwater	Terrestrial
Macrostomorpha	12	1	8	–
Polycladida	33	–	–	–
Prorhynchida	–	–	1	–
Gnosonesimida	1	–	–	–
Rhabdocoela	166	1	23	–
Proseriata	14	–	–	–
Tricladida	2	–	4	1
Prolecithophora	12	–	–	–
Bothrioplanida	–	–	1	–
Total	240	2	37	1

Spatial distribution

Marine and brackish water rhabdocoels were collected in 21 localities, varying from three to 51 species per locality. Most species (98; 59%) were found in a single locality, and 48 were found in two or three localities (29%). Only 20 species (12%) showed a wider distribution in four to six localities. However, for most species with a wider distribution, their localities are not far from each other (located along 30 km on the south-eastern coast of Santiago de Cuba). In eastern Cuba, we found 156 species, 136 collected on the south-eastern coast and 34 on the north-eastern coast (15 shared species; 10%). Nineteen species were found in western Cuba, including nine species (5%) shared with the eastern coasts of the archipelago.

The other flatworm groups were collected only in eastern Cuba. However, these taxa were not as well studied as the marine Rhabdocoela. Therefore, it is difficult to describe distribution patterns for these groups. For Polycladida, six species were collected on the north-eastern coast and 33 species on the south-eastern coast. Species richness of polyclads varied from one to ten species per locality. Gnosonesimida, Prolecithophora and the marine Tricladida were collected strictly on the south-eastern coast, and a single species each of Macrostomorpha and Proseriata were collected on the north-eastern and north-western coast, respectively. Freshwater microturbellarian richness varied from one to ten species per locality. The species of Gnosonesimida and Bothrioplanida were collected in only one locality, and the species richness of freshwater triclads varied between one and three in each locality.

DISCUSSION

This is the first broad study of free-living flatworms in Cuba to include marine, brackish, freshwater and terrestrial species. It is also the most intensive research on the microturbellarians in the Caribbean. Excluding our published results, in the Caribbean, we know of only nine marine, two brackish and one freshwater rhabdocoel (Marcus, 1960; Curini-Galletti & Puccinelli, 1994; Therriault & Kolasa, 1999; Willems *et al*., 2004; Artois & Tessens, 2008; Reygel *et al*., 2011; Van Steenkiste & Leander, 2018, 2022), 25 proseriates (Curini-Galletti, 1991; Martens & Curini-Galletti, 1993; Curini-Galletti & Martens, 1996; Scarpa *et al*., 2017; Curini-Galletti *et al*., 2019) and three macrostomorphs (Rieger, 1971; Therriault & Kolasa, 1999). The rhabdocoel fauna of Cuba is now the best known of the Caribbean by far, even in the entire Western Atlantic (excluding Brazil). In contrast, few species of polyclads have been collected in Cuba, considering that > 130 species have been recorded in the Tropical Western Atlantic (Quiroga *et al*., 2004, 2008; Bolaños *et al*., 2006, 2007).

The high number of new species and new records for Cuba is primarily because we have conducted the first intensive study of turbellarians for the archipelago and because of the inclusion of the neglected microturbellarians. However, given that our knowledge in other tropical areas and archipelagos is still very scant, we cannot draw any firm conclusions regarding species richness in the different areas. Furthermore, the statistical estimator shows an underestimated species richness for the three taxa considered (marine Rhabdocoela, freshwater Rhabdocoela + *Macrostomum* spp. and marine Polycladida).

In general, meiofauna are neglected in biodiversity studies because they are very small and often hard to study, or at least they are conceived as such (Hutomo & Moosa, 2005). Marine microturbellarians have been systematically ignored in meiofaunal studies because of the traditional sampling methods, which include fixing samples in formaldehyde before extraction, destroying all soft-bodied meiofauna, including flatworms. Also, morphological identification of meiofauna is time consuming and requires rapidly disappearing taxonomic expertise (Fonseca *et al*., 2018). These limitations have biased the studies of meiofauna in Cuba to the best-known taxa, such as nematodes and copepods. Several studies have been conducted on meiofaunal diversity in Cuba (e.g. Armenteros *et al*., 2014, 2018; Pérez-García *et al*., 2015); however, records of microturbellarians did not exist until recently.

In the context of the current extinction of species, accelerated by climate change and human economic activities, it is necessary to understand more of the ecology of free-living flatworms, such as their local distribution and tolerance to biotic and abiotic factors (Leasi & Cline, 2022). Most studies on the ecology and distribution of free-living flatworms have been conducted on triclads (see Vila-Farré & Rink, 2018), and very few have investigated the ecology of microturbellarians (e.g. Jouk *et al*., 1988; Balsamo *et al*., 2020; Leasi & Cline, 2022). The habitat preferences of the Cuban free-living flatworms do not show any typical pattern compared with these taxa in other regions. However, our study was focused on taxonomy, and only the type of habitat of each species was recorded. Some factors driving the distribution of microturbellarians have been described by Leasi *et al*. (2016), Jörger *et al*. (2021) and Leasi & Cline (2022), for example.

Analyses of biogeographical patterns in the distribution of free-living flatworms, mainly microturbellarians, are very difficult (Artois *et al*., 2011). There are very few areas of the world with a well-known microturbellarian fauna, such as Western and Northern Europe and the Mediterranean [see Schockaert *et al*. (2008) and Balsamo *et al*. (2020) for freshwater species and Curini-Galletti *et al*. (2020) for marine species]. For the rest of the world, very few areas have been studied in detail (e.g. Brazil and Galapagos Islands). In general terms, most microturbellarians are known only from the type locality; however, higher taxa show a worldwide distribution. This is probably related to two factors: microturbellarians have direct development, hence they lack pelagic larvae (see Martín-Durán & Egger, 2012); and their small and soft bodies are very sensitive to changes in temperature and salinity (Purschkeg, 1981; Armonies, 1986, 1988). Notwithstanding, these factors themselves do not explain the known distribution of turbellarians, the study of which is hampered by the lack of global studies. Furthermore, some species show a very wide distribution and others represent species complexes (Tessens *et al*., 2021). Historical biogeographical processes have probably also determined the present distribution of turbellarians, evidenced, for example, in the bipolar distribution of some marine species (Volonterio & Ponce de León, 2021, and references therein). Recently published studies suggest that the distribution of freshwater triclads (*Dugesia* spp.) is a result of vicariance and dispersal events, including transoceanic dispersal (Solà *et al*., 2022).

The high species richness of Cuba and the Caribbean, mainly known for conspicuous terrestrial and marine species, was also found in small and understudied free-living flatworms. We expect a strong increase in the known species richness of the area in the future. This study forms a base from which to continue exploration of the free-living flatworms in the Caribbean and other tropical areas.

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DATA AVAILABILITY

The data underlying this work are available in the Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article on the publisher's website.

Table S1. Data on the sampling campaign for free-living flatworms in Cuba (2016–2022).
Table S2. Habitat and distribution of the marine and brackish water free-living flatworms collected in Cuba (2016–2022). Abbreviations: b, brackish; l, rotten leaves; m, marine; r, rocky bottoms; s, sediments; v, vegetation; 1, Flores; 2, Playa Caletón; 3, Gibara Bay; 4, Naranjo Bay; 5, Morales; 6, Guardalavaca; 7, Macabí; 8, Nicaro; 9, Las Sardinas; 10, El Guaimaral; 11, Chivirico; 12, Hotel Guamá; 13, El Masío; 14, Bahía Larga; 15, Bueycabón; 16, Santiago Bay; 17, Aguadores; 18, Sardinero; 19, Siboney; 20, Juraguá; 21, Taco Bay; 22, Mata Bay; 23, Baracoa Bay; 24, Punta de Maisí.
Table S3. Habitat and distribution of the freshwater and terrestrial free-living flatworms collected in Cuba (2016–2022). Abbreviations: f, freshwater; s, sediments; t, terrestrial; v, vegetation; 1, Tacajó River; 2, Los Pinos River; 3, Arroyo Veguita; 4, Bayamo River; 5, Cauto River; 6, El Aterrizaje; 7, Jarahueca River; 8, Sardinero River; 9, Siboney River; 10, Juraguá River; 11, Carpintero River; 12, San Juán River; 13, Gascón River; 14, Dajao River; 15, Duaba River; 16, Bano River; 17, Jaibo River.