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Rapid Communication

First record of the land planarian *Bipalium kewense* Moseley, 1878 (Tricladida: Geoplanidae: Bipaliinae) from Cuba

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Abstract

The cosmopolitan land planarian *Bipalium kewense* Moseley, 1878 (Tricladida: Geoplanidae: Bipaliinae) is recorded for first time from Cuba on the basis of specimens from the Gran Parque Natural Topes de Collantes, Sancti Spíritus province. Both external morphology and internal anatomy agree with *B. kewense* features. Maximum likelihood and Bayesian inference analysis of mitochondrial cytochrome c oxidase I (COI) sequences obtained from Cuban individuals form a well-supported clade with other specimens of *B. kewense* from Europe. This constitutes the first Cuban land planarian identified to species level based on morphological and molecular studies.

Key words: bipaliid, DNA barcoding, flatworm, invasive species, West Indies

Introduction

Species of the land planarian subfamily Bipaliinae (Tricladida: Geoplanidae) are easily identified by having auricles on their heads, giving them an expanded, lunate appearance. This group presents its highest diversity in Asia and Africa, with records from China, India, Japan, Korea, Madagascar, the Russian Far East and Taiwan (Kawakatsu et al. 2005). However, some bipaliid species have been recorded from beyond their native distribution: *e.g. Diversibipalium multilineatum* (Makino and Shirasawa, 1973), *Bipalium adventitium* Hyman, 1943; *B. kewense* Moseley, 1878; *B. pennsylvanicum* Ogren, 1987 and *B.* cf. *vagum*. The first aforementioned species (native to Japan) was recently recorded in Europe (Mazza et al. 2016). *B. adventitium*, *B. pennsylvanicum* and *B.* cf. *vagum* occur in USA (Ducey et al. 2007; Ducey and Noce 1998; Hill and Merickel 2011; Ogren 1987). *B. kewense* is more widespread, having a cosmopolitan distribution (Pérez-Gelabert 2010; Winsor 1983).

The native range of *B. kewense* appears to be southeastern Asia, from northern Vietnam to southern Cambodia (Winsor 1983). Hitherto, this species is recorded from more than 40 countries of North, Central and South America, The Caribbean, Africa, Europe, Asia and Australasia, being one of the most widespread land planarian. The primary mode of *B. kewense* dispersal (along with about 30 other invasive species of land planarians) appears to be by transport of exotic potted plants, with the soil containing the land planarians or their cocoons (Justine et al. 2014; Lago-Barcia et al. 2015; Winsor 1983).

The first recorded land planarians in Cuba were described as *Bipalium* sp. and *Geoplana* sp. based only on external features (Reinés 1996). These individuals were associated with earthworm cultures. The next record is that of Suárez (2012), who recorded a case of malacophagy on two species of Cuban land snails by a land planarian identified as belonging to family Rhynchodemidae. More recently, Alegre and Barba (2014) recorded several specimens

of an unidentified land planarian in a cave from Baracoa, Guantánamo province, Eastern Cuba.

The present study constitutes the first detailed record of the species *B. kewense* in Cuba.

Material and methods

Specimens collecting and fixation

Flatworm specimens (n = 2) were collected by hand in rotting logs and litter from La Chispa and the Caburní River, Gran Parque Natural Topes de Collantes, Sancti Spíritus province, Cuba. They were photographed alive and killed with hot water (80 ºC). A small fragment of the posterior end was cut from each specimen and stored in 96% ethanol for DNA studies. Planarians were fixed in 4% neutral phosphate-buffered formaldehyde and transferred to 70% ethanol for storage.

Histological studies

One specimen was selected for histological studies. Tissue blocks of the pre-pharyngeal region, pharynx (which internal morphology was not described in the present study) and copulatory apparatus were dehydrated in an ascending ethanol series and embedded in paraffin wax. Transversal (pre-pharyngeal region) and sagittal (pharynx and copulatory apparatus) 5–10 µm sections were stained with Cason´s trichrome (Carbayo 2005) and mounted in Canada balsam. For the pre-pharyngeal region, Cutaneous Musculature Index (CMI) and Parenchymal Musculature Index (PMI) (Froehlich 1955) were calculated. Micrographs were taken with an AxioCam digital camera attached to a Carl Zeiss AxioScop 2 Plus compound microscope. Scale bars of all plates are given in micrometers. The studied material is deposited in the Colección Helmintológica de las Colecciones Zoológicas (CZACC), Instituto de Ecología y Sistemática, La Habana, Cuba.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from both specimens with the DNEasy® Blood & Tissue kit (Qiagen, USA) following manufacturer´s protocol. Partial sequences (~900 bp) of the popular DNA barcode mitochondrial gene (cytochrome c oxidase subunit I, COI) were amplified with the primer set BarS [5´-GTT ATG CCT GTA ATG ATT G-3´ (Álvarez-Presas et al. 2011)] and COIR [5´-CCW GTY ARM CCH CCW AYA GTA AA-3´ (Lázaro et al. 2009)]. PCR reactions were performed in a total volume of 20 µL with the KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). PCR cycling parameters consisted of an initial denaturalization at 94 ºC by 2 min followed by 35 cycles of 98 ºC by 10 s, 45 ºC by 30 s and 68 ºC by 30 s and a final extension step of 68 ºC by 5 min. The results of the PCR reactions were checked by agarose gel electrophoresis. Then PCR products were excised from gel and purified with the NucleoSpin® Gel and PCR Clean Up kit (Macherey-Nagel, Düren, Germany), following manufacturer´s protocol. Samples were submitted to Hokkaido System Science Co., Sapporo, Japan for sequencing from both strands, using the same PCR primers.

Raw sequences were manually edited with Sequencher 4.1.4 (http://genecodes.com). Sequences were deposited in GenBank NCBI (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KX349431 (specimen from La Chispa) and KX349432 (specimen from Caburní River).

Phylogenetic analysis

A multiple sequence alignment was made using ClustalW with default gap opening and extension costs as implemented in MEGA 5.10 (Tamura et al. 2011). The same software was also used to determine the optimal model of sequence evolution (GTR+G) following the Akaike Information Criterion (AIC) and performs the Maximum likelihood analysis (ML). Branch support for the ML tree was inferred by bootstrap using 1,000 iterations. Bayesian inference analysis (BI) was made with MrBayes v3.2.0 (Ronquist et al. 2012), with 3×10^6 generations, sampling every 100 generations and discarding the 25% of samples as burn-in. The convergence statistics of the BI was checked with the software Tracer v1.6 (Rambaut et al. 2014). Average standard deviation of split frequencies was lower than 0.01 at the end of the analysis.

Results and discussion

Material examined

Voucher CZACC 18.001; Cuba, Sancti Spíritus province, Gran Parque Natural Topes de Collantes, La Chispa, 80º01′17″N, 21º55′44″W; 10/X/2014; J. Morffe and N. García coll.; entire specimen, a small portion of the posterior end removed for molecular studies.

Voucher CZACC 18.002; Cuba, Sancti Spíritus province, Gran Parque Natural Topes de Collantes, path to Caburní River, 80º00′15″N, 21º55′13″W; 12/X/2014; J. Morffe and N. García coll.; pre-pharyngeal region, transverse sections on 8 slides (10 µm thick); pharynx, sagittal sections on 9 slides (10 µm thick); copulatory apparatus, sagittal sections

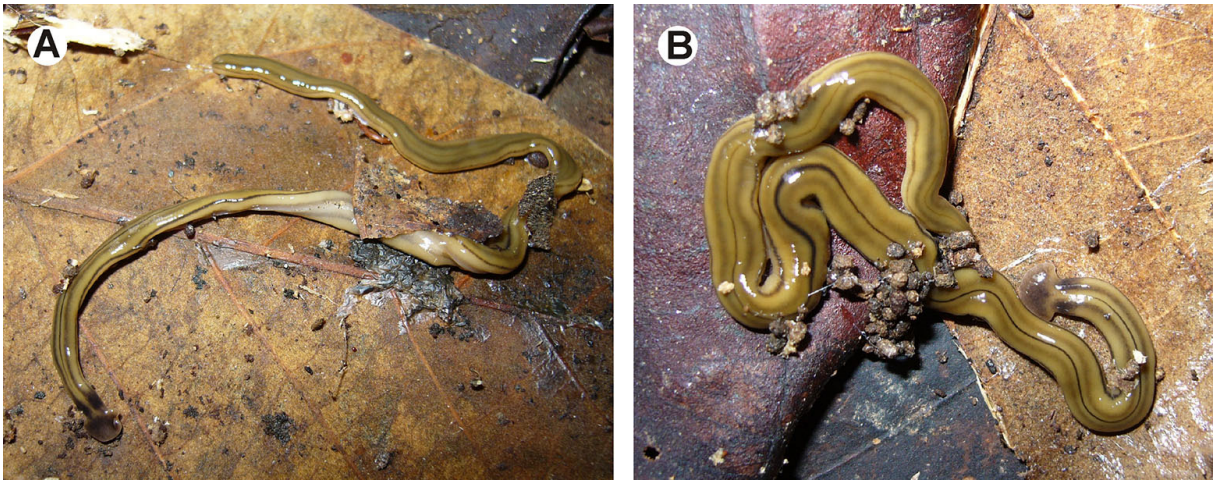

Figure 1. Specimens of *Bipalium kewense* Moseley, 1878 (Tricladida: Geoplanidae: Bipaliinae) from two localities from Gran Parque Natural Topes de Collantes, Sancti Spíritus province, Cuba. A. Specimen from the path to Caburní River (CZACC 18.002). B. specimen from La Chispa (CZACC 18.001). Scale bars not available. Photographs by Jans Morffe.

on 10 slides (5–10 µm thick); a small portion of the posterior end removed for molecular studies; rest of the body fragments stored on 70% ethanol.

The external morphology of the specimens (Figure 1) is consistent with the Winsor (1983) re-description: body elongate, its sides almost parallel. Dorsum convex, ventral side almost flat. The anterior end is expanded, forming a semilunate head plate. Posterior end bluntly rounded. The color pattern is the dorsum pale ochre with five longitudinal stripes: one median, dorsal; two sub-lateral and two marginal. The median stripe is black, evident, wider at level of the pharynx. The sub-lateral stripes are grayish, wide, with diffuse margins. Marginal stripes are thin, black, with well-defined margins. Sub-lateral and marginal stripes link together behind the head plate forming a collar, dorsally interrupted. Ventral surface very pale ochre, creeping sole white, delineated at both sides by diffuse grayish-violet stripes. Such creeping sole comprises *ca.* 28% of the body width at level of the pre-pharyngeal region; a value similar to the 25% previously recorded by Winsor (1983). Most of the body measurements of both specimens (Table 1) are within the range of the sexual specimens studied by Winsor (1983). Only the distance of the mouth to gonopore, expressed as percentage of the body length is slightly lower in the specimen from La Chispa (6.4 *vs.* 7.7–23.2).

The musculature arrangement at pre-pharyngeal level also accords with Winsor´s studies. The cutaneous musculature is not strong (Figure 2A), with a CMI value of *ca.* 4.7%. CMI values previously recorded (Winsor 1983) range 2.4% to 5.3%. Parenchymal musculature is more developed, with two ventral plates of longitudinal muscles located beneath the nerve cords, and intersected by transverse muscles. In addition, longitudinal muscle bundles with transverse muscle fibres form a ring zone that extends dorsally and laterally. The ends of the ring zone contact the margins of the ventral plates. PMI value of *ca.* 12.8% was recorded for the Cuban specimen, being in the range of 10.0% to 18.8% observed for the species (Winsor 1983). At level of the creeping sole the parenchymal musculature is more specialized, with a pack of longitudinal fibres arranged as a dorsoventral wedge that divides the longitudinal plates of muscles (Figure 2B).

Table 1. Body measurements of two fixed specimens of *Bipalium kewense* Moseley, 1878 (Tricladida: Geoplanidae: Bipaliinae) from La Chispa and the path to Caburní River, Gran Parque Natural Topes de Collantes, Sancti Spíritus province, Cuba. Values are given in millimeters unless otherwise indicated.

Measurements	La Chispa	Path to Caburní River
Body length	94.0	91.0
Body width	5.0	5.0
Mouth-anterior end	44.0	36.0
Gonopore-anterior end	50.0	47.0
Mouth-gonopore	6.0	11.0
Mouth-anterior end (% body length)	46.8	37.1
Gonopore-anterior end (% body length)	53.2	48.5
Mouth-gonopore (% body length)	6.4	11.3

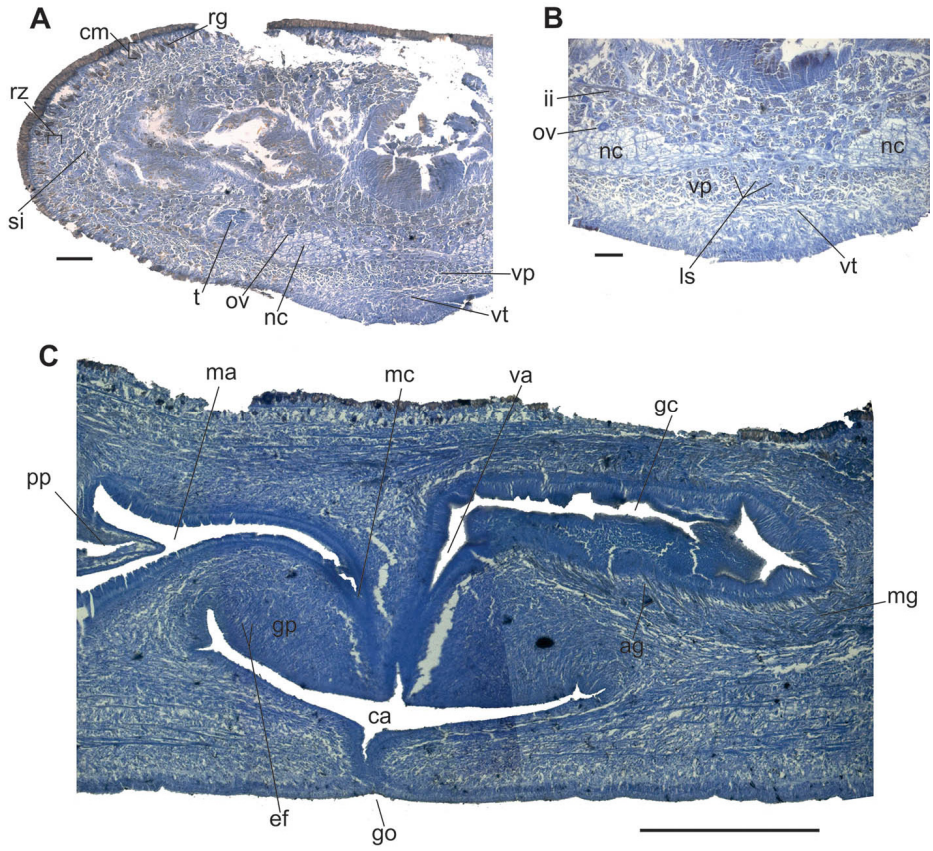

Figure 2. *Bipalium kewense* Moseley, 1878 (Tricladida: Geoplanidae: Bipaliinae). Internal anatomy. A. Transverse section at level of the pre-pharyngeal region. B. Detail of the creeping sole. C. Sagittal section of the copulatory apparatus, anterior to left. Scale bars: A 200 µm. B 100 µm. C 500 µm. Abbreviations used: **ag**, acidophil glands; **ca**, common genital atrium; **cm**, cutaneous musculature; **ef**, ectal fibres; **gc**, glandular canal; **go**, gonopore; **gp**, glandular pad; **ii**, infra-intestinal transverse muscles; **ls**, longitudinal muscles over creeping sole; **ma**, male atrium; **mc**, male copulatory canal; **mg**, musculature of glandular canal; **nc**, nerve cord; **ov**, ovovitelline duct; **pp**, penis papilla; **rg**, rhammite gland; **rz**, ring zone of longitudinal and transverse muscles; **si**, supra-intestinal transverse muscles; **t**, testis; **va**, vagina; **vp**, ventral plate of longitudinal muscles; **vt**, ventral transverse muscles. Micrographs by Jans Morffe.

The specimen sectioned for histological studies presented a fully developed copulatory apparatus (Figure 2C), which arrangement was similar to the species re-description. The ovaries are located above and slightly included in the ventral nerve cords. The penis papilla is well developed and conical, projecting into the funnel-like male atrium. The epithelium covering the penis papilla is thin, cuboidal, becoming thicker and columnar in the male atrium. The female glandular canal possesses its proximal part horizontal and the distal part (vagina) descends through the genital pad. In both, glandular canal and vagina are covered on a ciliated columnar epithelium. There are many acidophil glands in the parenchyma, surrounding the glandular canal. The genital pad is covered on a thin, flattened epithelium, finelly papillated and presents a well-developed musculature, which includes weak ectal fibres underlain by a thin layer of longitudinal muscles. The common genital canal opens to a common atrium covered by a columnar epithelium.

Comparing both Cuban specimens with the available sequences of four *B. kewense* individuals from Spain and Açores BLAST search showed 100% of similarity. Moreover, in the ML and BI analysis the specimens from the present study formed a well-supported monophyletic group with the aforementioned *B. kewense* sequences (Figure 3). Thus, the molecular data support the morphological and histological studies and confirm the present specimens as *B. kewense.* Concordant with Álvarez-Presas et al. (2014), based on the COI gene *B. adventitium* comprises the sister taxon to *B. kewense*, but in a clade with low support values.

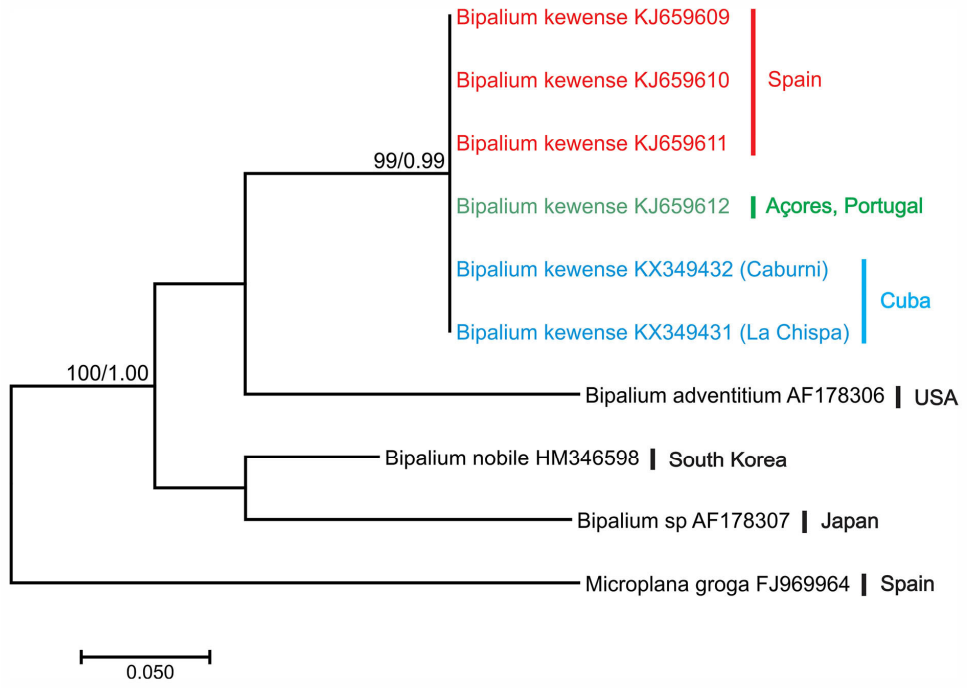

Figure 3. Maximum likelihood tree inferred from cytochrome c oxidase 1 (COI) gene for several species of *Bipalium*. One species of *Microplana* was used as outgroup. Values at the nodes correspond to bootstrap (>75)/posterior probability (>0.95).

The current study extends the distribution of *B. kewense* to now include all islands of the Greater Antilles. Previously, the species was recorded from Jamaica (Cockerell 1897), Puerto Rico (Winsor 1983) and Hispaniola (Pérez-Gelabert 2010). This is also the northernmost record of the species in the West Indies and the Caribbean.

Within its native distribution in southeastern Asia, *B. kewense* inhabits highlands (1300–2000 masl) with rainforests (Winsor 1983). Out of its native range, the species has been recorded usually from man-modified areas such as hot-houses, gardens, nurseries, plantations and earthworm cultures (Álvarez-Presas et al. 2014; Justine et al. 2014; Sánchez-García 2014; Winsor 1983). As with previous reports, our specimens were also found in disturbed areas. The individual from La Chispa was collected under litter and rotting logs in a mixed plantation (coffe, guava and banana), while the one from Caburní River was found under rotting logs in a cleared area next to a path used for hiking. Other alien species of land planarians have been also recorded for anthropogenic environments, as *Caenoplana coerulea* Moseley, 1877 in Argentina, Spain and Balearic Islands (Álvarez-Presas et al. 2014; Breugelmans et al. 2012; Luis-Negrete et al. 2011); *Platydemus manokwari* de Beauchamp, 1963 in France, USA, Puerto Rico, Singapore, Australia, New Caledonia, Solomon Islands and Guam (Justine et al. 2014; Justine et al. 2015) and more recently *D. multilineatum* in Italy (Mazza et al. 2016).

Reinés (1996) recorded the presence of specimens of *Bipalium* sp. in earthworm cultures from Western and Eastern Cuba. These specimens were identified only on the basis of external features and described as large flatworm having three dorsal stripes. However, the drawing provided by the author contradicts such description and shows the characteristic pattern of *B. kewense*: the fine median stripe, both sub-lateral stripes wide, with diffuse margins and the fine marginal stripes. Also, it is possible to distinguish the collar dorsally interrupted and posterior to the head plate. Thus, these specimens appear to belong to *B. kewense*. This further suggests a wide distribution of the species in the country, especially in areas disturbed by human activities. Further support for this statement will require morphological and molecular studies on a more broadly representative geographic sampling effort.

The current study constitutes the first record of a Cuban land planarian identified to species level. Also, for the first time for the country, histological studies and molecular analyses were combined with external morphology for the purpose of identification.

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