

## Research Article

**First finds in North and South America of *Pentacoelum kazukolinda* (Platyhelminthes: Tricladida), a worldwide invasive flatworm**Constanza Vásquez-Doorman<sup>1,\*</sup>, Francisco Brusa<sup>2,3</sup>, Jhoe Reyes<sup>4</sup>, Ronald Sluys<sup>5</sup>, Miquel Vila-Farré<sup>6</sup> and Miguel L. Allende<sup>1</sup><sup>1</sup>Center for Genome Regulation, Facultad de Ciencias, Universidad de Chile, Santiago, Chile<sup>2</sup>Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, Argentina<sup>3</sup>División Zoología Invertebrados, Facultad de Ciencias Naturales y Museo (UNLP), La Plata, Argentina<sup>4</sup>Facultad de Ciencias de la Vida y de la Salud, Universidad Científica del Sur, Lima, Perú<sup>5</sup>Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, The Netherlands<sup>6</sup>Department of Tissue Dynamics and Regeneration, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany

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## OPEN ACCESS

**Abstract**

Invasive species form an important threat to autochthonous biodiversity and ecosystems, as they may negatively affect native species. *Pentacoelum kazukolinda* is an invasive maricolan flatworm with a freshwater ecology that was already known to be widely distributed in the Northern hemisphere: USA (Hawaii), Spain, Germany, The Netherlands, and Japan. Here we detail the first records from Chile, Peru, and continental USA. These new records from North and South America were substantiated by observations on the very characteristic external appearance of the specimens, their anatomy, and/or molecular data. We demonstrate for the first time that the animals are capable of regeneration. At the newly discovered localities in South America, this flatworm co-occurs with introduced snails, which may constitute its prey species, and with introduced aquatic plants that may have facilitated its introduction into the New World.

**Key words:** introduced species, new records, anthropochory dispersal, New World, regeneration, ecology**Introduction**

One of the major threats to biodiversity and ecosystems are invasive species (Kumschick et al. 2015; Bellard et al. 2016; Pyšek et al. 2020). The planarian *Pentacoelum kazukolinda* (Kawakatsu & Mitchell, 1984) is an invasive flatworm that thus far has been reported from the USA (Hawaii), Spain, Germany, The Netherlands, and Japan (Sluys et al. 2015). However, its impact on the ecosystem has not been demonstrated yet. Phylogenetically, this triclad belongs to the suborder Maricola or marine planarians, of which most species occur in marine habitats. However, the genus *Pentacoelum* Westblad, 1935, is an exception, as its currently known four species inhabit brackish water or freshwater environments (*P. fucoideum* Westblad, 1935, *P. punctatum* (Brandtner, 1935), *P. kazukolinda*, *P. sinensis* Wang & Zhao, 2016 (cf. Sluys 1989, He et al. 2016)).

The taxonomic history of *P. kazukolinda* is complex. The Hawaiian members were collected in 1966–1967 from Manoa Stream, Honolulu, on Oahu Island and were described as *Oahuhawaiiiana kazukolinda* Kawakatsu & Mitchell, 1984. Thereafter, other specimens were described from near Valencia, Spain, under the name *Pentacoelum hispaniense* Sluys, 1989. Later it was discovered that specimens also occurred at other localities in Spain, Germany, The Netherlands, and Japan. These all concerned the same species as described from Hawaii and Valencia. Therefore, based on morphological and anatomical similarities, it was concluded that the correct species name is *P. kazukolinda* and that the species has an extensive distributional range (Sluys 1989; Sluys et al. 1990, 2015; Vila-Farré et al. 2008).

Circumstantial evidence suggested that *P. kazukolinda* had acquired such a wide distributional range through anthropochore dispersal, and it was predicted that the species may also occur in North and South America (Sluys et al. 2015). In the present paper, we detail records for the occurrence of *P. kazukolinda* in two South American countries, Chile and Peru, and in continental North America (Florida, USA), thus considerably expanding its already wide distributional range. Furthermore, we have examined under laboratory conditions the regenerative capacity of these worms, as this may contribute to their ability to invade new areas. The associated aquatic flora and fauna at the new sites in North and South America were examined for potential prey species of the flatworms, as well as for aquatic plants that may have functioned as possible means of transport through which the worms colonized these areas.

## Materials and methods

### *Collection of specimens*

Chile: Specimens were collected from under rocks in a freshwater spring that flows into an artificial pond at the recreational O'Higgins Park (−33.468861; 70.977472) in Santiago, Metropolitan Region, during summer season on December 18<sup>th</sup>, 2020. Specimens, and a rock with egg capsules attached to them, were transported in clean zipper plastic bags to the laboratory and then maintained in Montjuïc planarian water (1.6 mM sodium chloride, 1.0 mM calcium chloride, 1.0 mM magnesium sulphate, 0.1 mM magnesium chloride, 0.1 mM potassium chloride, 1.2 mM sodium bicarbonate) (Cebrià and Newmark 2005).

Peru: Specimens were collected from two localities: (1) vegetated, secondary channels of the Chillón River near Trapiche, Canta Province, Lima (−11.73455; 76.972367 and −11.733833; 76.9721) on August 29<sup>th</sup>, 2015 and October 29<sup>th</sup>, 2017; (2) the regional conservation area Humedales de Ventanilla in the central-western region of Ventanilla district, Lima (−11.875222; 77.145139 and −11.872278; 77.138583) on September 13<sup>th</sup>, 2014 and October 1<sup>st</sup>, 2016. Samples of filamentous algae and floating macrophytes

were collected and filtered through a plankton net with a mesh size of approximately 80 microns. The filtered material was then placed in a wide mouthed jar of 500 ml and taken to the laboratory. In addition, filamentous algae were manually sampled, after which the material was put into similar jars with water from their habitat. In these last-mentioned samples, more *Pentacoelum* turned out to be present than in the filtered ones.

USA: A single individual was collected in February 2016 from a freshwater artesian well 2 km distant from the Florida coast (29.653861; 81.23275).

### *Preservation and histology*

Some specimens from Peru were relaxed in ~ 4% magnesium chloride for approximately 30 min. Once they did no longer respond to stimulation by a needle, the medium was removed and replaced with Bouin's fluid. Samples were kept in Bouin's for 24 h, then washed with 70% ethanol until the yellow colour disappeared, and, subsequently, stored in 70% ethanol. Other specimens were directly fixed in 10% formalin, followed by a cleaning, and storage process similar to that of the specimens fixed in Bouin's fluid. For histology, samples were dehydrated in an ascending series of ethanol and embedded in Paraplast®. Sagittal and horizontal serial sections were made at intervals of 6 µm and stained in Hematoxylin and Eosin or with a modified Masson's trichrome technique (Suvarna et al. 2013). The specimens studied were deposited in the Departamento de Protozoología, Helmintología e Invertebrados Afines, Museo de Historia Natural (MUSM-INV), Peru. Two specimens were collected alive from Chillón River, Peru; one of these was preserved in ethanol (MUSM-INV 4842), and the other was sectioned sagittally (MUSM-INV 4841a–n, 14 glass slides). From Humedales de Ventanilla, Peru, ten specimens were observed alive and collected. Six specimens were preserved in ethanol (MUSM-INV 4843–4845), while three specimens were sectioned sagittally (MUSM-INV 4847a–d, 4848a–c, 4849a–d), and one was sectioned horizontally (MUSM-INV 4846a–c).

### *18S sequencing and phylogenetic analysis*

DNA was extracted from a single, fresh and starved worm from Chile, according to specifications detailed in Grohme et al. (2018). In brief, the tissue was homogenized in GTC buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% v/v N-lauroylsarcosine, 7% v/v beta-mercaptoethanol) for 1–2 min on ice, occasionally mixing on a vortex to disrupt the tissue. This was followed by a phenol-chloroform-isoamyl alcohol extraction and glycogen-isopropanol precipitation, 70% ethanol wash and resuspension in 30 µL of AE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0).

The ribosomal subunit 18 (18S) gene was amplified by polymerase chain reaction (PCR) in full length from the isolated DNA by using the primers 18S\_1F 5'-ACCTGGTTGATCCTGCCAGTAG-3' and 18S\_9R 5'-GATCC

TTCCGCAGGTTTCACCTAC-3' (Carranza et al. 1996). The final PCR reaction volume for all molecules was 50 µL under the following conditions: 2 min at 95 °C, 35 cycles of 50 s at 94 °C, 45 s at 45 °C, and 50 s at 68 °C, and, finally, 4 min at 68 °C. Amplification products were sequenced by Macrogen Inc. (Seoul, Korea), using the primers mentioned above. Chromatograms were revised, and a consensus sequence was obtained by mapping to a reference clone (KM200926.1) with default parameters and 0.001 error probability limit, using Geneious version 2021.1.1. The resulting sequence was deposited in GenBank from the National Center for Biotechnology Information, NCBI (accession number OM293668). Basic Local Alignment Search Tool for Nucleotides (BLASTN) was run for analysis on NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using default settings (Zhang et al. 2000). To determine the phylogenetic position of our new specimens from Chile (18S sequence, OM293668.1), we built a phylogenetic tree with the Maximum Likelihood method (ML) on IQ-TREE (v 1.6.6) (Nguyen et al. 2015) using the same species and replicating the experimental conditions as in Li et al. (2021).

#### *Regeneration assay*

Two planarians from Chile were carefully dropped onto wet tissue paper and quickly cut pre- and post-pharyngeally using a sharp surgical knife. Head, trunk and tail fragments (6 pieces in total) were immediately transferred to a Petri dish containing Montjuïc planarian water and observed for 19 days. Regenerating pieces were kept continuously in the dark at 19 °C; the water was changed every 2–3 days. Photographs were taken with a digital camera attached to a stereo microscope.

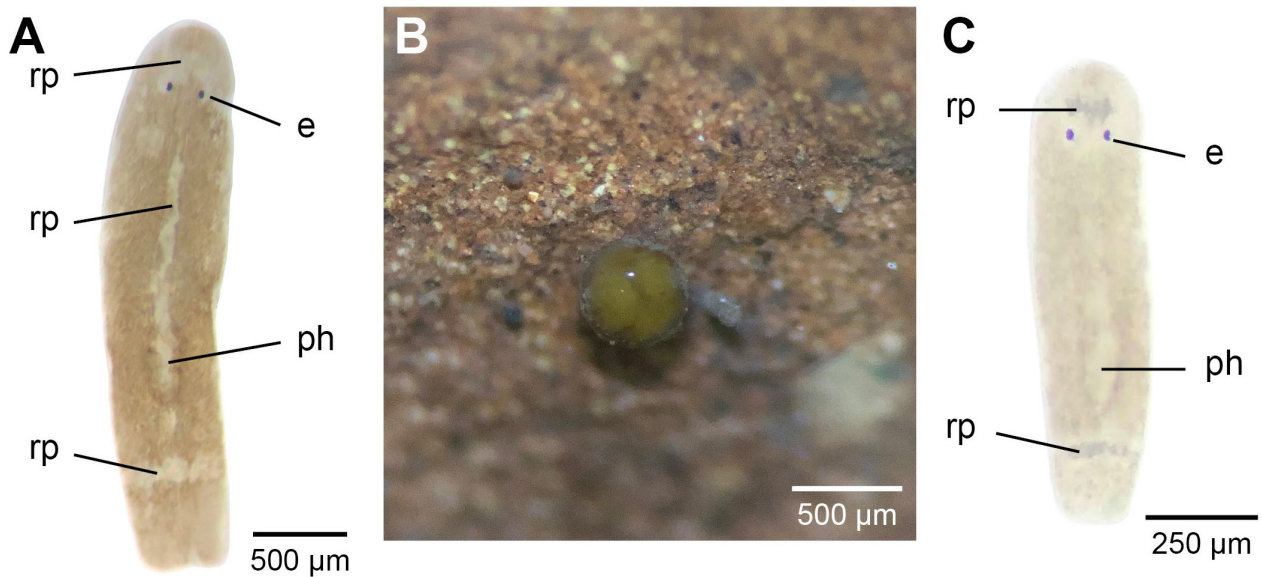
#### *Abbreviations used in the figures*

bc, bursal canal; bl, blastema; cb, copulatory bursa; cvd, common vas deferens; e, eyes; ec, egg capsule; ed, ejaculatory duct; eg, erythrophil glands; go, gonopore; in, intestine; lb, lateral bursa; lg, lateral gonopore; rp, refringent patch; sg, shell glands; od, oviduct; ph, pharynx; pb, penis bulb; pp, penis papilla; vd, vas deferens.

## **Results**

### *Records of *Pentacoelum kazukolinda* in Chile*

Identification: External appearance and molecular characterization. The external appearance of the specimens closely matched what is documented in the literature for *P. kazukolinda*, with their rounded anterior end and blunt posterior end. Live, sexually mature specimens measured 2.6–3.1 mm in length and 0.5–0.7 mm in width, while hatchlings measured 0.9–1.1 mm in length. The dorsal body pigmentation was light brown, with the three characteristic refringent spots on the dorsal surface: a transverse patch anterior



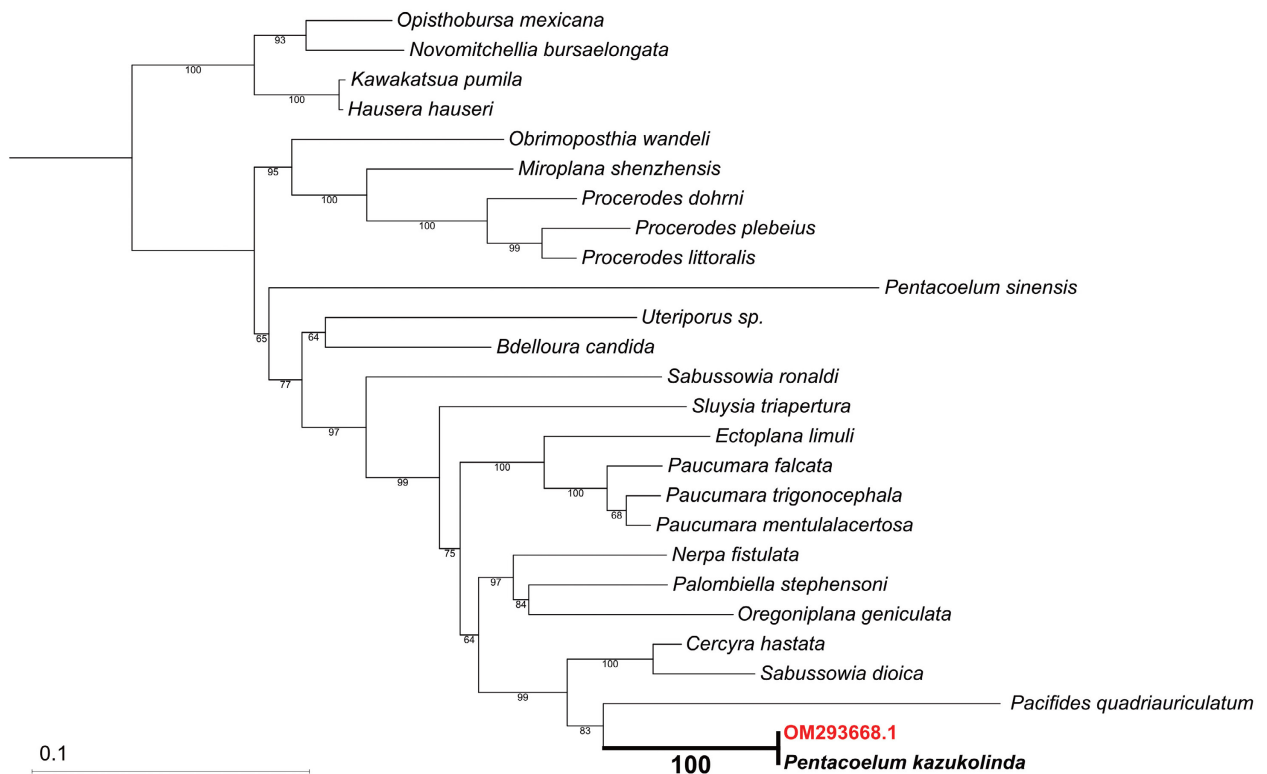
**Figure 1.** *Pentacoelum kazukolinda* from Chile. (A) Dorsal view of a live adult, showing external anatomy; (B) Stalkless, translucent egg capsule attached to a rock; (C) Dorsal view of a hatchling, showing refringent patches under transmitted light. Photos by CVD.

to the eyes that might be composed of one or two stripes; a mid-dorsal longitudinal stripe; and a more or less continuous transverse patch posterior to the pharynx (Figure 1A). These patches are opaque under transmitted light. Animals laid stalkless, translucent egg capsules that measured 0.45–0.55 mm in diameter (Figure 1B); 3–4 juvenile worms hatched from each capsule and presented light body pigmentation and refringent patches (Figure 1C). Two pigmented eyecups were located in unpigmented spots in the anterior region of the body. The unpigmented pharynx was located in the posterior third of the body. Locomotion was remarkably fast.

The 18S rDNA gene sequence identified the specimens from Santiago as *Pentacoelum kazukolinda*. The consensus sequence showed 97–100% identity to other *P. kazukolinda* sequences through BLASTN analysis, while a maximum likelihood phylogenetic tree showed that the newly sequenced Chilean individual (OM293668.1, highlighted in bold red, Figure 2) clusters with the published sequence of *P. kazukolinda* (highlighted in bold black, Figure 2). It is noteworthy that *Pentacoelum sinensis*, a Chinese member of the same genus, is situated far apart from *P. kazukolinda* (Figure 2), which clusters with *Pacifides quadriauriculatum*. Thus, in our phylogeny, the genus *Pentacoelum* is not monophyletic. Unfortunately, our single-gene analysis lacks the phylogenetic resolution to clarify the relations within the Maricola. Nevertheless, the non-monophyly of the genus *Pentacoelum* has been recovered in previous phylogenies, inferred also from a limited number of genes, based on concatenated datasets of 18S and 28S (Li et al. 2019, 2021).

Location and associated flora and fauna. The O'Higgins Park is the second-largest urban park in Santiago, Chile; it dates from the nineteenth century and encompasses an area of about 75 hectares. Planarian specimens (3 adults,



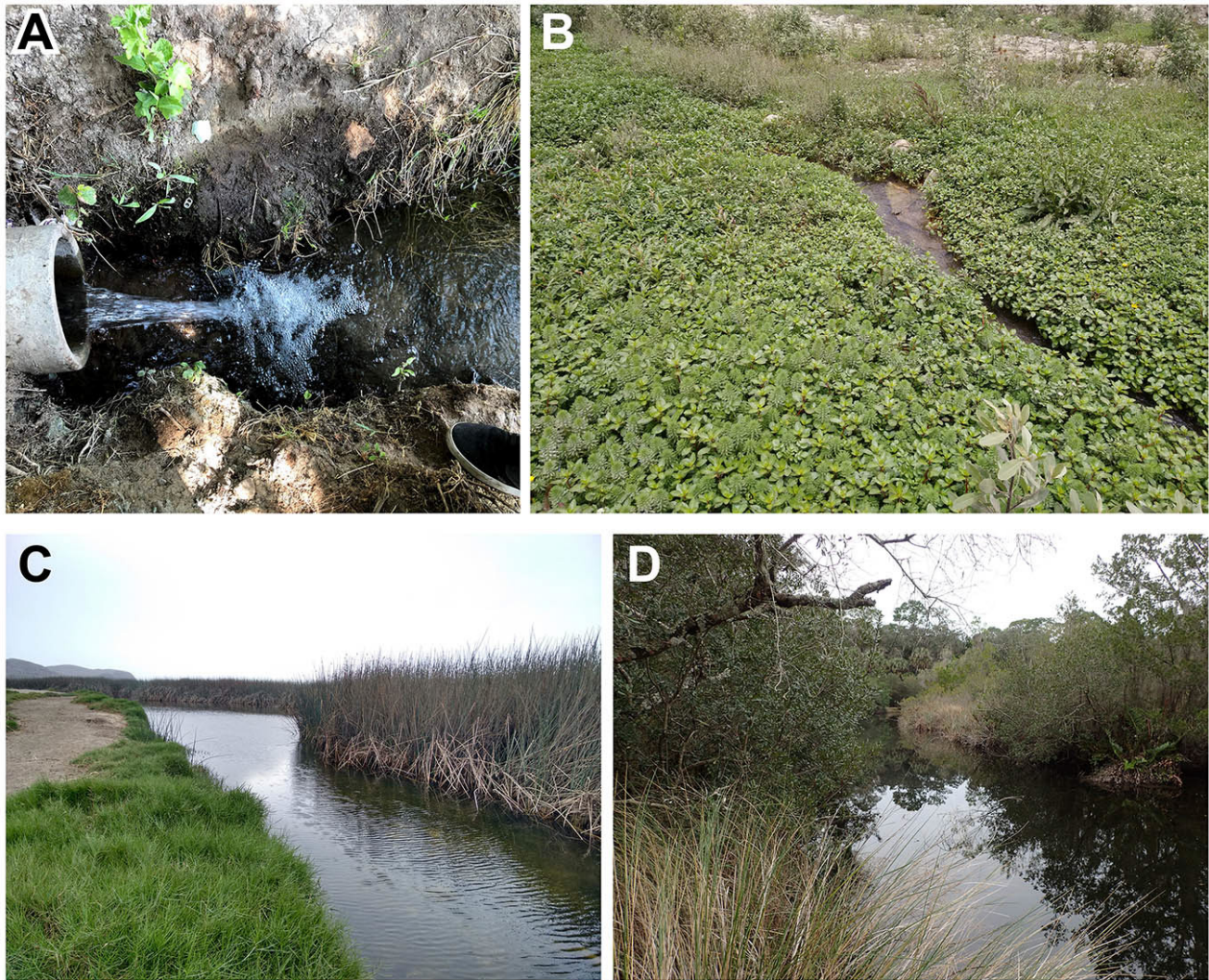


**Figure 2.** Maximum likelihood phylogenetic tree topology based on the 18S rDNA dataset. Numbers at nodes indicate support values (bootstrap). The newly sequenced Chilean individual (highlighted in bold red) clusters with the published sequence of *P. kazukolinda* (highlighted in bold black), with a bootstrap value of 100.

12 juveniles, and 5 egg capsules attached under a rock) were collected from a human-modified freshwater spring (Figure 3A), running into an artificial pond that holds a Chinese botanical garden with macrophyte aquatic plants. Water conductivity reached 1010  $\mu\text{S}/\text{cm}$ ; pH:  $\sim 7$ . At this sampling locality, *P. kazukolinda* coexists with specimens of the freshwater triclad *Girardia* sp., as well as leeches (Glossiphoniidae), hydras, and the snails *Potamopyrgus antipodarum* (Gray, 1843) and *Physella acuta* (Draparnaud, 1805), as reported by Collado (2014, 2017).

#### *Records of Pentacoelum kazukolinda in Peru*

**Identification: External appearance and anatomy.** Mature, live animals measured 1.54–3.8 mm in length and 0.73–0.85 mm in width (Figure 4A, B). The anterior end was rounded and the posterior end truncated, but the latter may attain a forked shape when animals adhere to the substratum (Figure 4A). The ventral surface was pale and slightly translucent (Figure 4B), while the dorsal surface was light brown, although intestinal branches were visible. The body margin was pale and slightly translucent, especially at the posterior end (Figure 4C). The dorsal body surface exhibits three refringent patches: (1) a transverse, more or less continuous and broad patch anterior to the eyes; (2) a longitudinal, mid-dorsal stripe located in the anterior half of the body (this stripe may be composed of two or three short stripes); (3) a transverse, more or less rectangular, narrow patch located approximately



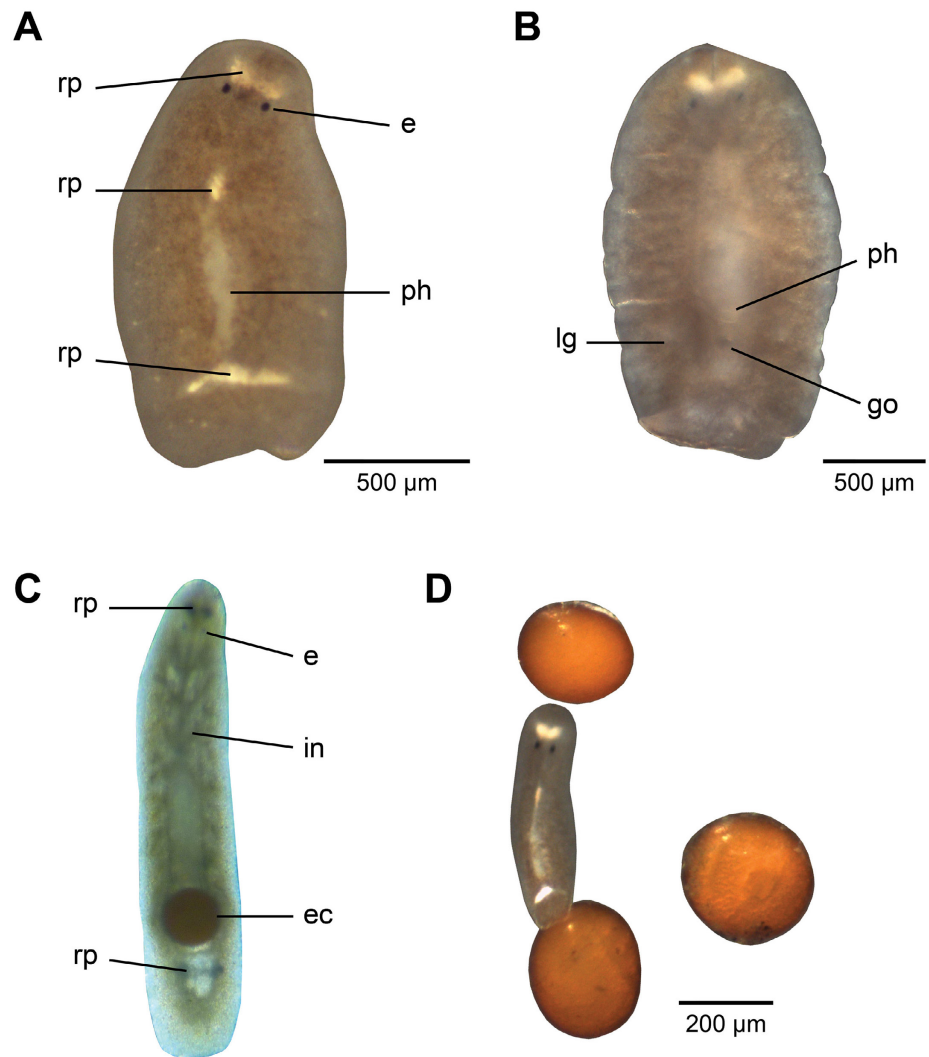
**Figure 3.** Sampling localities. (A) O’Higgins Park’s artificial pond, Santiago, Chile; (B) Chillón River’s secondary channels, Lima, Peru; (C) Humedales de Ventanilla, Lima, Peru; (D) Artesian well, Florida, USA. Photos (A) by CVD, (B, C) by JR, and (D) by Jeremias Brand and Lukas Schärer.

halfway between the pharyngeal pocket and the posterior body end (this patch may be composed of two or four small patches). These patches are opaque when observed under transmitted light, while the intestinal branches are visible (Figure 4C). Preserved animals were similar in shape and size to live animals. However, the dorsal refringent patches become very pale and lose their refringent property.

In the mid-ventral body region, extending from behind the copulatory bursa to almost the end of the tail, the ventral epidermis is pierced by the numerous openings of erythrophilic glands, which stain bright red (Figure 5A, C); this structure may correspond to the mid-ventral adhesive band. The pharyngeal pocket is located in the posterior third of the body (Figure 4A). The pharynx has two types of glands, viz., (a) xanthophilic ones, of which the cell bodies are located outside of the pharynx and discharge at the tip of the pharynx, and (b) erythrophilic glands, located in the stroma of the pharynx that also discharge at its tip.

The testicular follicles are located dorsally and are arranged into two rows on either side of the body, from the region of the ovaries to the posterior part



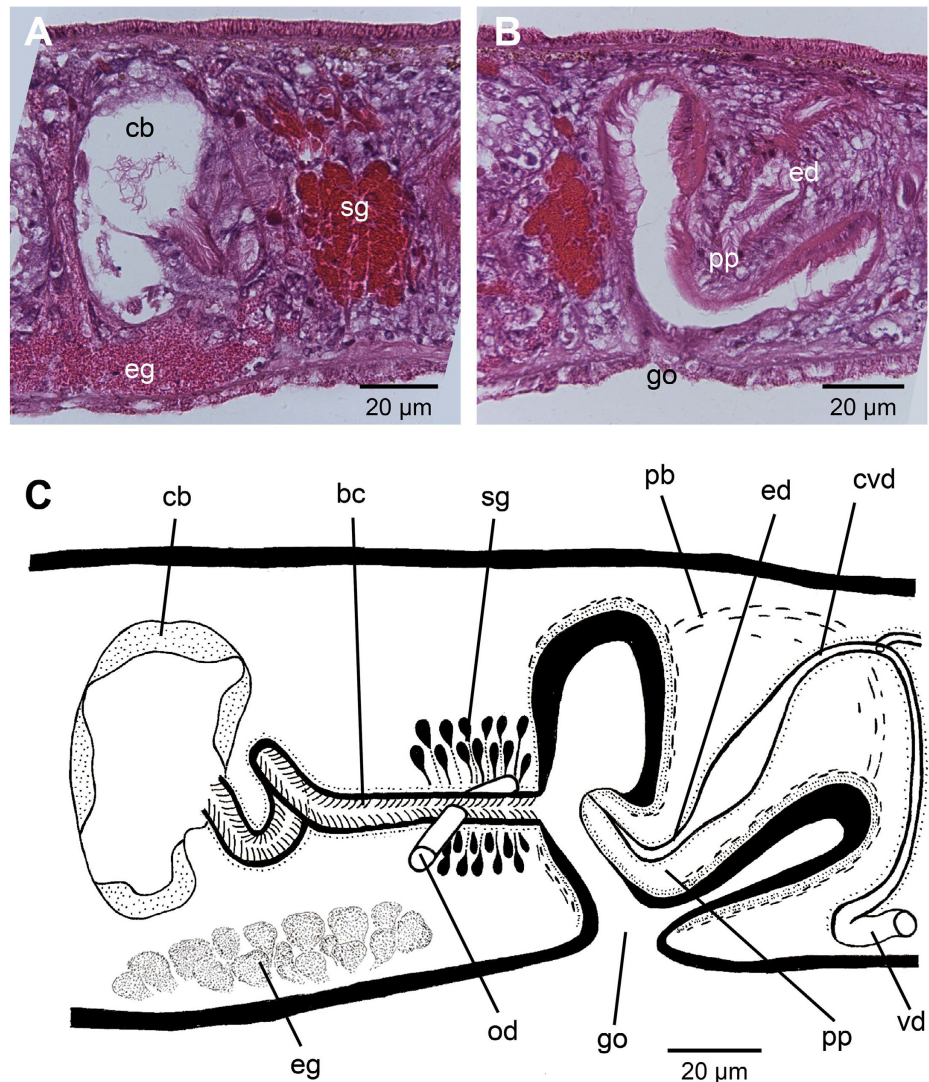


**Figure 4.** *Pentacoelum kazukolinda* from Peru. (A) Dorsal view of a live animal showing the refringent patches under reflected light; (B) Ventral view of a live animal showing the common and lateral gonopores; (C) Dorsal view of a live animal, showing egg capsule and refringent patches under transmitted light (scale bar not available); (D) Egg capsules and a juvenile with refringent patches. Photos by JR.

of the body. The sperm ducts are expanded to form spermiducal vesicles packed with spermatozoa. At the level of the penis bulb, the two sperm ducts gradually narrow and, subsequently, curve dorsally and then unite before entering the penis bulb, thus forming a common vas deferens (Figures 5B, C). The hemispherical penis bulb comprises an inner layer of circular muscle and a weak outer layer of longitudinal fibres. The common vas deferens penetrates the anterodorsal wall of the penis bulb. It widens for some distance and continues as an ejaculatory duct, which opens at the tip of the penis papilla (Figures 5B, C). The latter is lined with a nucleated epithelium, underlain by a thick, subepithelial layer of circular muscle, followed by a layer of longitudinal fibres.

Vitellaria extend from the pre-ovarian region to the posterior region of the body. Two ventral ovaries are located at  $1/3^{\text{th}}$  to  $1/4^{\text{th}}$  of the distance between the brain and the root of the pharynx and situated immediately

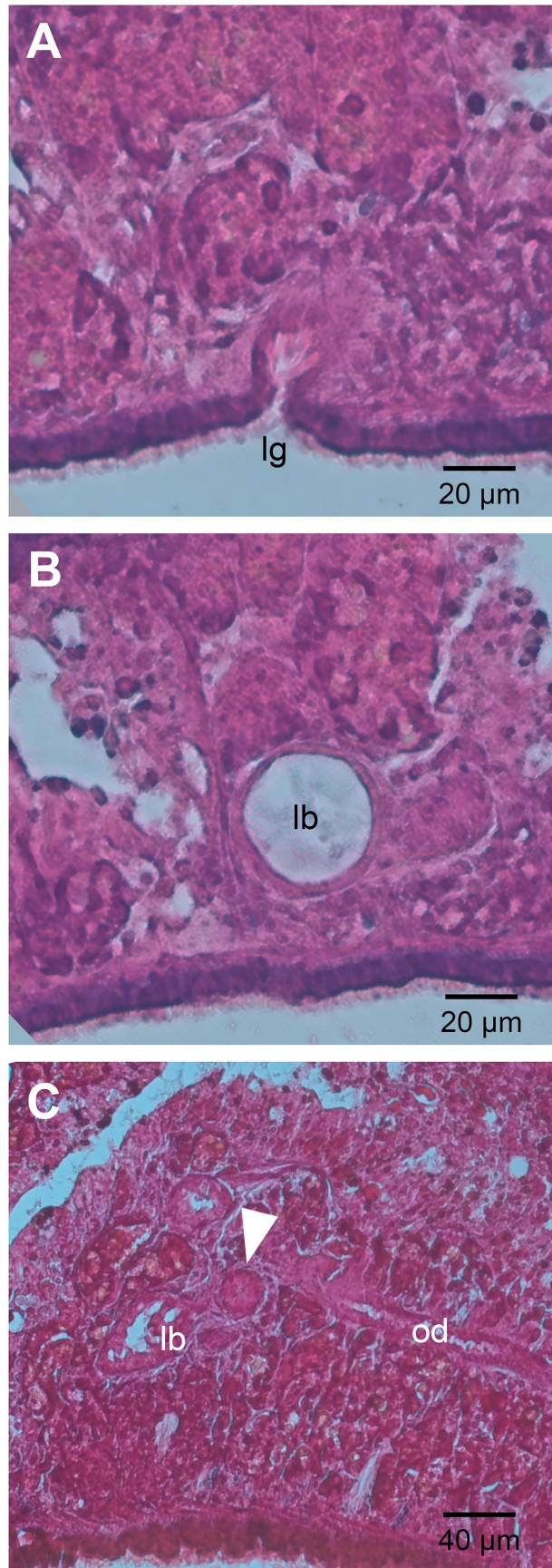




**Figure 5.** *Pentacoelum kazukolinda* from Chillón River, Peru, specimen MUSM-INV 4841a–n, sagittal sections; anterior to the right. (A) Photomicrograph of the copulatory bursa; (B) Photomicrograph of the penis papilla; (C) Reconstruction of the copulatory apparatus of *P. kazukolinda*. Photos by JR.

dorsally to the ventral nerve cords. The oviducts originate from the ventrolateral wall of the ovaries. At the level of the copulatory apparatus, the oviducts give rise to a branch that runs to the bursal canal (Figure 5C) and another branch that turns towards the lateral bursa, which opens to the ventral exterior via a lateral gonopore (Figure 6A, B). At the point of communication between oviduct and lateral bursa there is a transition zone with rather large, circularly arranged, occluding cells (Figure 6C). Thus, there are three gonopores located between the pharyngeal pocket and the posterior end of the body, viz., the primary, mid-ventral gonopore, and a lateral gonopore on either side of the body (Figure 4B).

The horizontally oriented bursal canal arises from the posterior wall of the genital atrium and, thereafter, runs posteriad in a sinuous trajectory to open into the copulatory bursa (Figure 5A, C). The bursal canal is lined with a ciliated, nucleated epithelium and is surrounded by a layer of circular muscle;



**Figure 6.** *Pentacoelum kazukolinda* from Peru, photomicrographs of specimens MUSM-INV 4848a–c, sagittal sections (A, B), and MUSM-INV 4846a–c, horizontal section (C). (A) Lateral gonophore; (B) Lateral bursa; (C) Transition zone with a group of large, occluding cells (white arrowhead). Photos by FB.

it receives the openings of erythrophilic shell glands at its proximal portion, near its communication with the atrium and the oviducts (Figure 5A, C).

Some specimens had an egg capsule in their genital atrium. The brown egg capsules were located just in front of the posterior refringent patch (Figure 4C) and measured about 0.26–0.29 mm in diameter (Figure 4D).

Location and associated flora and fauna. Specimens were collected from two localities in Peru, viz., (1) vegetated, secondary channels in the Chillón River (Figure 3B) and (2) ponds at Humedales de Ventanilla (Figure 3C), a protected wetland area where groundwater is fed by three sources: rainfall, marine infiltration, and freshwater from the Chillón river basin (Gobierno Regional del Callao 2009). Interestingly, these ponds are next to the ocean, and, thus, the water is brackish, with conductivities measuring up to 18 mS/cm (Román 2018). In the river Chillón, *P. kazukolinda* was found together with the freshwater planarian *Girardia* sp. and species of microturbellarians (Reyes and Brusa 2017; Reyes et al. 2022), in association with the algae *Enteromorpha* sp. and *Cladophora* sp., which were present on the muddy littoral between rocks. At Humedales de Ventanilla, *P. kazukolinda* was highly associated with the algae *Chara* sp., which grows in the muddy littoral zone (Reyes and Brusa 2017), and on which we found the egg capsules of *Pentacoelum* to be deposited, which gave rise to 4–5 hatchlings per capsule. We did not find any mollusc species at these locations.

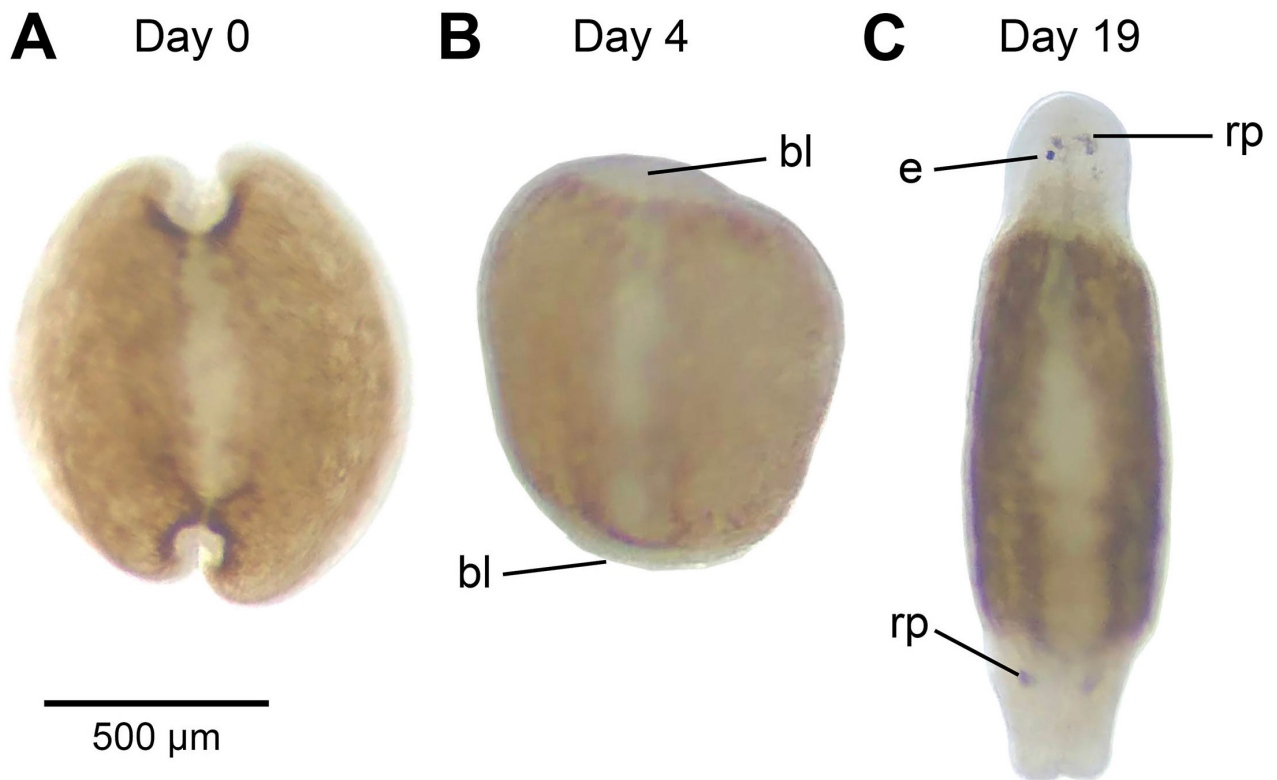
#### *Record of Pentacoelum kazukolinda in the USA*

Identification, location, and habitat. A single worm was collected from a freshwater artesian well that is located 2 km from the Florida coast (Figure 3D); the smell of the water suggested that it was presumably rich in sulphur. When still alive, the animal showed the characteristic pattern of refringent dorsal patches and the dimensions and body shape of *P. kazukolinda*. The specimen did not survive under laboratory conditions and, after that, deteriorated. The animal was identified by MVF, who is well-acquainted with the habitus of the species, solely based on its external appearance.

#### *Regeneration*

Worms did not survive longer than 30–35 days under laboratory conditions. Nevertheless, this gave us about 20 days to observe the regeneration process, during which regenerating head, trunk, and tail pieces were observed for several days. Trunk fragments were able to regenerate anterior and posterior regions. Head and tail fragments were also capable of regeneration. Wound contraction occurred about 30 minutes after amputation (Figure 7A). Anterior and posterior blastema formation was visible from day 3–4 post-amputation (Figure 7B). By day 19, regenerating pieces showed visible eyes and refringent patches (Figure 7C). Although the animals are well capable of regeneration, we did not find evidence in the field of asexual reproduction by fission.





**Figure 7.** *Pentacoelum kazukolinda*, regeneration. (A) Live trunk piece at the day of head and tail removal, showing wound contraction; (B) Live trunk piece 4 days after amputation, showing anterior and posterior regeneration blastemas; (C) Regenerating, live trunk piece 19 days after amputation, showing regenerated refringent patches and eyes. Photos by CVD.

### Discussion

Molecular data incontrovertibly identified the animals from Chile as specimens of *P. kazukolinda*, while anatomical features did the same for specimens from Peru. From these molecular and histological results, it is evident that the highly characteristic external appearance of *P. kazukolinda* coincides with its genetic and anatomical markers. Therefore, we feel confident that the single specimen from Florida, which was only observed while still alive and then showed the characteristic pattern of refringent dorsal patches, also represented *P. kazukolinda*. With respect to anatomical features, it is noteworthy that the group of occluding cells that is interpolated between the oviducts and their openings into the lateral bursae was also depicted in the reconstructions of the holotype and the paratype of the species (cf. Sluys 1989, figures 222, 223).

Based on their evaluation of the situation at that moment, Sluys et al. (2015) predicted the occurrence of *P. kazukolinda* in North and South America. Here, we corroborate this prediction by reporting the species from four localities in three countries: Chile, Peru, and continental USA. In their research, Sluys et al. (2015) were able to study the prey preference of *P. kazukolinda* by examining the gut content of the worms through an analysis of a mitochondrial gene sequence, the cytochrome c oxidase subunit 1 (COI), and a nuclear sequence, the ribosomal subunit 16 (16S).

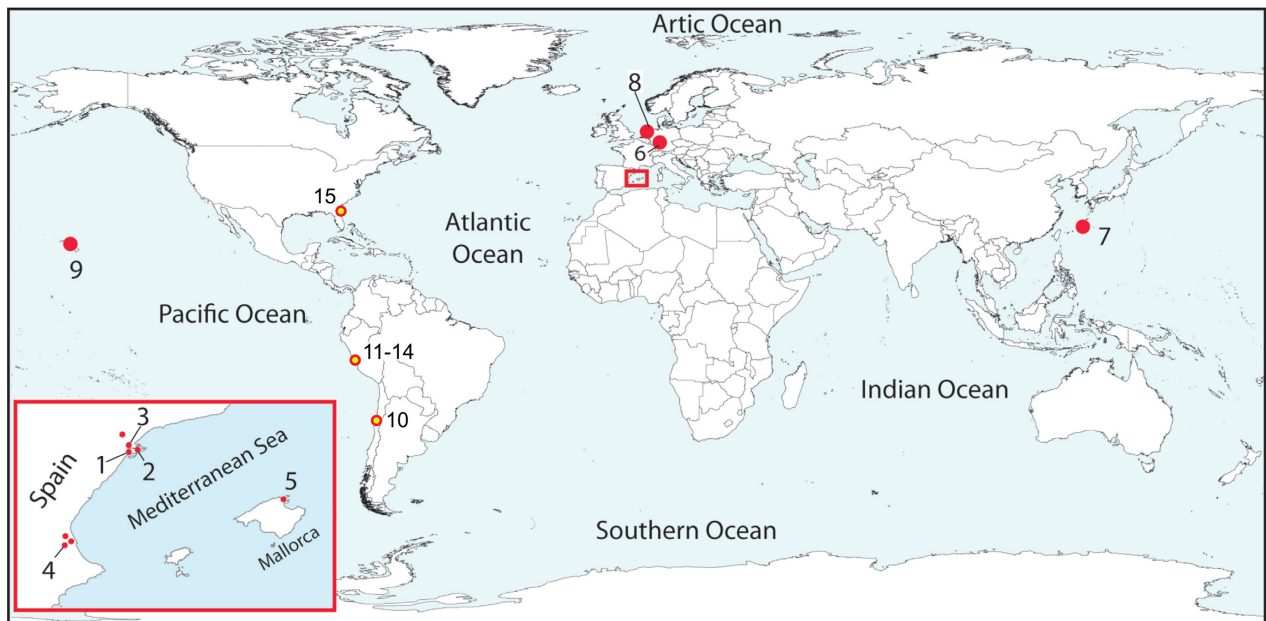
This revealed that the flatworms preferred gastropods, while in one population, they also fed on annelids. The New Zealand mud snail *P. antipodarum*, in particular, was abundantly present in the diet of the flatworms at all localities examined by these authors (Sluys et al. 2015). Although, in the present study, we were unable to obtain COI sequences from the gut content of the worms, it is interesting that specimens of the invasive molluscs *P. antipodarum* and *P. acuta* have been collected at the same sampling locality in Chile (Collado 2014, 2017), and an undetermined species of *Physa* Draparnaud, 1801 and the African snail *Melanooides tuberculata* (Müller, 1774) were collected at the same locality in Peru (Vizcardo and Gil-Kodaka 2015). Thus, it may well be the case that also at these localities, these molluscs, and notably *P. antipodarum*, form the food refuge of *P. kazukolinda*.

It is interesting to relate here the two facts that the presence of larval trematodes was established in specimens from Spanish populations of *P. kazukolinda* (Vila-Farré et al. 2008; Sluys et al. 2015) and that specimens of the snail *P. antipodarum* may be infested with several larval stages of trematodes as well (Gérard et al. 2003). In the planarians, the larval stages (metacercaria) were located in both the gut and the mesenchyme, and thus, they may have acquired the parasites through consumption of their mollusc prey. It was not clear if the planarians were hosts themselves or not.

It is noteworthy that the ponds at Humedales de Ventanilla in Peru are brackish, so that *P. kazukolinda* is capable of living under such conditions. Similarly, Vila-Farré et al. (2008) reported *P. kazukolinda* from brackish waters of the Ebro River.

Our observations that the egg capsules produced by *P. kazukolinda* are devoid of a stalk and measure 0.26–0.55 mm in diameter (Figures 1B, 4D) and that 4–5 juveniles hatch from each capsule, agree with earlier observations that the egg capsules measure 0.3–0.7 mm in diameter and that the number of hatchlings varies between 1 and 7, with an average of 4 juveniles (Vila-Farré et al. 2008). Although earlier studies reported that hatchlings were unpigmented, apart from the refringent spots (Vila-Farré et al. 2008; Sluys et al. 2015), juveniles from Chile (Figure 1C) and Peru turned out to be lightly pigmented.

The regenerative ability, typical of triclads, was previously mentioned by Vila-Farré et al. (2008) when preparing worms for karyological analysis. We observed that *P. kazukolinda* quickly contracts the amputation site and generates anterior and posterior blastemas within 3–4 days, as is the case in other planarian flatworms. Eyes and refringent patches were also regenerated and were visible from about day 15 of regeneration. We could not observe the complete regeneration process, resulting in a fully pigmented and proportional organism, because the animals did not survive for long periods under laboratory conditions. This may have resulted from the fact that the Montjuïc planarian water for laboratory cultures is not optimally suitable



**Figure 8.** Distribution of *Pentacoleum kazukolinda*. Filled red circles: previously known localities. Red circles, filled yellow: new localities of the specimens presented in this study (Table S1). Rectangular inset, detail of the Iberian Mediterranean coast. 1–3. Tarragona, Catalunya; 4. Xuquer River, Antella, Valencia; 5. Torrent Sant Jordi, Pollença, Mallorca; 6. Neckar River, Heidelberg, Germany; 7. Okinawa, Japan (two sampling sites); 8. Harlingen, The Netherlands; 9. Manoa Stream, Oahu, Hawaii (type locality); 10. O’Higgins Park, Santiago, Chile; 11, 12. Chillón River near Trapiche, Canta Province, Lima, Perú. 13, 14. Humedales de Ventanilla, Ventanilla district, Lima, Perú. 15. Florida, USA. Localities 1–9 were extracted from Sluys et al. (2015).

for many planarian species. It was originally formulated to mimic the water composition of an artificial spring in Barcelona, hosting a population of the model species *Schmidtea mediterranea* (Benazzi et al. 1975).

In view of its worldwide distribution (Figure 8, Supplementary material Table S1), we can conclude that *P. kazukolinda* has successfully overcome potential limitations of its transport, establishment, and spread. Sluys et al. (2015) suggested that the dispersal of *P. kazukolinda* is facilitated by the introduction of aquatic plants, such as Taro *Colocasia esculenta* (Linnaeus, 1753) Schott. Although *C. esculenta* occurs at a locality near Humedales de Ventanilla, Peru, we did not find *P. kazukolinda* at this particular spot. Furthermore, *C. esculenta* is not present at the Chinese botanical garden in Santiago, Chile. Nevertheless, our new findings lend support to the hypothesis that the dispersal of *P. kazukolinda* is facilitated by the introduction of aquatic plants, such as macrophytes, present at the Chile and Peru localities, and species of *Chara* Linnaeus, 1753. *Chara* plant species are mainly distributed in the Northern Hemisphere (the USA and Europe), but were also found in the Humedales de Ventanilla, Peru, associated with *P. kazukolinda*, which uses these plants as a substrate for depositing its egg capsules. These plants can potentially host both the planarian and its prey species during transport. In this way, the flatworm is transported with its ecosystem, thus, having available the main component of its diet immediately upon introduction. As the planarians appear to feed on several species of gastropods and annelids, the presence of such potential prey species at the introduction site may greatly facilitate the settlement of *P. kazukolinda*.



Interestingly, the sampling site of *P. kazukolinda* in Florida is also of artificial origin, a characteristic shared by all other localities inhabited by the species. This supports the notion that *P. kazukolinda* reached continental USA also by means of human-mediated dispersal.

The snails *P. antipodarum*, *P. acuta* and *M. tuberculata* currently are globally invasive species (Vaz et al. 1986; Taylor 2003; Alonso and Castro-Díez 2008; Collado 2014, 2017), being native to New Zealand, North America, and NE Africa and SE Asia, respectively. At this moment it remains an open question whether *P. kazukolinda* was originally also a native of New Zealand and from there started its spread over the world, effectuated by the international plant trade. The first step in the elucidation of this enigma is to determine whether presently the flatworm occurs in New Zealand.

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### Authors' contribution

CVD, FB, and JR prepared material, all authors contributed to the analysis of the data. CVD prepared figures and the first draft of the manuscript. All authors contributed to subsequent versions of the manuscript, with RS editing the text. All authors read and approved the final manuscript.

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### Supplementary material

The following supplementary material is available for this article:

**Table S1.** Geographic coordinates of sampling localities.

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