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From Korea to Croatia: the first record of the polyclad flatworm *Notocomplana koreana* in the Mediterranean

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Abstract

Polyclads are large-bodied, free-living flatworms that occur in diverse marine habitats. They are known predators of crustaceans, ascidians, snails, bivalves, polychaetes, and other flatworms. The acotylean genus *Notocomplana* Faubel, 1983 currently comprises 32 species that primarily inhabit the Pacific but are also found in the Atlantic and Indian Oceans. Here, we report the discovery of *Notocomplana koreana* (Kato, 1937) in the Mediterranean Sea. *N. koreana* was originally described from Korean and Japanese waters but was recently found to have expanded its range to the Atlantic. In this report, we shed light on the likely route of migration of *N. koreana* to the Mediterranean and discuss the challenges in identifying such an inconspicuous species. Through an integrative taxonomic approach, which merged morphological trait analysis using live animals and histology with a comparative genetic analysis of partial 28S ribosomal DNA and mitochondrial cytochrome c oxidase subunit I (*COI*) barcoding markers, we uncovered the characteristics of this introduced species and provide insights into its potential effect on Mediterranean ecosystems.

Keywords: integrative taxonomy; morphological identification; genetic characterisation; invasive species.

Introduction

Polyclads are free-living, predatory flatworms that primarily occur in marine environments. More than 1,000 species of polyclads have been described worldwide (Tyler *et al*., 2006-2024) in various habitats, from rocky shores to coral reefs. The Polycladida are divided into two suborders, Cotylea and Acotylea, based on the existence or lack of a sucker (cotyl) and marginal tentacles (Lang, 1884; Dittmann *et al*., 2019a).

The genus *Notocomplana* belongs to the suborder Acotylea and is part of the superfamily Leptoplanoidea Faubel, 1984. It is characterised by paired tentacular and cerebral eye clusters and the presence of a single male copulatory apparatus consisting of a true seminal vesicle and an elongate or blunt unarmed conical penis papilla and separate gonopores. The female copulatory apparatus possesses a Lang's vesicle, which functions as a seminal receptacle and a muscular vagina bulbosa (Faubel, 1983). The genus belongs to the monotypic family Notocomplanidae Litvaitis, Bolaños & Quiroga, 2019, which unites all former Notoplanidae that lack a penis stylet. Currently, 32 species are recognised as belonging to the genus (Tyler *et al*., 2006-2004).

The species *Notocomplana koreana* was first described from the coast of Korea by Kato (1937) and recently revised by Oya & Kajihara (2017) along with several other molecularly and morphologically similar species found in Japan (Oya & Kajihara, 2017). Based on these descriptions –and combined with a comparative genetic analysis of partial 28S ribosomal DNA and mitochondrial cytochrome c oxidase subunit I (*COI*) barcoding markers– new material from the Mediterranean was determined to be *N. koreana*. While *N. koreana* originates from the Northwest Pacific Ocean, it was recently introduced to the Atlantic (Gittenberger *et al*., 2023), but this is the first record of the species in the Mediterranean.

Materials and Methods

Animals

Adult animals were obtained in Rovinj, Croatia on 29 March 2017 (#1-#6) and 13-18 May 2019 (#7-#8). They were collected from the underside of rocks from the intertidal zone of the Mediterranean Sea. Eight animals were used for molecular investigations (#1-#8), of which five (#1, #2, #5, #6 and #8) were selected for histological research based on their large size and superior fixation quality. Histological sections and drawings were based on specimens #5 and #6, as the other sectioned individuals were inadequate.

Histology

After removing a small tissue sample from the side for molecular analysis, specimens #1, #2, #5, #6 and #8 were fixed using techniques from Lee *et al.* (2006) and Dittmann *et al*. (2019b). First, the specimens were placed on filter paper with a frozen fixative consisting of 3.5% formaldehyde in phosphate-buffered saline (PBS). Cold fixative was added to prevent dehydration, and a soft brush was used to keep the specimens flat. They were left for 12 days at 4 °C and then washed with PBS and distilled water before being dehydrated with an increasing ethanol series and cleared using methyl benzoate overnight and benzene for 30 minutes. They were left overnight in a 1:3 benzene:paraplast solution and embedded in paraplast (Leica, Germany). The five embedded specimens were then sagitally sectioned at approximately a 5-7 µm thickness and stained with an Azan trichromic stain, according to Romeis (1968).

Documentation

A live animal was photographed with a Windows Phone 7 (Fig. 1A). The stained sections were photographed with a Leica DM 5000B compound microscope equipped with a Leica DFC 490 digital camera. Images were processed in Adobe Photoshop 7, and drawings were produced on a Samsung Galaxy S3 tablet.

DNA extraction, PCR amplification and sequencing

DNA was extracted from the tissue samples of eight specimens kept at -20 °C in 99% ethanol. A phenol-chloroform DNA extraction was used following Chen *et al.* (2010).

Previously published primers, 28S_1F and 28S_6F (Álvarez-Presas *et al*., 2008), were used to amplify partial 28S rDNA markers from specimens #1–#8 (see Table 1). The polymerase chain reaction (PCR) used Taq DNA

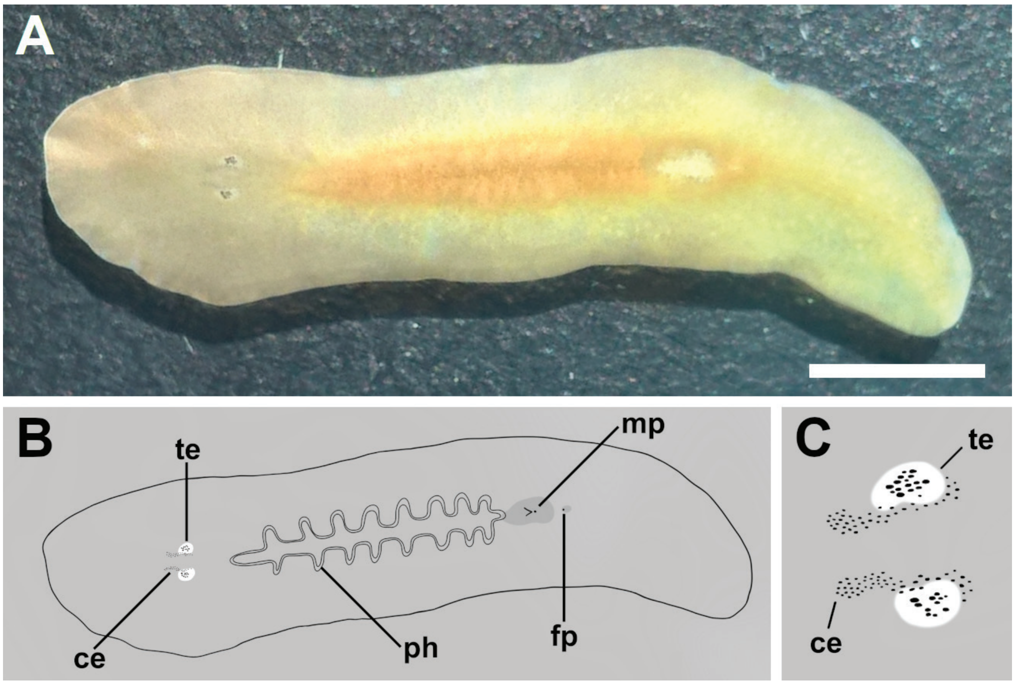

Fig. 1: Overview of *Notocomplana koreana* found in the Mediterranean Sea. A- Picture of a live animal. B- Schematic drawing of the same specimen. C- Detail of the eyes in the region of the brain. All panels are directed anteriorly and to the left. *ce* cerebral eyes, *fp* female gonopore, *mp* male gonopore, *ph* pharynx, *te* tentacular eyes. Scale bar in A: 2.5 mm.

Table 1. Primer sequences used for genetic investigation.

Primer name	Gene	Primer sequence (5’–3’)	Source
28S_1F	28S	TATCAGTAAGCGGAGGAAAAG	Álvarez-Presas *et al*. 2008
28S_6R	28S	GGAACCCCTTCTCCACTTCAGT	Álvarez-Presas *et al*. 2008
Acotylea_COI_F	*COI*	ACTTTATTCTACTAATCATAAGGATATAGG	Oya & Kajihara, 2017
Acotylea_COI_R	*COI*	CTTTCCTCTATAAAATGTTACTATTTGAGA	Oya & Kajihara, 2017

polymerase (New England BioLabs, USA), 1 µl each of forward and reverse primers and between 1 µl and 4 µl of DNA template. The 28S-standard PCR protocol was as follows: 5 minutes of denaturation at 94 °C and 35 cycles of the following steps: 30 seconds of denaturation at 94 °C; an annealing temperature of 53 °C for 30 seconds; and a 2-minute extension at 72 °C. This was followed by a final extension at 72 °C for 10 minutes. Two specimens, #4 and #7, were used to obtain *COI* sequences. For *COI* markers, previously published Acotylea_COI_F and Acotylea_COI_R primers (Oya & Kajihara, 2017) were used with the following *COI* PCR protocol: 94 °C for 5 minutes; then 35 cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1.5 minutes and a 7-minute extension at 72 °C. The amplicons were then purified with the Wizard SV gel and PCR clean-up system (Promega, USA) following the manufacturer's protocol. Finally, PCR products were sequenced by Microsynth Austria GmbH using the PCR primers. All obtained sequences were submitted to NCBI GenBank.

Phylogenetic reconstructions

Published sequences on Genbank of Notocomplanidae and *Notoplana delicata* as an outgroup were aligned using MAFFT v7.490 (Katoh & Standley, 2013) with the parameters '--auto' and '--maxiterate 10000'. After alignment curation using Gblocks 0.91b (Talavera & Castresana, 2007) with the least stringent parameters, the resulting matrix for 28S was 670 nt and for *COI*, it was 434 nt in length. Phylogenetic trees using maximum likelihood (ML) were generated with IQTREE v2.1.3 (Minh *et al.*, 2020) with 100 bootstrap replicates using the parameters '-m MFP -nt AUTO'. Phylogenetic reconstructions using Bayesian inference (BI) were calculated with MrBayes v3.2.7a (Ronquist *et al.*, 2012). Trees were visualised with FigTree 1.4.3.

Results

Taxonomy

Rhabditophora Ehlers, 1985
Order Polycladida Lang, 1881
Suborder Acotylea Lang, 1884
Superfamily Leptoplanoidea Faubel, 1984
Family Notocomplanidae Litvaitis, Bolaños & Quiroga, 2019
Genus ***Notocomplana*** Faubel, 1983
Notocomplana koreana (Kato, 1937) (Figs. 1-2)

Material examined. N. koreana specimens obtained in Rovinj (45.0833° N, 13.6333° E) in 2017 and 2019.

Appearance. The live animal is approximately 20 mm long and 8 mm wide. The body is oval-shaped, narrowing posteriorly but broad at the anterior end with small tentacular knobs. The dorsal surface exhibits a white-yellowish colour with brown spots and a darker brown area indicating the pharynx. The male gonopore was observed as a white spot behind the pharynx, followed by the separate female pore as a smaller white spot (Fig. 1A). Marginal areas are translucent, and the digestive tract is highly branched. The animal possesses tentacular and cerebral eye clusters along both sides of the median line (Fig. 1B, C).

Reproductive system. The male gonopore is directly posterior to the pharynx (Fig. 2A). It consists of an unarmed penis papilla, a true seminal vesicle and an interpolated prostatic vesicle (Fig. 2C, E).

The male genitals are directed anteriorly (seen from the gonopore). The common sperm duct ends in a muscular, kidney-shaped seminal vesicle connected to the muscular prostatic vesicle. The prostatic vesicle contains a glandular epithelial lining folded into six longitudinal tubular chambers enclosing the intra-prostatic ejaculatory duct (Fig. 2B). The ejaculatory duct projects into the penis papilla, which is without a stylet. The penis papilla can be pushed through a large, ciliated male atrium and out through the male gonopore (Fig. 2C, E).

Situated posteriorly to the male pore is the female genital opening (Fig. 2E). The female genitals are directed posteriorly –as seen from the gonopore (Fig. 2D, E). The common oviduct runs upward dorsally to join the vagina. Posterior to the oviduct, Lang's duct leads to a relatively small, spherical Lang's vesicle. Anterior to the oviduct, the internal vagina leads into the muscular external vagina (vagina bulbosa) that ends in the female gonopore (Fig. 2D, E).

Sequence analysis

We obtained eight partial 28S rDNA sequences (1,575 bp long) for specimens #1–#8 with consecutive GenBank accession numbers (PQ164443–PQ164450), which were identical after trimming ambiguous parts at the 5' and 3' ends. The BLASTn of the trimmed sequence returned 99.9%–100% similarity with published sequences of *Notocomplana japonica*, *Notocomplana hagiyai* and *N. koreana* (data not shown).

We also obtained partial *COI* mtDNA sequences for specimens #4 and #7 (GenBank accession numbers PQ165297.1 and PQ165298.1), which differed in 2 of 769 bps (0.26%) after trimming ambiguous parts at the 5' and 3' ends. The BLASTn of the trimmed sequences returned 98.6%-99.58% identity with published *N. koreana* sequences, followed by up to 95.22% identity with published *N. hagiyai* sequences and up to 91.16% identity with published *N. japonica* sequences.

Phylogenetic analysis

We performed separate phylogenetic analyses with the 28S and the *COI* sequences using ML and BI approaches, with similar results. For simplicity, only the ML trees (Fig. 3) are shown here. We found that 28S was unsuitable for determining *Notocomplana* at the species level, as the 28S trees showed that *N. japonica*, *N. koreana* and

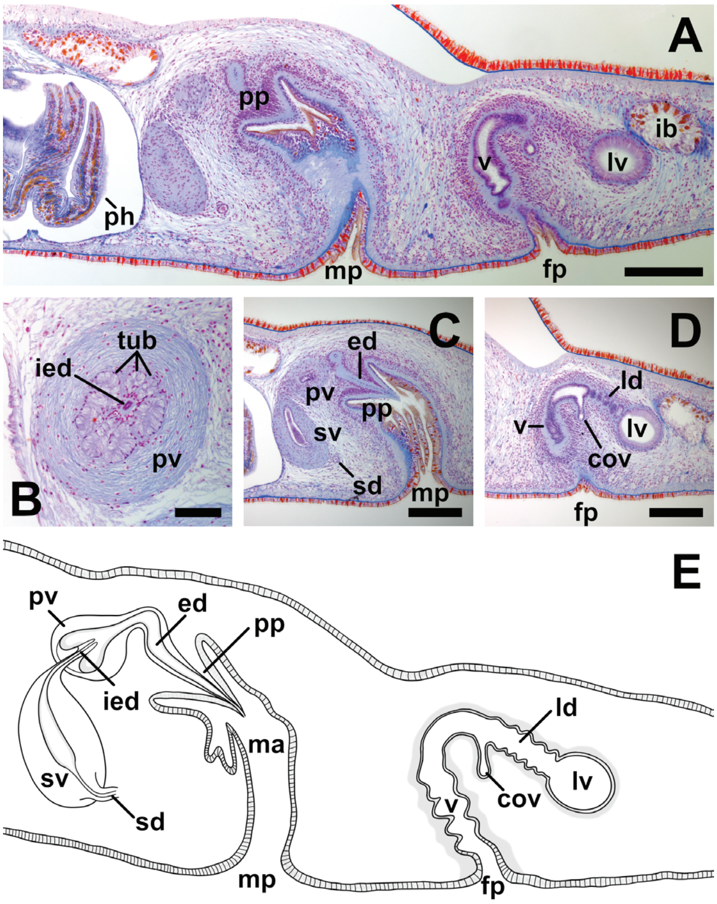

Fig. 2: Histology of the male and female genital apparatus of *Notocomplana koreana*. Sagittal sections. A- An overview of the male and female genital apparatus. B- Prostatic vesicle. C- Male genital apparatus. D- Female genital apparatus. E- Reconstructions of the male and female genital apparatus. All panels are directed anteriorly and to the left. *cov* common oviduct, *ed* ejaculatory duct, *fp* female gonopore, *ib* intestinal branch, *ied* intra-prostatic ejaculatory duct, *ld* Lang's duct, *lv* Lang's vesicle, *ma* male atrium, *mp* male gonopore, *ph* pharynx, *pp* penis papilla, *pv* prostatic vesicle, *sd* spermiducal duct, *sv* seminal vesicle, *tub* tubular chambers in the prostatic vesicle, *v* vagina. Scale bars in A, C and D 200 µm and in B 100 µm.

N. hagiyai were grouped together and indistinguishable (Fig. 3A). However, the *COI* trees provided clear resolution between the congeners (Fig. 3B).

Discussion

Species determination

Morphologically, the presence of paired tentacular and cerebral eye clusters, a single male copulatory apparatus with a true seminal vesicle and an unarmed penis papilla, as well as the female apparatus possessing a Lang's vesicle and a vagina bulbosa led to the determination of *Notocomplana* (Faubel, 1983) as the genus.

This determination was further narrowed down to four comparable species based on similar body size and colour, the presence of small tentacular knobs, the amount and positioning of the eyes, the distance between the male and female genital pores and the approximate shape of Lang's vesicle (Oya & Kajihara, 2017) to *N. japonica*, *N. koreana*, *N. septentrionalis* and *N. hagiyai*. All other described congeners did not fit the description of one or more of these organs.

N. hagiyai was newly described by Oya & Kajihara in 2017, and the other three species were revised alongside it. All four species are difficult to distinguish based on their external morphology. They are similar in overall shape, position of the eyes and colouration. However, one morphological trait that separates *N. koreana* from the other three species is the presence of a common sperm duct. Another distinctive trait is the shape and size of Lang's vesicle (see Fig. 2A, E). This vesicle is very small and spherical in *N. koreana* compared to its congeners, all of which possess a much larger and less uniform Lang's vesicle. Further, the authors have distinguished the species by the structure of the prostatic vesicle, specifically the number of tubular chambers. In *N. koreana*, there are six (see also Fig. 2B), while the other three species have more, ranging from eight chambers in *N. japonica* to eleven in *N. septentrionalis* (Oya & Kajihara, 2017).

Phylogenetic analyses support the morphology-based differences between these four species. However, *N. humilis*, the type species of *Notocomplana*, and not *N. septentrionalis*, is a sister group to *N. hagiyai*, *N. koreana* and *N. japonica* (Fig. 3A) (Oya & Kajihara, 2020). Analogous to Oya & Kajihara (2017), the molecular analysis of our specimens based on partial 28S rDNA yielded

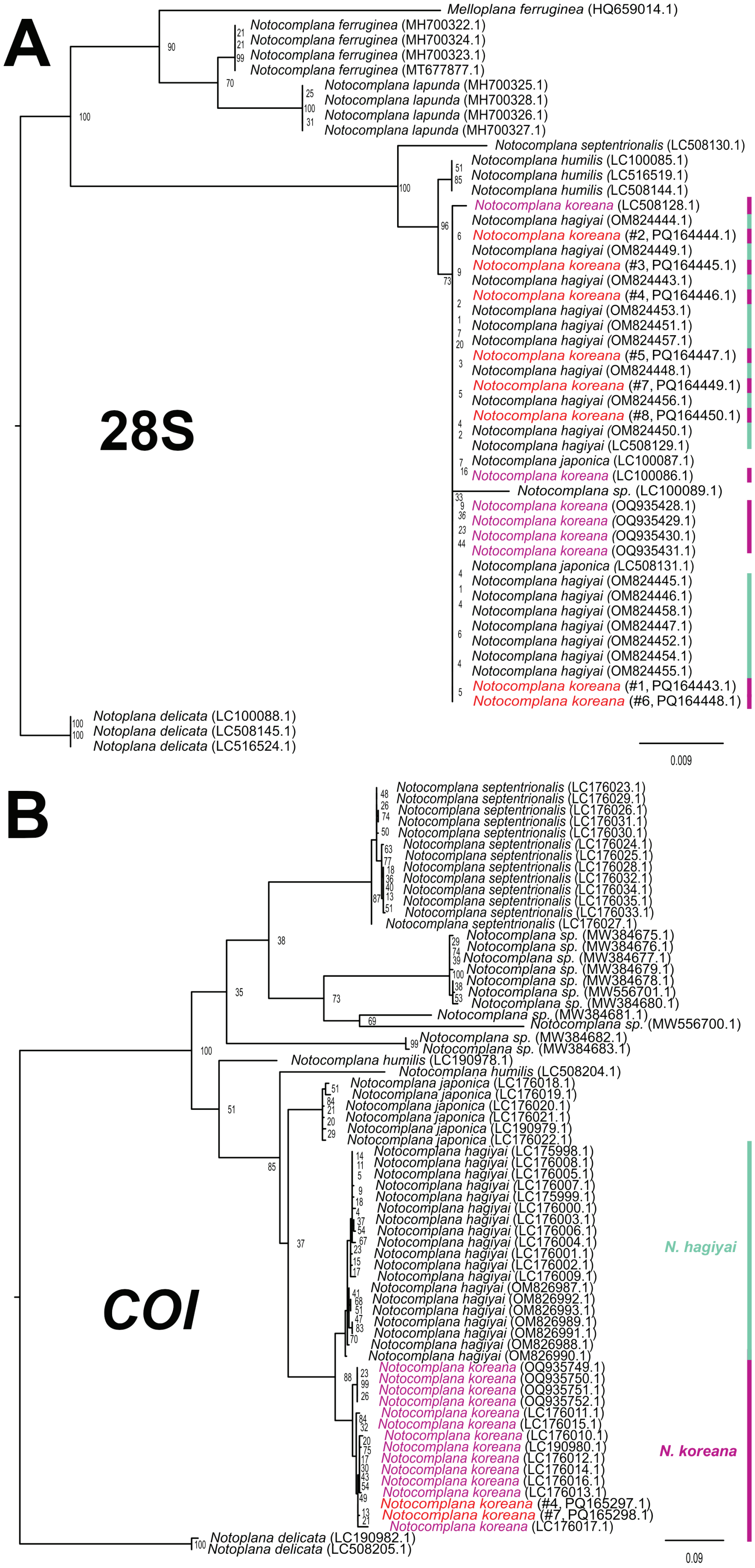

Fig. 3: Phylogenetic maximum likelihood reconstruction of Notocomplanidae using A- partial 28S sequences and B- partial *COI* sequences. Genbank accession numbers are in brackets. Scale bars indicate the number of substitutions per site.

less resolution than the analysis based on *COI* markers (Fig. 3A, B). This suggests a close relationship between *N. koreana*, *N. japonica* and *N. hagiyai*, where only the faster-evolving mitochondrial marker gene *COI* contains enough phylogenetically informative sites to distinguish between the species (Fig. 3A, B). The above-mentioned species –and several other *Notocomplana* species– have been described from Japan, and it is unknown whether they occupy separate ecological niches. Geographical, morphological and molecular data suggest a relatively recent speciation of *N. hagiyai*, *N. japonica* and *N. koreana*, and it is unknown if they are interbreeding.

Introduction to the Mediterranean

N. koreana is the only *Notocomplana* species reported to have been introduced to European waters, specifically in the Dutch North Sea (Gittenberger *et al*., 2023). In their broad survey of non-indigenous marine species, the authors remarked that flatworms are often especially difficult to determine based on their morphology, which leads to an underestimation of the number of introduced species. Human activity, namely ship traffic, likely plays a vital role in the dispersal and introduction of non-indigenous flatworm species. Ship hull fouling and ballast water are known pathways for flatworm displacement. However, establishing the exact time and locality of their primary introduction into European waters is challenging (Gittenberger *et al*., 2023). Confirming their presence in the Mediterranean makes it likely that they are also distributed across European waters apart from the Dutch and Croatian coasts. They could have spread from the Northern Atlantic to the Mediterranean either through human vectors or via natural dispersal through Gibraltar. Another possibility is that Mediterranean colonisation occurred independently from the North Atlantic colonisation, possibly via the Suez Canal. It is interesting to note that the sequences from the Adriatic and Japanese Seas are clustered together in our phylogenetic analysis, with the sequences from the North Sea (OQ935749-OQ9357552) forming a sister group (Fig. 3B). This suggests that the populations from the North Sea and the Adriatic have an independent origin and are not part of the same staggered colonisation event.

There are a number of other well-known polyclad species that have been introduced to the Mediterranean Sea. One is *Boninia neotethydis* Curini-Galletti & Campus, 2007, a species that originated from the Red Sea and likely entered the Mediterranean through the Suez Canal via Lessepsian migration (Curini-Galletti & Campus, 2007). This phenomenon describes the unidirectional migration of organisms from the Red Sea into the Mediterranean and has been observed since the opening of the Suez Canal in 1869 (Por, 1978). As *B. neothethydis* preys on Mediterranean mussel species, it can be regarded as an invasive species in the Mediterranean, affecting autochthonous mussel populations and aquacultures (Curini-Galletti & Campus, 2007). Other introduced species in the Mediterranean include *Pseudobicerus duplicinctus* Prudhoe, 1989, *Pseudobiceros stellae* Newman & Cannon, 1994 and *Maritigrella fuscopunctata* Newman & Cannon, 2000, which originally inhabited the Indian and Indo-Pacific Oceans (Velasquez *et al*., 2018). The acotylean polyclad *Echinoplana celerrima* Haswell, 1907 was first described in Australia and, more than seven decades later, was discovered in the Mediterranean (Galleni, 1978), where it is now commonly found (Gammoudi *et al*., 2012; Gammoudi *et al*., 2017).

Gathering information about the presence of introduced flatworm species is increasingly relevant given their roles in their respective ecosystems, either as predators or partners of symbiotic relationships. Like all polyclads, *Notocomplana* species are carnivorous and feed on small invertebrates such as crustaceans, molluscs and ascidians (McNab *et al*., 2021). This implies that their predation habits may also affect the populations of these communities in European waters. In contrast, they might influence the autochthonous species of polyclads through novel niche competition. Some flatworm species have already gathered attention as commercial pests, endangering the yields of shellfish and coral industries. Other species are important symbionts to other invertebrates, such as molluscs, echinoderms or corals (McNab *et al*., 2021). Further studies are needed to uncover the specific implications of *N. koreana* as an introduced species in Europe.

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