

## Research Article

# The Tanaidacea challenge to invasion science: taxonomic ambiguities and small size result in another potential overlooked introduction to the Iberian coast and nearby areas

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## Abstract

A major challenge in invasion science is detecting overlooked introductions, their pathways of introduction and spread. One of the most successful introduced taxa in aquatic ecosystems are peracarid crustaceans. There are a growing number of reports of accidental introductions of peracarids worldwide, mostly related to human transport hubs (e.g., ports and marinas). Tanaidaceans are especially abundant in these communities. Most frequently given examples of natural and anthropogenic passive dispersers belong to the family Tanaididae. However, their wide distribution requires confirmation. Most records come from 70–80' of last century, when identification of the species relied only on morphological characters. The small size and large intraspecific variation of tanaidids generate a high taxonomic uncertainty, as in the case of *Zeuxo turkensis*. Population of this species was previously known from Turkish, Japanese, and Australian coasts. In the two last places this tanaidid was identified as *Hexapleomera sasuke*, despite there were some premises that it should be synonymized with *Z. turkensis*. Here we investigate specimens that resembled both *Hexapleomera sasuke* and *Zeuxo turkensis* collected in marinas around the Iberian and Moroccan coasts. Integrating morphological and molecular methods (barcoding) we confirmed: (1) the first record and presence of well-structured populations of *Z. turkensis* in Spain, Portugal and Morocco, representing the first record of the species for Atlantic waters; (2) the conspecificity between *H. sasuke* and *Z. turkensis*, which should be synonymized; and (3) the wide distribution of *Z. turkensis* associated with human transport hubs (i.e. marinas) in the study area, showing its potential for introduction and spread. Integrated approaches and greater taxonomic support are key to advancing knowledge on the origin and invasion patterns of this and other small and poorly known human-mediated widespread species.



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**Key words:** Peracarida, Mediterranean, North Atlantic, morphology, non-indigenous species, COI barcoding

## Introduction

Non-indigenous species (NIS) are considered a form of biological pollution that is one of the greatest threats to world's oceans (Carlton and Geller 1993; Ruiz et al. 2000; Elliott 2003; Hayes and Sliwa 2003; Occhipinti-Ambrogi 2007). Significant efforts have been made in the last two decades to understand the pathways of introduction and spread of NIS and to develop reliable inventories on marine NIS. However, important knowledge gaps still exist in many areas, including European seas (Streftaris et al. 2005; Galil et al. 2014; Ros et al. 2015; Bailey et al. 2020), hindering our understanding of the global impact of this phenomenon.

One of the most important vectors for unintentional introductions in marine ecosystems are ships, since about 90% of the world trade is transported by this way (Kuhlenkamp and Kind 2018; Bailey et al. 2020). A wide variety of organisms are inadvertently transported either attached to the hulls or in ballast water tanks (Ruiz et al. 2000; Carlton 2003; Kuhlenkamp and Kind 2018; Desiderato et al. 2021). Upon arrival, transported species may (or not) adapt to human transport hubs (e.g., ports and marinas) of novel localities, and start to develop self-sustaining populations. Successful establishment and subsequent spread of NIS in these habitats depends on several factors, but fouling communities play a pivotal role on this process. Several studies have shown that fouling communities growing on artificial structures support an increased richness and abundance of NIS compared to those growing on natural hard substrates, such as nearby reefs of rocks, shellfish, or polychaete tubes (Wasson et al. 2001; Glasby et al. 2007; Tyrrell and Byers 2007; Marraffini et al. 2017). Habitat forming species of fouling communities act as reservoir of potential introduced epibenthic species, including small mobile epibionts that are frequently overlooked in monitoring studies of NIS (Ros et al. 2012; Johnston et al. 2017; Marraffini et al. 2017; Tempesti et al. 2022). One of the dominant groups of such assemblages, both in number of species and abundance, are peracarid crustaceans (Martínez-Laiz et al. 2018; Beermann et al. 2020; Saenz-Arias et al. 2022). Within peracarids, one of the most challenging taxa for NIS detection and monitoring are Tanaidacea. This is mainly due to their small size, “cryptic” behaviour, taxonomic uncertainties, and lack of taxonomic expertise among collectors.

Tanaidacea are small crustaceans commonly occurring in almost all marine habitats (Larsen 2005; Błażewicz-Paszkowycz et al. 2012). Like other representatives of Peracarida they lack planktonic larvae; their development takes place in marsupium. Majority of adults, mainly from one of two suborders of Tanaidacea: Tanaidomorpha spend most of their life hidden in self-constructed tubes (Sieg 1983). Due to semi-sessile lifestyle, development without free-living larvae and limited mobility capacity, majority of species are supposed to have narrow geographic ranges (Bamber 1998; Larsen 2005). Despite this general suggestion, many tanaids, mainly representatives of the shallow water family Tanaididae, are recorded from places separated by hundreds kilometres and/or by geographical barriers (Edgar 2008; Martínez-Laiz et al. 2019; Del Pasqua et al. 2022; Gagnon et al. 2022). Worldwide distribution of some species might be questionable, especially for records coming from XIX century or the beginning of the last century (e.g. *Hexapleomera robusta* (Moore, 1894), *Sine-lobus stanfordi* (Richardson, 1901), *Tanais dulongii* (Audouin, 1826), *Zeuxo coralensis* Sieg, 1980), when recognition of species was based only on basic morphological characters, thus they often could have been misidentified (Bamber 2012, 2014). However, as stated above, tanaidids are often found in marinas and ports, suggesting their potential for long-distance human-aided passive dispersal (attached to the ship hulls or via ballast water) (Hutchings et al. 1993; Larsen 2005, 2014). One of the most widespread genera is *Hexapleomera* (Edgar 2008; Morales-Vela et al. 2008;

Esquete and Fernandez-Gonzalez 2017; Wi et al. 2018). For example, the wide distribution of *Hexapleomera sasuke* Tanabe & Kakui, 2019 was recently confirmed using morphological and molecular methods by Tait et al. (2021).

*Hexapleomera sasuke* was originally described from Japan (from Yakushima Island, Ishigaki Island and two public aquaria) (Tanabe and Kakui 2019). The authors also provided the cytochrome *c* oxidase (COI) barcode of the species (655 bp). Two years later, the species was recorded in a harbour of southern Australia and sequences of five Australian specimens appeared to be identical to the Japanese ones, while another one differed by a single mutation (Tait et al. 2021). However, the sequences of the Australian individuals matched not only with *Hexapleomera sasuke* but were also identical with the sequence of *Zeuxo turkensis* Larsen, 2014 described from the Turkey coast of the Mediterranean (Larsen 2014). Tait et al. (2021) provided detailed morphological comparison of their material with original descriptions of both species. But due to only partial overlap of the COI sequences (Mediterranean sequence is 389 bp long), the lack of sequences of other genes and no possibility to study the type material, they decided not to synonymize the two species.

An extensive sampling campaign of fouling peracarids in marinas around the Iberian Peninsula and Moroccan coasts provided a large collection of Tanaididae, including animals morphologically resembling both *H. sasuke* and *Z. turkensis*. Integrating morphological, molecular, demographical, and biogeographical data we aim to address the following goals:

1. Confirmation of the identity of this challenging tanaidid through morphological and molecular analysis as well as resolving the problem of possible synonymization between *H. sasuke* and *Z. turkensis*.
2. Determination of the establishment success of this tanaidid populations through demographic analysis along Iberian Peninsula and Moroccan coast.

Moreover, we compiled biogeographical data of *H. sasuke* and *Z. turkensis* to discuss the potential for wide distribution and human-mediated dispersal of the species.

## Material and methods

### Sampling surveys

The material was collected during two sampling campaigns, I in 2011 and II in 2017, conducted for the study of mobile epibionts associated to fouling communities in marinas along the Iberian Peninsula and nearby areas (Ros et al. 2015). Here we only consider those marinas where Tanaidacea was present. Sampling campaign I was carried out between May and July 2011. During this campaign material was collected from marinas along Atlantic and Mediterranean coasts of Spain and Portugal as well as along northern coast of Morocco (Figure 1, Table 1). In each marina, at least three colonies of the habitat-forming Bryozoa *Bugula neritina* Linnaeus, 1758, a common component of fouling communities of marinas and harbours, was collected by hand together with their associated epifauna (Ros et al. 2015). Tanaididae was present in 73 colonies collected from 32 marinas.

Sampling campaign II was conducted in April 2017. During this campaign mobile epibionts were collected from artificial passive collectors made of bath puffs and deployed in marinas of the southern coast of the Iberian Peninsula (Table 1). A total of 18 collectors were deployed at 1-meter depth for one month in each marina (see Revanales et al. 2022 for more details about methodology). Tanaididae was present in the three marinas, in a total of 40 collectors.

Additionally, a complementary campaign was carried out at a fishing port in the Sado estuary, Portugal, over the summer and spring of 2021. In order to capture mobile species associated with fouling communities, eight samples were scraped from distinct artificial structures, four in each season. Tanaididae were found in all the samples from both seasons.

All samples were preserved in 90% ethanol. Epifauna was sorted and identified to higher taxonomic level and Tanaidacea were taken for further analysis. The measurements of specimens were made with the help of a camera connected to the microscope (Nikon Eclipse Ci-L) using NIS-Elements View software ([www.nikoninstruments.com](http://www.nikoninstruments.com)). The following abbreviations were used for morphological description: “A1” – Antenna 1, “A2” – antennule, “P1” – “P6” – pereopod 1 – pereopod 6, “Pl” – pleopod, “NL:W” means “N times as long as wide”.

### Morphological and demographic analysis

For the analysis tanaidids from the two first sampling campaigns (in 2011 and 2017) were used. Tanaids were identified with help of relevant taxonomic literature and specimens were classified according to sex and development stage as: manca, neutrum, female with oostegites, brooding female, male or juvenile male. Term “manca” describes juveniles without pereopod-6 or only with buds of pereopod-6. Neutrum is a developmental stage after manca which cannot be determined as female or male and juvenile male is a stage in which the beginning of modification of antennules and cheliped can be observed (Larsen 2005).

Ten specimens: one neutrum, three females with oostegites, four brooding females, one male, one juvenile male and one not specified (only head available) were dissected, put into slides and compared with each other as well as with the descriptions of *Zeuxo turkensis* given by Larsen (2014) and *Hexapleomera sasuke* by Tanabe and Kakui (2019). For comparison, the list of 53 characters created by Tait et al. (2021) was used. Three characters were not considered (Pereonites 1 to 3 L:W, Pl 1 to 3 lateral setation and cheliped dactylus medial seta) due to lack of information (e.g., setation not well seen, broken individual etc.). All dissected individuals were later successfully used for DNA barcoding.

Demographic analysis was presented as percent contribution of particular stages and sex per marina. Differences in contribution of stages between areas of sampling (Atlantic and Mediterranean Sea) were checked using Kruskal-Wallis test.

### Molecular analysis

To provide molecular characterization of the studied taxon, a representation of individuals from each station (from one to five specimens) from the 2011 and 2017 campaigns was chosen. Total DNA was extracted from one chela with a mixture of 50 µl pure H<sub>2</sub>O with 0.005 g Chelex® (SIGMA-ALDRICH Co.) and 3.3 µl proteinase K. The digestion at 55°C lasted for 6 hours. The DNA barcoding fragment of cytochrome *c* oxidase subunit I gene (COI) (658 bp) was amplified using the degenerated LCO1490-JJ (5'-CHACWAAYCATAAAGATATYGG-3') and HCO2198-JJ (5'-AWACTTCVGGRTGVCCAAARAATCA-3') primer pair (Astrin and Stüben 2008) with the use of DreamTaq Green PCR Mastermix (Thermo Scientific) polymerase. Because amplification of the desired fragment often failed, an internal primer (m1COIint, 5'-GGWACWGGWTGAACWGTWTAYCCYCC-3', Leray et al. 2013) that allows to obtain the 313 bp long 3'-end fragment of the barcoding region of the COI was used. In both cases the reaction conditions followed Hou et al. (2007). Sequences were obtained by Macrogen Inc., the Netherlands on the Applied

Biosystems 3730xl capillary sequencer. The short fragments were sequenced in both directions (reverse sequencing for two individuals failed) while for the long ones only forward sequencing was successful. From the collection of 2021 four whole individuals were used for molecular analysis manipulation being done with tweezers, previously sterilized using an open flame. DNA extraction was done through kit – E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek), following manufacturer's protocol. The barcoding fragment of the COI gene was amplified after four attempts. Originally the pair of degenerate primers – LoboF1 and LoboR1 published by Lobo et al. (2013) and their recommended PCR protocol were used without any success. This led us to the reduction of the main annealing temperature from 54°C to 50°C for the third attempt but again without success. In the final, fourth trial the Lobo primers were exchanged by the universal LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TGATTTTGGTTCACCCTGAAGTTTA-3') primers (Folmer et al. 1994), as they were successfully used for Tanaididae (with emphasis on *Zeuxo* spp. and *Hexapleomera sasuke*) before (Larsen et al. 2014; Tanabe and Kakui 2019; Tait et al. 2021). The total PCR reaction volume was 25 µl, consisting of: 12.5 µl of Taq DNA Polymerase 2X Master Mix 1.5 mM MgCl<sub>2</sub> (VWR), 4 µl of DNA extract, and 1 µl of each primer. Ultrapure water was added to achieve the total reaction volume. Successful reactions conditions were the following: one minute of initial denaturation at 95°C, followed by 35 cycles of 60 seconds at 50°C and 90 seconds at 72°C. The final step of elongation was performed at 72°C for seven minutes. Purification of PCR products was done using Diffinity RapidTip25 (Diffinity Genomics®) purification tips, following manufacturer's protocol. Purified PCR products were sequenced by Eurofins Genomics, bi-directionally, using a BigDye Terminator 3 Kit (Applied Biosystems™), coupled with an ABI 3730XL DNA analyser (Applied Biosystems™).

DNA of one individual, characterized by the COI sequence, was used for the amplification of the histone 3 (H3) nuclear gene. It was amplified with the primer pair H3aF (5'-ATGGCTCGTACCAAGCAGACACGGC-3') and H3aR (ATATCCTTAGGGCATAGATAGGTGAC) (Colgan et al. 1998) and the following conditions: initial denaturation in 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 60 s and final elongation in 72°C for 15 min. Sequencing was performed bidirectionally in MacroGen Inc.

Electropherograms were viewed in Geneious 10.1.2 (sequences from the 2011 and 2017 collections) or MEGA-X (Kumar et al. 2018, sequences from 2021 collection) and primer sequences as well as ambiguous positions were trimmed. Sequences were initially blasted using default parameters on NCBI BLASTn (Johnson et al. 2008) and translated into amino acid sequences to confirm that no stop codons were present.

All sequences were deposited in GenBank with the accession numbers: [OQ922007–OQ922017](#) and [OR038200](#) (COI) and [OQ922006](#) (H3). Relevant voucher information, taxonomic classifications, and sequences are deposited in the dataset “DS-ZTURIP” (<https://dx.doi.org/10.5883/DS-ZTURIP>) in the Barcode of Life Data System (BOLD) ([www.boldsystems.org](http://www.boldsystems.org)) (Ratnasingham and Hebert 2007). The sequences were subjected to the Barcode Index Number (BIN) System (Ratnasingham and Hebert 2013) in BOLD.

The distribution of species was plotted on the World map using QGIS 3.16 (QGIS.org 2021). All graphics were adjusted for publication with the Adobe Illustrator CS6.

## Results

In total, 586 specimens were collected during the campaign I in 32 marinas along the Iberian and Moroccan coasts, and 297 specimens in the three marinas surveyed

in southern Spain during campaign II (Figure 1, Table 1). In this second survey the target species was recorded in two sites (Rota and Sancti Petri) in 2017 where it was absent in 2011. The survey in Sado estuary resulted in 1004 individuals but the demographic study of this collection will be provided later elsewhere. In the present work only the molecular data from this survey are used.

**Table 1.** Detailed information about sampled marinas and station number within marina.

	Marina	Number of stations within marina	Sampling date	Latitude, longitude (decimal degrees)	Salinity (psu)	Temp. (C)	Number of specimens
Atlantic 2011	Santander	5	02.05.2011	43.45, -3.82	34.27	18.43	14
	Gijón	1	04.05.2011	43.54, -5.67	35.57	16.97	1
	Grana	5	07.05.2011	43.48, -8.26	34.57	16.17	39
	A Coruña	2	04.05.2011	43.37, -8.40	35.67	15.97	1
	Cascais	1	09.05.2011	38.69, -9.42	34.40	19.53	3
	Albufeira	6	10.05.2011	37.08, -8.27	35.80	19.87	27
	Faro	2	11.05.2011	37.01, -7.94	36.33	21.03	3
	Isla Canela	3	16.05.2011	37.19, -7.34	36.07	23.00	44
	Chipiona	3	17.05.2011	36.74, -6.43	35.37	19.27	13
	Cádiz	2	17.05.2011	36.54, -6.38	36.30	19.90	8
	Conil	3	17.05.2011	36.29, -6.14	36.67	19.57	37
	Barbate	2	17.05.2011	36.19, -5.93	35.67	19.73	9
	Fuengirola	3	15.05.2011	36.54, -4.62	36.40	20.77	23
Mediterranean 2011	Benalmádena	1	13.05.2011	36.60, -4.51	36.40	20.47	11
	Málaga	2	15.05.2011	36.72, -4.41	36.40	24.00	88
	Caleta Vélez	2	03.07.2011	36.75, -4.07	36.53	24.33	21
	Reqvelis	3	01.07.2011	36.76, -2.61	36.90	26.13	31
	Almería	1	11.07.2011	36.83, -2.46	36.77	25.70	1
	Alicante	5	29.06.2011	38.34, -0.49	38.23	28.07	9
	Dénia <sup>1</sup>	1	28.06.2011	38.85, 0.11	37.03	26.70	90
	Valencia	1	28.06.2011	39.43, -0.33	37.77	27.80	1
	Borriana	2	28.06.2011	39.86, -0.07	37.70	26.37	1
	Orpesa <sup>1</sup>	1	28.06.2011	40.08, 0.13	37.90	25.37	1
	Tarragona <sup>1</sup>	1	27.06.2011	41.11, 1.25	38.00	25.83	2
	Vilanova Geltrú <sup>1</sup>	1	27.06.2011	41.21, 1.73	37.80	24.43	6
	Barcelona <sup>1</sup>	5	26.06.2011	41.38, 2.18	37.80	23.77	37
	L'Estartit <sup>1</sup>	3	25.06.2011	42.05, 3.21	36.60	22.20	20
	Tánger	2	31.05.2011	35.79, -5.81	36.03	19.37	5
	Ceuta	2	29.05.2011	35.89, -5.32	36.13	21.87	4
	Marina Smir	1	30.05.2011	35.75, -5.34	36.37	20.23	2
M'Diq	3	30.05.2011	35.68, -5.31	36.20	21.37	34	
Atlantic 2017	Cádiz, Puerto América	12	14.05.2017	36.54, -6.38	NA	NA	81
	Rota	18	14.05.2017	36.62, -6.35	NA	NA	214
	Sancti Petri	14	13.05.2017	36.40, -6.21	NA	NA	2
Portugal 2021	Setúbal, Portugal <sup>2</sup>	15	07.07.2021	38.51, -8.90	36.12	23.65	274

<sup>1</sup> Error in coordinates published in the original paper by Ros et al. 2015 corrected here, <sup>2</sup> only the sample from which the molecular data were obtained is included here.



**Figure 1.** General body appearance of studied tanaidid species collected from Iberian Peninsula. Photograph by Macarena Ros.

### Morphological analysis

Within 53 analysed characters 23 were shared between specimens from Iberian Peninsula and Moroccan coast and descriptions of *H. sasuke* and *Z. turkensis* (Table 2): labial palp was small, setulose and oval. On maxilliped coxa two setae were present, on palp article 1 no distal expansion was observed, on article 2 one lateral marginal seta was present. Lacinia mobilis of left mandible was wide, but of right mandible was reduced and peg-like. Females were bearing ventrodistal indent on cheliped merus. In both sexes ventral process was observed on cheliped

**Table 2.** Comparison of morphological characters based on Tait et al. (2021) between description of *Hexapleomera sasuke* from Japanese coast (from Tanabe and Kakui 2019), *H. sasuke* from Australia (from Tait et al. 2021), description of *Zeuxo turkensis* from Turkish coast (Larsen 2014) as well as individuals collected along Iberian Peninsula and used for molecular study (present data).

	<i>Zeuxo turkensis</i> Larsen, 2015	<i>Hexapleomera sasuke</i> Tanabe & Kakui, 2019 Japanese population	<i>Hexapleomera sasuke</i> Tanabe & Kakui, 2019 Australian population	<i>Zeuxo turkensis</i> Iberian population
<b>Antennule</b>				
Antennule article 1 length (L):width (W)	3 (f); 3.8 (m)	3.06	1.3–4.1 (f)	1.3–3.2 (f); 4.1 (m)
Antennule article 1 L to article 2 L	2.91	2.39	nd	0.7–2.9
Antennule terminal aesthetascs	4 (f); 5 (m)	3 (f); 6 (m)	nd	1–6 (f); 4 (m)
<b>Antenna</b>				
Antenna 7 <sup>th</sup> article presence	absent	present	absent	present
Antenna article 2 L:W	1.5 (f); 1.8(m)	1.3 (f); 2.2 (m)	nd	1.3–1.8 (f); 1.7 (m)
<b>Mouthparts</b>				
*Z Labial palp on outer lobe (size, shape)	present, small setulose oval	present, small setulose oval	nd	present, small setulose oval
Labial palp fused to outer lobe or not	unspecified	partly fused	nd	partly fused
*H Maxilliped coxa setae	2	2	nd	2
Maxilliped basis setae	2	1	nd	1–2
Maxilliped palp article 1 lateral margin expanded	rather not	rather not	nd	rather not
*Z Maxilliped palp article 1 lateral marginal setae	3	1	nd	1–2
*Z Maxilliped palp article 2 lateral marginal setae	1	1	nd	1
*H Maxilliped endite distal spiniform setae (each with)	2	2	nd	2 (hard to observe in some specimens)
Maxilliped endite distal plumose setae (each with)	2	2	nd	2 (hard to observe in some specimens)
*Z Left mandible lacinia mobilis	wide, digitate distally with 5 processes	wide, digitate distally with 5 processes	nd	wide, digitate distally with 5 processes
*Z Right mandible lacinia mobilis	reduced, peg like	reduced, peg like	nd	reduced, peg like
*Z Mandible setal row	2	2	nd	1–2
Maxillule palp articles	2	2	nd	1–2
Maxillule palp setae	5	4	nd	3–5
Maxillule endite terminal spiniform setae	8	8	nd	7–8
<b>Cheliped</b>				
Cheliped merus with ventrodistal indent	no (f); yes (m)	yes (m); yes (f)	nd	yes (m); yes (f)
Cheliped carpus L:W, (f)	1.4	1.5	nd	1.2–1.5
Cheliped carpus L:W, (m)	1.3	1.1	nd	1.3
Cheliped carpus L: propodus L, (f)	0.8	0.78	nd	0.6–0.8
Cheliped carpus L: propodus L, (m)	0.6	0.6	nd	0.6
Cheliped propodus W:carpus W, (f)	1	0.9	nd	1.0–1.3
Cheliped propodus W:carpus W, (m)	1.1	1.1	nd	1.3
*Z Cheliped carpus ventral process (f)	present	present	nd	present
*Z Cheliped carpus ventral process (m)	present	present	nd	present
Cheliped fixed finger proximal tooth-like apophysis (m)	present	present and absent	nd	present
Cheliped fixed finger cutting edge shape (f)	proximal invagination present; distal expansion into lamella with undulating dorsal surface	proximal invagination present; distal expansion into lamella with undulating dorsal surface	nd	proximal invagination present; distal expansion into lamella with undulating dorsal surface

	<i>Zeuxo turkensis</i> Larsen, 2015	<i>Hexapleomera sasuke</i> Tanabe & Kakui, 2019 Japanese population	<i>Hexapleomera sasuke</i> Tanabe & Kakui, 2019 Australian population	<i>Zeuxo turkensis</i> Iberian population
Cheliped propodus proximoventral process	present (f); present (m)	present (m); slight process present (f)	nd	present (f); present (m)
Cheliped dactylus ventral spinulation (f)	present	present	nd	present and absent
Cheliped dactylus ventral spinulation (m)	present	present	nd	present
Cheliped dactylus ventral apophysis (f)	absent	absent	nd	present and absent
<b>Pereopods</b>				
*Z Coxa 1 apophysis presence	absent	absent	nd	absent
Pereopod 1 (P1) propodus serrate or plumose strong medial seta	present	present	nd	present
Pereopods 2 & 3 carpal dorsodistal crotchet length cf. Propodus	less than 1/3	less than 1/3	nd	less than 1/3
Pereopods 2 & 3 propodus ventral setae	P2: 3; P3: 2	P2: 1; P3: 2	nd	P2: 2–3; P3: 1–3
Pereopods 2 & 3 propodus ventrodiscal spiniform seta	absent	absent	nd	absent
Pereopods 2 & 3 crotchets distal on carpi	4	4	nd	P2: 3–4; P3: 4–5
*Z Pereopods 4 to 6 crotchets distal on carpi	5	5	nd	4–5
Carpal crotchets form	with ancillary apical spine	with ancillary apical spine	nd	with ancillary apical spine
Pereopod 6 propodus leaf-like setae in mediodistal row	7	9	8–11	9–11
Pereopod 6 propodus dorsodistal setae (excluding subdistal setae)	2 longer	2 longer	nd	2 longer
<b>Pleopod</b>				
*Z Pleopods 1 & 2 basal article medial setae (plumose)	1	1	1	1
*Z Pleopods 1 & 2 basal article lateral setae (plumose)	6	5	5 to 7	4–7
*Z Pleopods 1 & 2 endopods medial setae	1	1	1	1
*H*Z Pleopod 3 basal article medial setae (plumose)	0	0	0	0
Pleopod 3 basal article lateral setae (plumose)	3	3	2–3	3–4
Pleopod 3 endopod medial setae (plumose)	1	1	1	1
Pleopod 3 versus 1 & 2	slight	slight	slight	slight
<b>Uropod</b>				
*H*Z Uropod segments (including basal segment, mature specimens)	4	4	4	4

\*Z diagnostic character of *Z. turkensis*; \*H diagnostic character of *H. sasuke*. “nd” – no data.

carpus. On males cheliped dactylus ventral spinulation was present. No apophysis was observed on coxa of P1, but in all specimens medial seta on propodus was present. Length of dorsodistal crotchet on carpus of P2 and P3 was less than 1/3 of propodus. Similar form of carpal crotchet, with ancillary apical spine, was observed within all tanais. On propodus of P2 and P3 no ventrodiscal spine was observed. On propodus of P6 two long setae on dorsodistal margin were present. P11 and P12 basal article were bearing one plumose seta, on endopod one medial seta was present, on P13 basal article no seta was present, while on endopod one

plumose seta was present. In all observed specimens reduction in size and in setation of P13 was visible. Uropod had four segments (including basal segment) in all observed specimens.

Within analysed characters 33 were variable among studied specimens. Variability was observed for: proportion of L: W on A1 and A2 articles (A1 article L:W 1.3–4.1; A1 length of article 1: article 2 0.7–2.9; A2 article 2 L:W 1.3–1.8), number of setae on maxilliped basis and article 1 (1–2), maxillulae palp and endite setation and articulation (palp 1–2 articles, 3–5 setae; endite 7–8 setae). Differences in ratio of L: W in cheliped carpus and propodus (female carpus L: W 1.2–1.5; male carpus L:W 1.1–1.3; length of female carpus to propodus 0.6–0.8 and width of carpus to propodus 1.0–1.3 in both male and female), spinulation and ventral apophysis on dactylus in females was observed only in some specimens. Moreover, there were differences in number of ventral setae on propodus of P2 and P3 (1–3 setae), number of crotchets on carpus of P2 to P6 (P2 with 3–4 P3 to 6 with 4–5), and number of leaf-like setae in mediodistal row on propodus of P6 (7–11). Variability was also observed in number of lateral setae on basal article of P11, 2 (4–7 setae) and P13 (3–4 setae).

The following characters were observed only in *Z. turkensis*, but not in other specimens: antenna with six articles (vs. seven in the rest of individuals), labial palp not fused (vs. partly fused), three setae on maxilliped palp 1 (vs. one – two) and seven leaf like setae on propodus P6 (vs. 8–11). Two characters were found only in *H. sasuke*: maxillule palp with four setae and P2 propodus with one ventral setae.

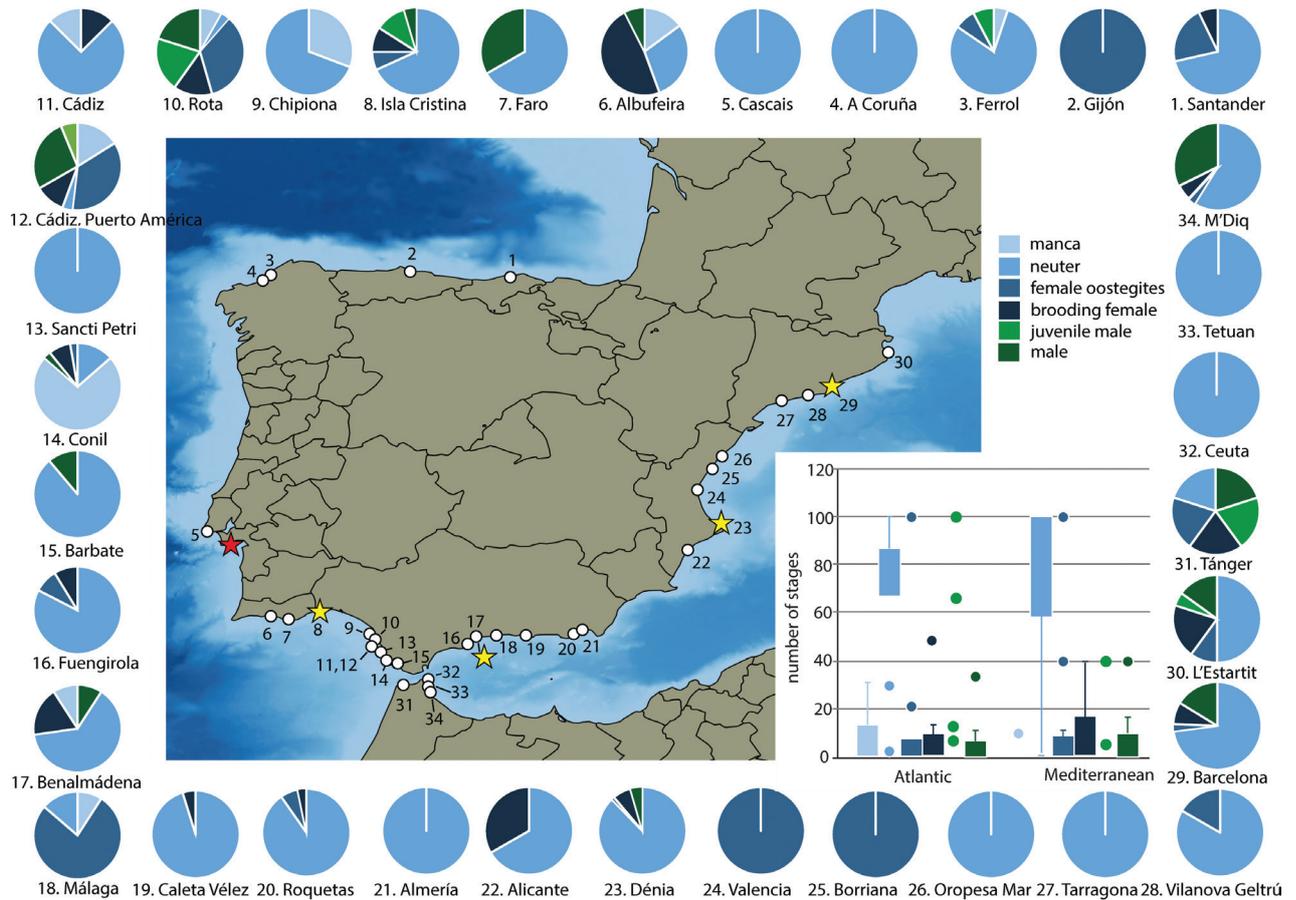
## Demographic analysis

Populations from the Iberian and Moroccan coast (campaign I) were dominated by neutrums, which represented 74%, female with oostegites constituted 2%, brooding females 10%, males 5%, juvenile males 1% and manca 4%. Figure 2 presents ratio of the six stages at each marina. No differences in the mean contribution of particular stages between marinas collected from Atlantic and Mediterranean were found (Figure 2).

Proportion between sexes and stages from campaign 2017 was: 45% of neutrums, females with oostegites constituted 4%, brooding females 11%, males 7%, juvenile males 13% and manca 18%.

## Molecular analysis

Although much effort was put into the molecular study of the COI of the available collection (103 individuals from 2011 and 2017 taken for DNA extraction) only eleven sequences were obtained. The more, only three of them were amplified with LCO1490-JJ/HCO2198-JJ primers making the final sequence over 600 pb long. In the case of the 2021 collection from Sado estuary in Portugal, after four attempts presented in the Methods section, only one sequence was obtained. The sequences were identical between themselves and showed 100% similarity to the sequence of *Zeuxo turkensis* (Larsen 2014; Larsen et al. 2014 – presented as *Zeuxo* sp. B) and all but one sequences of *Hexapleomera sasuke* (Tanabe and Kakui 2019; Tait et al. 2021) (Suppl. material 1). We managed to obtain molecular data for both Mediterranean and Atlantic coasts of Iberian Peninsula confirming the wide distribution of the studied species in this region and providing its first record from the Atlantic waters (Figures 2, 3; Suppl. material 2). The H3 sequence was identical with the already published sequence of *Zeuxo turkensis* (published by Larsen et al. 2014).



**Figure 2.** Distribution of *Zeuxo turkensis* at marinas along Iberian Peninsula and Moroccan coast with ratio of six stages of development: manca, neutrum, female with oostegites, brooding female, juvenile male and adult male at particular marinas and mean distribution calculated for Mediterranean and Atlantic Ocean. Star indicates the stations from where molecular data were obtained, red star – station sampled in 2021 from Sado estuary, Portugal (results of demographic study not presented here).

## Discussion

Our results show that the studied tanaidid species, which is recorded for the first time in Spain, Portugal, and Morocco, is a widely distributed species associated to human transport hubs (marinas and fishing ports in this case), and therefore it should be considered as a neocosmopolitan species. The species was identified as *Z. turkensis* that is conspecific to *H. sasuke*. Distribution of the species was so far confirmed in Mediterranean as well as Pacific (coast of Japan) and Indian (coast of Australia) oceans. Our findings also represent the first record of the species in Atlantic waters.

## Resolving taxonomic issues

Morphological analysis of 53 characters coupled with molecular analysis of two genetic markers revealed that *Hexapleomera sasuke* Tanabe & Kakui, 2019 shall be synonymized with *Zeuxo turkensis* Larsen, 2014.

All but one individuals studied by different working teams appear to be characterized by identical COI sequences (Suppl. material 1). Also, the H3 sequence of *Z. turkensis* published by Larsen et al. (2014) is the same as obtained by us for one individual. While apparently Tanabe and Kakui (2019) did not blast their sequences and as a result did not discuss the molecular identity between *Z. turkensis* and the species described by them, Tait et al. (2021) identified the molecular similarity of the individuals

collected in Turkey, Japan, and Australia. However, they decided not to synonymize the two taxa mostly because they could not examine specimens of *Z. turkensis*.

There are no species-specific characters neither for *Z. turkensis* nor *H. sasuke* (Table 2). The diagnostic features for *Z. turkensis* (Larsen 2014) were also found in the present study as well as in the Japanese and the Australian populations of *H. sasuke* (e.g., right mandible reduced in size, setation of labium, maxilliped, cheliped and pereopods, pleopods). At the same time, diagnostic characters of *H. sasuke* given by Tanabe and Kakui (2019) overlap with definition of *Z. turkensis* and were observed in individuals collected along the Iberian and Moroccan coast (maxilliped coxa with two setae, endite with two distal setae; pigmentation of pleopods, three outer setae on third pleopod basal article, uropod with four articles).

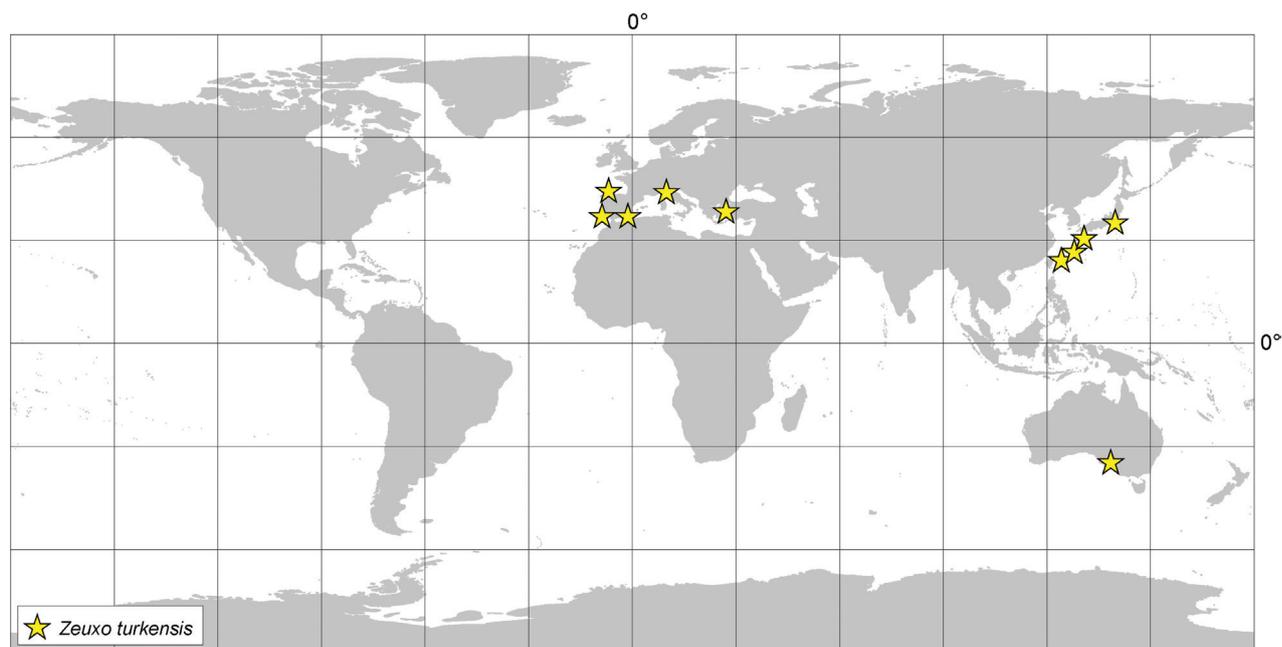
Careful comparison of our specimens with the description of Japanese, Australian and Turkish specimens, with character list by Tait et al. (2021), showed that, out of 53 characters, four were observed exclusively in *Z. turkensis* (e.g., articulation of antenna, fusion of labial palp, setation of maxilliped and pereopod 6). However, these characters were pointed out as not reliable for diagnosis, as number of articles in antenna is hard to detect, and fusion of labial palp with outer lobe depends on the viewing direction (Tait et al. 2021). Moreover, Larsen (2014) made description of his species basing only on one male and one female, so morphological intraspecific variability of Turkish population is not known. Setation of maxilliped and pereopod 2 distinguished *H. sasuke* from other individuals. Our observations indicate that number of setae on pereopods, maxilliped and maxillule palp is highly variable. Variability of number of setae was noticed not only within individuals collected from different marinas, but also between different replicates within the same marina or even between left and right appendages of the same individual. Consequently, the number of setae should not be used as diagnostic character.

To summarize, the combination of molecular identity noted for both mitochondrial (COI) and nuclear (H3) genes coupled with the high morphological variability observed even within single replicate proves that our target species is a single widely distributed tanaidid. Because Larsen (2014) provided its first formal description, the name *Zeuxo turkensis* proposed by him shall be prioritized, while *Hexapleomera sasuke* (Tanabe and Kakui 2019) shall be treated as junior synonym of that taxon.

Our study highlights also another taxonomic problem – lack of well-defined characters separating the two genera: *Hexapleomera* Dudich, 1931 and *Zeuxo* Templeton, 1840, that is the basis for further wrong species identification. Tait et al. (2021) already discussed genus specific features and admitted that separation is only possible for fully developed, strongly sexually dimorphic males.

### Biogeographic distribution and long-distance dispersal of *Z. turkensis*

*Zeuxo turkensis* was previously known from Turkey, Italy, Japan, and Australia (in the two last areas as *H. sasuke*) (Suppl. material 3). In the present study we add new records of the species from the Mediterranean and the first record from the Atlantic (Figures 2, 3). Larsen (2014) originally described this tanaidid species based on specimens collected in 2011 in Alsancak Port (Turkey). This early association with human transport hubs suggests both a potential for human-mediated dispersal and a cryptogenic origin. In 2016, Tempesti et al. (2020) found it in the fouling communities of the port of Livorno (Italy), supporting a cryptogenic status for the species in the Mediterranean Sea. Previously, *Z. turkensis* was found in Japan and described as *H. sasuke* from specimens collected between 1999 to 2017 associated to algae covering carapace of loggerhead sea turtle (*Caretta caretta* (Linnaeus, 1758)) bred in aquarium, aquarium tanks and tether lines in two ports (Tanabe and Kakui 2019; Tanabe et al. 2017).



**Figure 3.** Worldwide occurrence of *Zeuxo turkensis* species (in the Iberian Peninsula only some stations shown for clarity).

Such findings may also imply a potential for long-distance natural dispersal in association with turtles. However, as stated by Tanabe and Kakui (2019), tanaidaceans and algae may have entered the aquarium along with the unfiltered seawater. Moreover, the species has not been found associated to natural populations of turtles, so its dispersal through this pathway must be confirmed in future studies. Tait et al. (2021) found the species in 2009, 2010 and later in 2015 in floating docks of marinas in Adelaide (Australia). This, together with our records from marinas of Spain, Portugal, and Morocco, supports this tanaidid as a widespread human-mediated transported species of uncertain origin. Further studies are still necessary to understand the status and invasion patterns of this neocosmopolitan species.

### Establishment success of *Z. turkensis* and adaptations to novel environments

Species dispersion, no matter if natural or human-mediated, requires morphological, physiological and behavioural adaptations. Firstly, animals must survive the long journey. Tanaididae, that travel as passengers within algae, efficiently cling on the branches due to strongly setose body, spinose pereopods and curved dactylus on pereopods 4–6 (Johnson and Attradamal 1982, pers. obs.). The morphology and consequent clinging behaviour is a typical phenomenon observed in peracarids associated with fouling community (Aoki 1999; Guerra-Garcia et al. 2002; Ros et al. 2013; Cunha et al. 2018). It allows them not only to be passively transported, but also to make exchange between transport's vector and the new habitats easier (Martínez-Laiz et al. 2019). Another feature that may facilitate the long-distance dispersal and consequent colonization of new localities is building tubes on the surface of other organisms or on artificial substrate. It is known for two highly successful widespread fouling amphipod species *Jassa marmorata* Holmes, 1905 and *Jassa slatteryi* Conlan, 1990 (Beermann et al. 2020). Tube-building habit is typical for most of Tanaidacea from the suborder Tanaidomorpha, including *Z. turkensis*, and may contribute to its successful dispersal. Moreover, we found *Z. turkensis* in marinas associated to the widespread introduced bryozoan *Bugula neritina*, but

also to plastic passive collectors, suggesting both a high plasticity in its habitat use and a high ability to colonize artificial structures.

After arrival in novel habitats, newcomers must establish self-sustaining populations and spread to surrounding areas. This process depends on several factors such as propagule pressure, environmental and pollution tolerance, resource availability or biotic pressures in the recipient region (e.g., competition, facilitation) (e.g., Johnston et al. 2017; Leclerc et al. 2020). In this study, the population abundance of *Z. turkensis* was similar in the Atlantic and Mediterranean coasts, suggesting that it has wide tolerance to environmental parameters (like salinity, temperature). Furthermore, high abundance of *Z. turkensis* and its demographic structure with substantial number of neutrums through the year (Figure 2) indicate well established populations. Slightly decreasing number of neutrums and increasing number of brooding females from May to July are the indicators of successful reproductive season. Analogous situation was observed for this taxon in Australia, where large number of individuals were collected twice: in the first year of the study and five years later (Tait et al. 2021). Moreover, the presence in 2017 of two populations in Southern Spain where it was absent in 2011 suggests that the species is in expansion phase in the area. Furthermore, the Sado estuary population followed a similar pattern, since the species was not present until 2020 (Ribeiro RS pers. obs.), indicating the possibility for secondary dispersion by recreational boating from other nearby previously invaded national marinas.

Even though the populations of *Z. turkensis* along the Iberian Peninsula and nearby areas seems to be well adapted to the environment, no genetic variability was recorded. Such phenomenon is often associated with a recent colonization event as a consequence of genetic bottleneck (Dlugosch and Parker 2008). It is considered that native populations maintain higher diversity, contributing to recognize the origin of alien and invasive species (e.g., Brown and Stepien 2010; Cabezas et al. 2014; Rewicz et al. 2015; Jażdżewska et al. 2021). Our study showed that although relatively many sequences are presently available (35 sequences from different regions of the World) all but one represent single haplotype that does not allow to make any suggestions about the native range of *Zeuxo turkensis*. Reduced molecular diversity of members of fouling communities may be paired with complicated genetic structure of species expressed by haplotypes shared between very distant regions (e.g., Dias et al. 2016; Cabezas et al. 2019; Beermann et al. 2020; Nascimento et al. 2021) making difficult or even impossible to infer their native ranges. It may be the result of recent rapid expansion as observed for some freshwater species (e.g., Brown and Stepien 2010; Rewicz et al. 2015; Jażdżewska et al. 2021) or the evidence of long-lasting transfer of the fauna associated with marine transport (Beermann et al. 2020; Conlan et al. 2021). The study of *Z. turkensis* suggests rather the second case, an overlooked globally introduction associated with human-mediated transport on ships' hulls (alone or in combination with natural passive dispersal on turtles if this pathway is proven in the wild). The inclusion of collections from more localities and the use of other more rapidly evolving genes could shed more light into the origin and dispersal routes of the species.

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### Author Contribution

AS, AMJ, MR: Research conceptualization; RSR, RS, MR: Sampling design; AS, AMJ, RSR, RS Methodology; AS, AMJ, MR, RSR, RS: Investigation and data collection; AS, AMJ: data analysis and interpretation; Ethical approval: not applicable; AS, AMJ, RSR, RS, MR: funding provision; AS, AMJ, MR: Writing – original draft; AS, AMJ, MR, RSR, RS: Writing – review & editing.

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## Supplementary material 1

### Alignment of all presently available sequences of *Zeuxo turkensis*

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Data type: tif

Explanation note: Alignment of all presently available sequences of *Zeuxo turkensis* showing the length differences and position of the single mutation observed. \*in the work by Larsen et al. (2014) the individual was presented as *Zeuxo* sp. B, the formal description of the species was published later the same year (Larsen 2014). The sequence published by Larsen et al. (2014) was represented by three individuals.

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Link: <https://doi.org/10.3391/ai.2023.18.4.113092.suppl1>

## Supplementary material 2

### Barcode of Life Datasystem (BOLD) - data of all sequences produced during present study

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Data type: xlsx

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## Supplementary material 3

### *Zeuxo turkensis* distribution based on literature data

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Data type: xlsx

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