

Research Article

Presence of a second *Eriocheir* species in Europe as confirmed by molecular and morphological data

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Abstract

New Chinese mitten crab sequence data were obtained from eight European localities spanning France, The Netherlands, England and Wales. The Netherlands and Wales samples were taken from two areas where mitten crab populations had never before been sequenced. DNA results uncovered the presence of a second *Eriocheir* species coexisting sympatrically with *E. sinensis* H. Milne Edwards, 1853 in the Dee Estuary, Wales and at two sites sampled in The Netherlands. Molecular results and comparative morphology analyses suggest that the second species in European waters is likely to be an undescribed cryptic species. Additional evidence for the presence of a second European invasive mitten crab species is provided by confocal laser scanning microscopy of the male first gonopod. The origins of the Welsh introduction may date back to the early 2000s, when several Dutch dredging operators were active in the Dee Estuary. Despite several localities being sampled, the *Eriocheir* cryptic species was not found in England, which suggests that English and Welsh mitten crab populations originate from independent introductions. These results radically challenge current assessments of *Eriocheir* invasion in Europe and should be considered by stakeholders as a new baseline to implement efficient management strategies against invasive mitten crabs.

Key words: Chinese mitten crab, cryptic species, Dee Estuary Wales, dredging operations, phylogeography, molecular systematics, morphometrics, confocal laser scanning microscopy, first male gonopod morphology, carapace frontal margin

Introduction

The Chinese mitten crab, *Eriocheir sinensis* H. Milne Edwards, 1853, is on the world's list of 100 most invasive aquatic invertebrates (Lowe et al. 2000) and is cited in the handbook of alien species in Europe (<http://www.europe-alien.org>). Since the Chinese mitten crab was first recorded from Germany in 1912, favourable conditions plus an extensive network of canals have

facilitated its rapid spread and colonisation of North Eastern Europe coastal ecosystems and major catchments (Herborg et al. 2003, 2005). In comparison, *Eriocheir* establishment and dispersal across the watersheds of England and Wales has proceeded at much slower rates. Initial reports from the River Thames in 1935 (Anon 1936a, b; Harold 1935; Morritt et al. 2013) were followed by a long pause in new records until the late 1970s, when mitten crabs established and spread upstream through the River Thames (Ingle and Andrews 1976; Clark 1984, 2011; Clark et al. 1998; Morritt et al. 2013). Additional sightings outside the Thames catchment have significantly increased in recent years (see www.mittencrabs.org.uk). Although there is a plethora of information about the occurrence of the Chinese mitten crab in continental Europe (Herborg et al. 2003, 2005; Bentley 2011), little research has been undertaken with regard to the genetic diversity and connectivity of these mitten crab populations. Hänfling et al. (2002) suggested that European populations of Chinese mitten crab are genetically homogeneous (i.e., all haplotypes found were closely related). In contrast however, Herborg et al. (2007) found significant differentiation among the same European populations studied by Hänfling et al. (2002). Therefore, relationships among mitten crab populations in Europe remain uncertain, and several key marine-trafficking areas are underrepresented or not included in previous studies (e.g., Wales, France and The Netherlands).

The present study reports additional findings on the phylogeography of mitten crab populations from The Netherlands, France, England and Wales. After analysing the genetic diversity and genetic differentiation among samples from continental Europe and UK, molecular evidence uncovered previously unnoticed cryptic species of mitten crab in Europe. The molecular results were supported by male first gonopod morphology. Furthermore, the new genetic data suggest that English and Welsh populations originate from independent introductions.

Materials and methods

Sampling

A total of 411 mitten crabs were collected. Fyke nets were deployed at seven different locations between 2012 and 2015 (Table 1; Figure 1). These included five sites across the United Kingdom: two Thames River populations, Littlebrook (THL) and Kent (THK), Medway River, Allington (MDR), Great River Ouse, King's Lynn (KLY) and Dee Estuary, Chester, North Wales (DEE), and three sites located in continental Europe: Gironde Estuary, Bordeaux, France (GES), Hollands Diep, Rotterdam, The Netherlands (HDI), and Den Oever, Hollands Kroon, The Netherlands (DOV). Mitten crabs from Allington, River Medway (MDR) however, were caught in a fish pass using an elver trap. Individual crabs were frozen at -20°C to ensure good quality of the tissue for further manipulation.

Table 1. Location name and acronyms, sample size, GPS co-ordinates and year of sampling for each location. Nh: Number of haplotypes observed; Hd: haplotype diversity and π : nucleotide diversity.

Locality	Sample size (Nh)	Co-ordinates		Country	Year	Hd \pm sd	$\pi \pm$ sd
		Long.	Lat.				
River Thames, Littlebrook (THL)	23 (3)	51.47°	0.23°	England	2012	0.672 \pm 0.045	0.00197 \pm 0.00013
River Thames, Kent (THK)	8 (3)	51.47°	0.17°	England	2015	0.679 \pm 0.122	0.00199 \pm 0.00036
River Medway (MDR)	23 (4)	51.28°	0.50°	England	2012	0.684 \pm 0.062	0.00207 \pm 0.00021
King's Lynn (KLY)	15 (3)	52.70°	0.37°	England	2014	0.705 \pm 0.053	0.00201 \pm 0.00016
Dee Estuary (DEE)	21 (5)	53.18°	-2.88°	Wales	2013	0.767 \pm 0.055	0.01227 \pm 0.00445
Gironde Estuary (GES)	23 (2)	45.38°	-0.78°	France	2014–2015	0.237 \pm 0.105	0.00069 \pm 0.00031
Hollands Diep (HDI)	26 (3)	51.70°	0.53°	The Netherlands	2015	0.283 \pm 0.109	0.01171 \pm 0.00429
Den Oever (DOV)	6 (2)	52.93°	5.08°	The Netherlands	2015	0.333 \pm 0.215	0.01415 \pm 0.00914

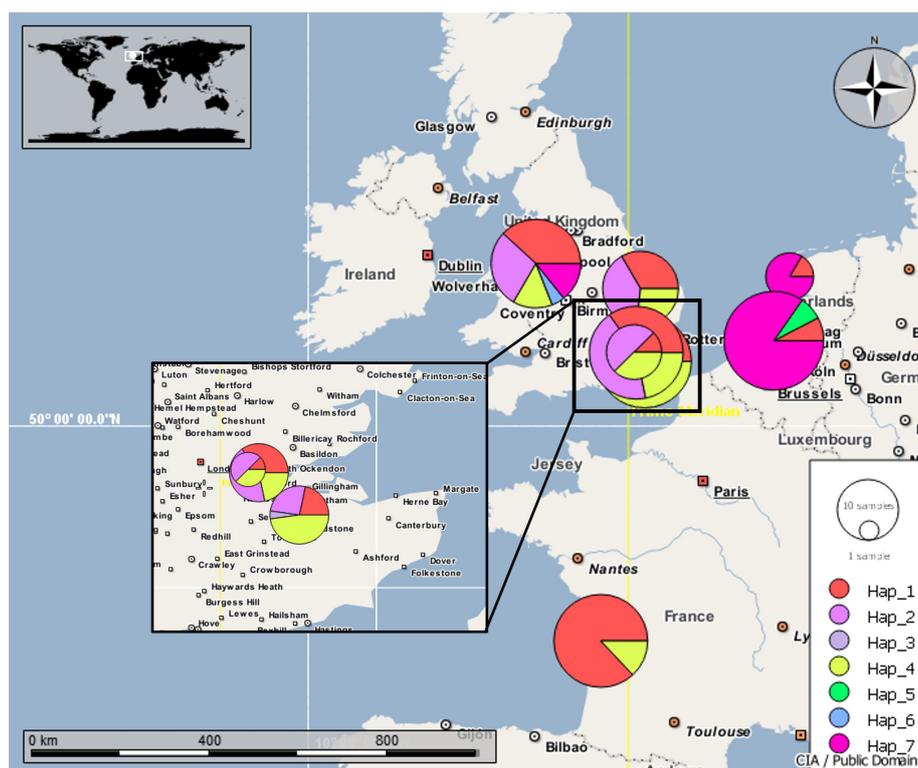


Figure 1. Map indicating the presence and frequency of each haplotype at each sampling location. Each colour represents a different haplotype (see legend), and size of the pie charts refers to the sampling size at each location (Table 1).

DNA extraction and quality assessment

A total of 145 individual crabs were used for DNA analyses, using a subsample from each locality (Table 1). Muscular tissue ($\sim 1 \text{ mm}^2$) from ambulatory legs of each frozen crab was extracted using forceps and kept in ethanol to preserve DNA. Total genomic DNA was extracted using the DNeasy® Blood and Tissue kit (QIAGEN). DNA quality and concentration ($\text{ng}/\mu\text{l}$) was checked using NanoDrop 8000™ v2.1 spectrophotometer and running 1% agarose gels including $1 \mu\text{l}$ of genomic DNA. Polymerase chain reactions were carried out with $\sim 30 \text{ ng}$ of genomic DNA, 1 U of Taq polymerase (Amersham), $1 \times$ buffer (Amersham), $10 \mu\text{M}$ of each standard universal primer for the COI gene (LCO1490 and HCO2198; Folmer et al.

1994) and 0.12 mM dNTPs. The thermal profile used was 95 °C for 15 min for initial denaturation, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 7 min. Amplified PCR products were purified using a MultiScreen®HTS vacuum manifold, re-suspending into ultrapure water before direct sequencing. DNA sequences were obtained using the Big-Dye Ready-Reaction kit v.3.1 (Applied Biosystems) on an ABI Prism 3770 automated sequencer from the Scientific and Technical Services of the Natural History Museum, London, England. The chromatograms for each DNA sequence were checked using BioEdit v.7.2.5 (Hall 1999).

Genetic diversity and genetic differentiation

The number of haplotypes (H_n), haplotype diversity (H_d), nucleotide diversity (π), and genetic differentiation among the sampled populations were calculated using the software DnaSP v.5.1 (Rozas et al. 2003). Genetic divergence among populations was estimated by Wright's fixation index (F_{st}), which can range from 0 (no genetic differentiation) to 1 (complete genetic differentiation among populations). For a more reliable interpretation of the genetic analyses, nucleotide sequence-based statistics (K_s and S_{nn}) and haplotype-based statistics (GammaST and H_s) were also obtained, as they represent more suitable estimates for mitochondrial haplotype data (Weir and Cockerham 1984; Hudson et al. 1992; Hudson 2000). The significance of the nucleotide sequence-based statistics was tested with a permutation (randomization) test with 1000 replicates, using the same software. Furthermore, a haplotype network was constructed using the program popART v.1.7 (Population Analysis with Reticulate Trees) (Leigh and Bryant 2015) to visualize relationships between individual haplotypes at the population level. The haplotype inference method used was minimum spanning network (MSN) (Bandelt et al. 1999).

Detection of a second invasive species of mitten crab in Europe

To allow for comparison with previous studies, new COI sequences were compared against GeneBank using BLASTN (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). COI sequences assigned to *Eriocheir* species were downloaded and analysed together with our new sequence data. Sequence alignment was conducted using Muscle v.3.6 (Edgar 2004) with default parameters. Model selection of nucleotide substitution was performed according to the BIC criterion as implemented in MEGA X (Kumar et al. 2018). Haplotype phylogenetic relationships were inferred using the Kimura 2-parameters (K2P) genetic distance and the Neighbor-Joining method. The percentage of replicate trees in which different haplotypes clustered together was estimated using the bootstrap test (200 replicates) as implemented in MEGA X (Kumar et al. 2018). The K2P genetic distance was selected following Matzen da Silva et al. (2011) comprehensive study on decapod crustaceans.

These authors obtained the frequency distribution of K2P genetic distances at the species, genus and family levels using COI sequences of 302 species, 154 genera and 58 families (Matzen da Silva et al. 2011). A recommended threshold of 2.2% was used for a rough differentiation of low and high intraspecific K2P distances (see also Ratnasingham and Hebert 2013; Raupach et al. 2015). An allied varunid crab, *Varuna litterata* (Fabricius, 1798) was selected for the outgroup comparison.

Confocal laser scanning microscopy (CLSM)

Material examined: *Eriocheir sinensis*, adult male, from the Bam Elms reservoir, Barnes, London, collected by Martin Honey, 2 October 1991, NHM reg. 1992.36.1; *Eriocheir japonica* De Haan, 1835, adult male, from a stream at Tsushima, Japan, presented by Duke of Bedford, 1907, NHM reg. 1907:12.9.1; *Eriocheir hepuensis* Dai, 1991, adult male, from Shatt Al-Basrah Canal, Iraq near the dam at 30°24'33.75"N; 047°46'32.32"E, collected by Murtada Naser, 30 November 2010, NHM reg. 2011.8035–8037; *Eriocheir* sp., adult male, from Den Oever, The Netherlands, 52°56'16"N; 005°02'26"E; collected by Michiel Kotterman, 2015, NHM; *Eriocheir* sp., adult male, from Hollands Diep, The Netherlands, 51°42'19.2"N; 004°32'42.5"E, collected by Michiel Kotterman, 2015, NHM.

The left first gonopod (G1) was dissected from mitten crab adult males and placed in separate Petri dishes and filled with deionised water. The distal tip of each G1 was cleaned using a fine paint brush and G1s were stained with a mixture of Congo red and acid fuchsin for 24 hrs at room temperature. G1s were rinsed in deionised water after the 24 hrs incubation in the stain. All G1s were too large to embed on a standard slide with coverslip, so a new platform was designed for scanning these samples using CLSM (Figure 2). The embedding platform comprised a glass gutter formed from 2 groups of 7 stacked microscope slides glued to each other using polyvinyl lactophenol. These were then fixed to a larger piece of glass to form a deep gutter which was dammed at each end by Blu Tack® (Bostik Ltd., England). The G1 was placed in the gutter ventral side up and firmly held using more Blu Tack® to keep it completely immobilised during the 10–12 hr scan. The gutter was then filled with 100% glycerine until reaching the highest level of the stacked slides and then examined for any air bubbles. If present, these were carefully removed using a fine tungsten needle. A rectangular coverslip (No. 1.5) was used to seal off the pleopods avoiding any additional inclusion of air bubbles. Avoidance of air bubbles was considered critical as they would expand during the 12 hr session due to the heat from the lasers and prevent a sharp image.

After embedding the G1, masking tape was fitted around the platform to further prevent glycerine leaking and the platform was carefully placed on the mechanical stage of the confocal microscope. To hold the large platform (Figure 2b) stable for the lengthy acquisition time, its edges were secured

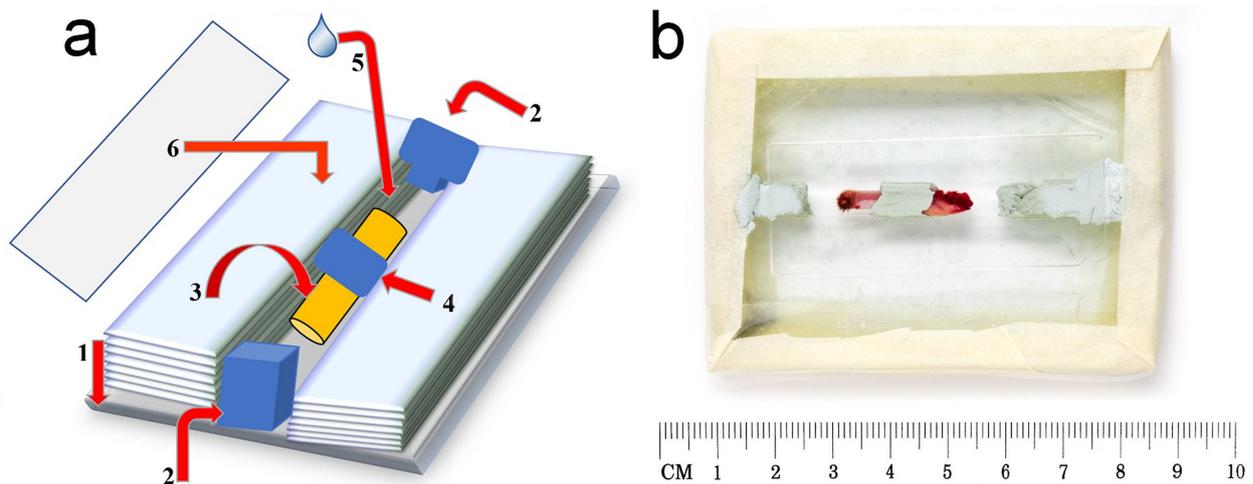


Figure 2. Preparation of *Eriocheir* G1 for Confocal Laser Scanning Microscopy (CLSM); a, Schematic illustration of the designed platform used to hold the male gonopod firmly during 10–12 hours of CLSM: (1) Two groups of 7 microscope slides were glued each other using polyvinyl lactophenol and these merged microscope slides were glued on each side of the glass/plastic platform; (2) Two sides of the new platform was sealed using Blu Tack®. The level of the Blu Tack® and microscope slides were levelled by using a cylindrical metal holder; (3) The male gonopod was placed and arranged inside the slide channel; (4) The gonopod was covered using Blu Tack® to immobilise it during long duration scanning; (5) The channel was filled with 100% glycerine until it reached the highest level of the adjacent slides; (6) The channel was covered with a long coverslip (avoiding the inclusion of air); b, A general view of designed platform to scan large appendages using CLSM. The G1 figured was dissected from an *Eriocheir* specimen captured in Hollands Diep, Netherlands.

using Blu Tack® before scanning. The G1s were then scanned using a Nikon A1-Si confocal microscope. A 10× dry objective with a numerical aperture of 0.3 was used to scan the surface of the gonopods together with the “large images” option of the confocal software (Kamanli et al. 2017, 2018). Four lasers at wavelengths of 405 nm, 488 nm, 561 nm, and 640 nm were used to produce a maximum intensity projection. Depending on the G1 size, between 16–25 tiles were required to scan their distal end. The Nikon software (NIS-Elements viewer version 4.20) was used for image-stitching to combine tiled areas. The final image was processed in Adobe Photoshop (CS6) to adjust brightness/contrast, to remove unwanted particles and to provide a black background.

River Dee dredging operations

The archives of the Port Mostyn authority were made available to this study for examination and details of the dredging operations in the area were extracted.

Results

DNA analysis

A total of 7 different haplotypes were recovered from the 145 putative *E. sinensis* individuals sequenced, with the most abundant haplotype (Hap_1; 34.5% of the specimens) being present in every locality sampled (Figures 1, 3; Table 2). BLAST results against the “nr/nt” GenBank database showed every haplotype recovered in this study to be also present in the native

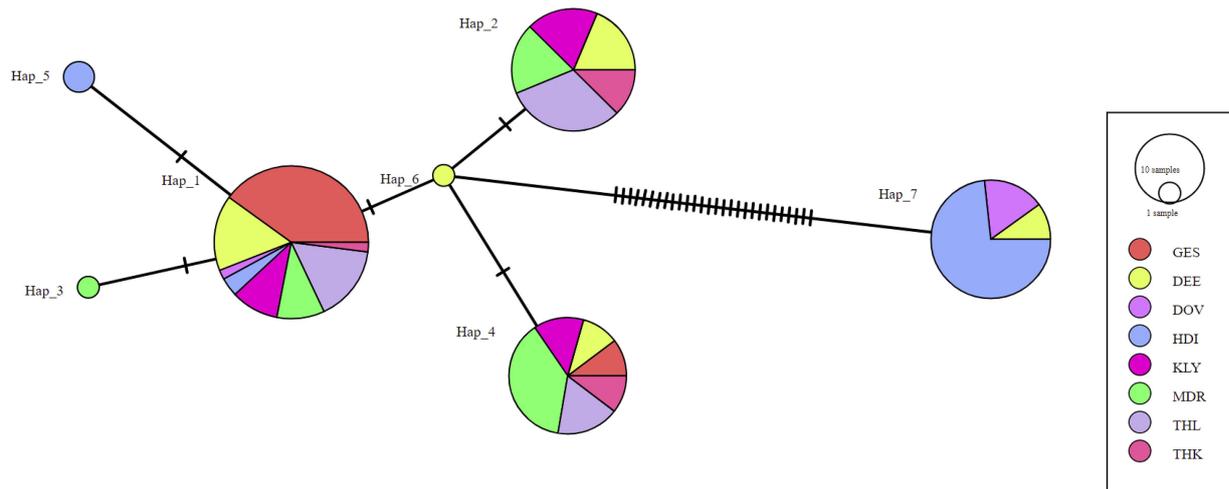


Figure 3. Haplotype network for sampled mitten crabs using the haplotype inference MSN (Minimum Spanning Network). Each pie chart represents a different haplotype, and each colour represents one population. Size of the pie charts indicates the overall frequency of the haplotype within the samples. Acronyms as described in Table 1.

Table 2. Geographical distribution of haplotypes obtained in the present study. For previous studies, both native and invasive ranges of the species are included (except for Haplotype-03 and Haplotype-05, reported for the first time from an invasive population). Locality abbreviation as in Table 1.

Haplotype	Locality	Previous studies
Hap_1	THL, THK, MDR, KLY, DEE, GES, HDI, DOV	River Changjiang (China) River Elbe (Germany); River Wesser (Germany), River Thames (England), River Tagus (Portugal)
Hap_2	THL, THK, MDR, KLY, DEE	River Changjiang (China); River Feiyunjiang (China); River Oujiang (China) River Elbe (Germany); River Wesser (Germany), River Thames (England), River Tagus (Portugal)
Hap_3	MDR	Tongan (China); River Geumgang (China)
Hap_4	THL, THK, MDR, KLY, DEE, GES	River Feiyunjiang (China); River Zhujiang (China) River Elbe (Germany); River Wesser (Germany), River Thames (England), River Tagus (Portugal); California (USA)
Hap_5	HDI, DOV	Okinawa (Japan)
Hap_6	DEE	Okinawa (Japan) River Weser (Germany)
Hap_7	DEE, HDI, DOV	Vladivostok (Russia) River Rhine (Germany)

range of *Eriocheir*, and some had been previously observed in Europe (Table 2). Haplotypes 3 and 6 were singletons (found in a single population, Medway River and Dee Estuary, respectively). Haplotype 4 was previously detected in San Francisco Bay and Sacramento (California, USA), but haplotypes 3 and 5 had never been observed in invasive populations before. Genetic distances between haplotype 7 and other *E. sinensis* haplotypes ranged between 4.6% and 4.8%, well above the intra-specific threshold observed in other decapod crustaceans and suggested the presence of a second *Eriocheir* species in Europe. Unexpectedly, haplotype 7 gave a 100% match in GenBank with a sequence described by Xu et al. (2009) as pertaining to *E. japonica* (Figure 4).

Genetic diversity and population genetic differentiation

The haplotype network constructed with the MSN algorithm (Figure 3) gives visual confirmation to the results obtained. The total number of mutations

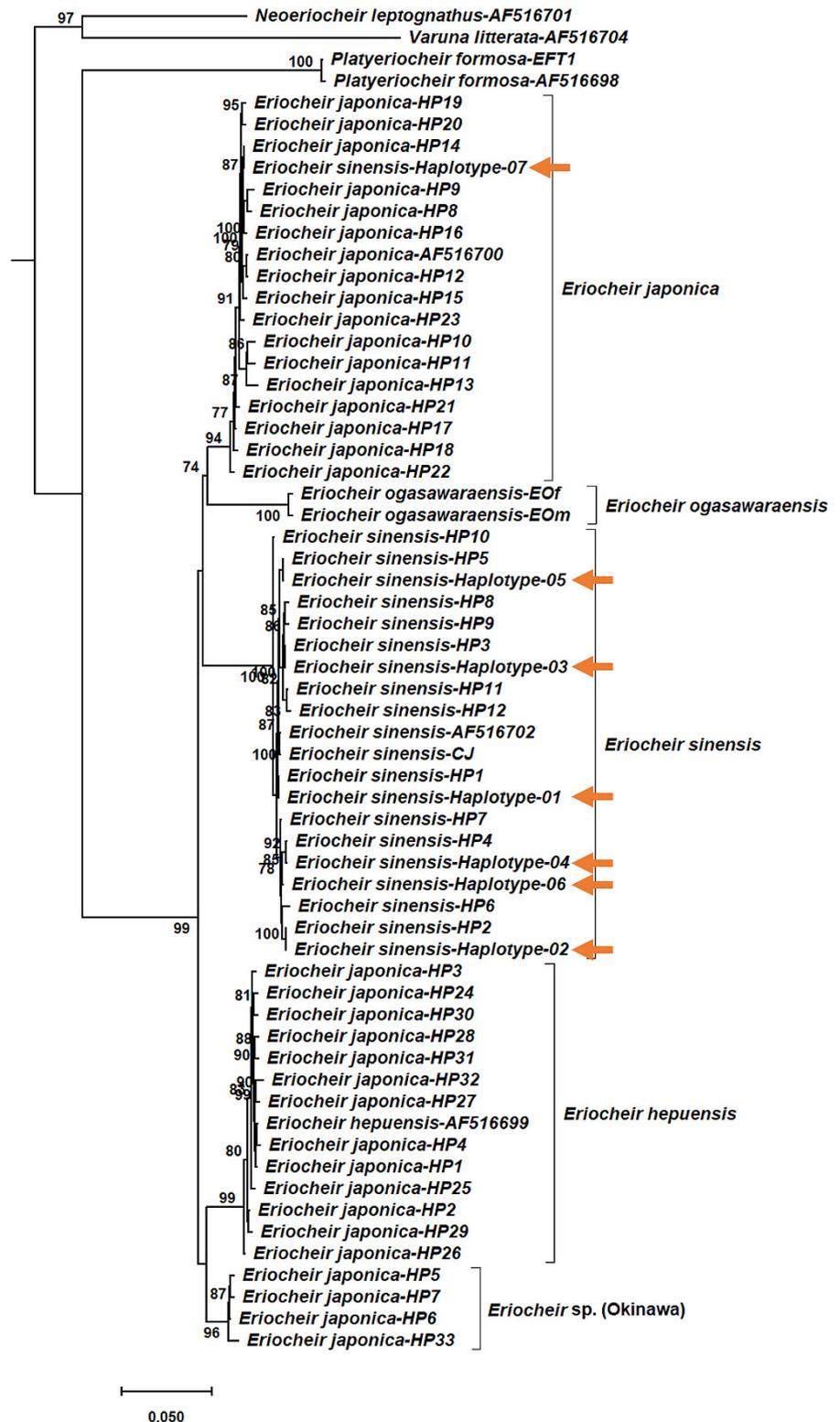


Figure 4. Evolutionary relationships among COI haplotypes. Orange arrows indicate the 7 haplotypes observed in this study.

among haplotypes ranged between 1 and 3 except for haplotype 7, where ≥ 28 mutations were observed compared with other haplotypes. This highlighted haplotype 7 as being a different lineage (Figure 3). Haplotype 1 was especially frequent in Gironde and Dee estuaries and Thames River-Littlebrook.

Table 3. Pairwise genetic distances between mitten crab localities. Fst: upper diagonal; GammaST: lower diagonal. Values in bold indicate significant differentiation assessed by permutation test ($P < 0.05$). Acronyms as described in Table 1.

	GES	DOV	HDI	DEE	THL	THK	KLY	MDR
GES		0.90063	0.82002	0.14152	0.32075	0.53979	0.33384	0.35519
DOV	0.764		0	0.59273	0.8706	0.7988	0.84334	0.86853
HDI	0.705	0		0.62572	0.8047	0.75254	0.78038	0.80392
DEE	0.099	0.368	0.470		0.07605	0.02534	0.04954	0.09302
THL	0.212	0.707	0.684	0.063		0	0	0.03347
THK	0.340	0.702	0.544	0.046	0.023		0	0
KLY	0.219	0.713	0.638	0.053	0.001	0.023		0
MDR	0.237	0.703	0.683	0.072	0.039	0.025	0.025	

Haplotype 2 was exclusively found in UK locations. In addition, specimens from Medway River, Hollands Diep and Dee estuary presented a private haplotype (Haplotypes 3, 5 and 6, respectively), while haplotype 7 was shared by the two Dutch and Dee estuary populations (Figure 1).

Haplotype diversity was higher in the UK (Table 1), ranging from 0.767 ± 0.055 in Dee estuary to 0.672 ± 0.045 in Thames River-Littlebrook. For continental populations, values ranged from 0.333 ± 0.215 in Den Oever to 0.237 ± 0.105 in Gironde estuary. Conversely, nucleotide diversity was highest in Den Oever ($\pi = 0.01415 \pm 0.00914$) and particularly low in Gironde estuary ($\pi = 0.00069 \pm 0.00031$). Overall genetic differentiation among localities was significant regardless of the statistics studied ($S_{nn} = 0.288$, $P = 0.000$; $K_s = 3.781$, $P = 0.000$; $H_s = 0.537$, $P = 0.000$). Genetic distances were higher when comparing samples from UK against continental Europe ($F_{st} = 0.66 \pm 0.2$; $\text{GammaST} = 0.574 \pm 0.194$) and significant pairwise comparisons mainly involved those population comparisons (Table 3). F_{st} and GammaST estimates were consistent (Pearson's $r = 0.98$, $R^2 = 0.96$), although GammaST values were always lower than F_{st} . Both estimates were close to zero among UK populations, between Dee and Gironde estuaries and between Den Oever and Hollands Diep, indicating low genetic divergence. Moderate genetic structuring was found between the Ouse River-King's Lynn population and the Gironde estuary, and between Dee estuary and the Dutch populations. Considerable genetic differentiation, with both GammaST and F_{st} values close to 1, was found when comparing UK and Gironde estuary populations against Den Oever and Hollands Diep.

Phylogenetic reconstruction

Some of the new sequences matched 100% with those from previous studies, revealing the presence of our haplotypes all over Northern Europe and Portugal. In addition, haplotype 4 was also detected in San Francisco Bay and Sacramento (California, USA). Nonetheless, haplotypes 3 and 5 had never been described in Europe before. K2P pairwise distances among the haplotypes Hap_1 to Hap_6 and *E. sinensis* data were as expected, ranging between 0.2% and 0.5% and indicating low intraspecific nucleotide variation. However, the K2P model revealed high nucleotide differentiation between haplotype 7 and the other haplotypes, well above the 2.2% recommended

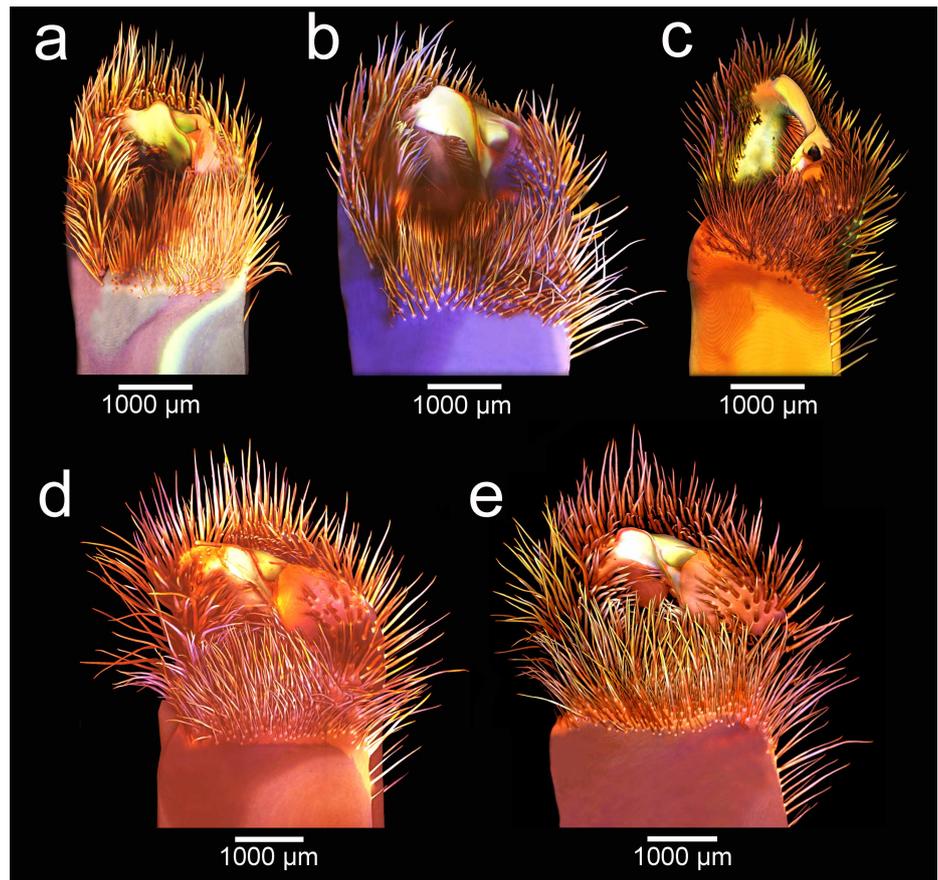


Figure 5. CLSM images of *Eriocheir* G1 distal tip morphology in dorsal view from five localities. a, *E. sinensis*, River Thames, London; b, *E. hepuensis* from Shatt Al-Basrah Canal, Iraq, NHM 2011.8035-8037; c, *E. japonica* from Tsushima, Japan, NHM 1907.12.9.1; d, e, the undescribed cryptic species, collected from Den Oever and Hollands Diep, respectively.

threshold (pairwise distances between 4.6% and 4.8% with respect to the other haplotypes described in this study). Haplotype 7 gave a 100% match with a sequence by Xu et al. (2009) described as pertaining to the Japanese mitten crab, *Eriocheir japonica*, but clearly distinct from COI sequences pertaining to *E. japonica* from other authors (Figure 4).

CLSM G1 images

The CLSM dorsal view of *Eriocheir* G1 distal tip images from five localities (Figure 5) revealed distinct morphology types for each known species namely, *Eriocheir sinensis* from River Thames, London; *E. hepuensis* from Shatt Al-Basrah Canal, Iraq; *E. japonica* from Tsushima, Japan and for the second European *Eriocheir* invasive species detected from Den Oever and Hollands Diep, The Netherlands. The detailed morphology examination of the two European invasive mitten crab species indicated that *E. sinensis* (Figure 5a) from the River Thames are morphologically distinct from the Dutch *Eriocheir* specimens (Figure 5d, e). The G1s of specimens from The Netherlands showing haplotype 7 were all identical among themselves and different from *E. sinensis* specimens, confirming the DNA results and suggesting the presence of a second invasive species yet to be identified.

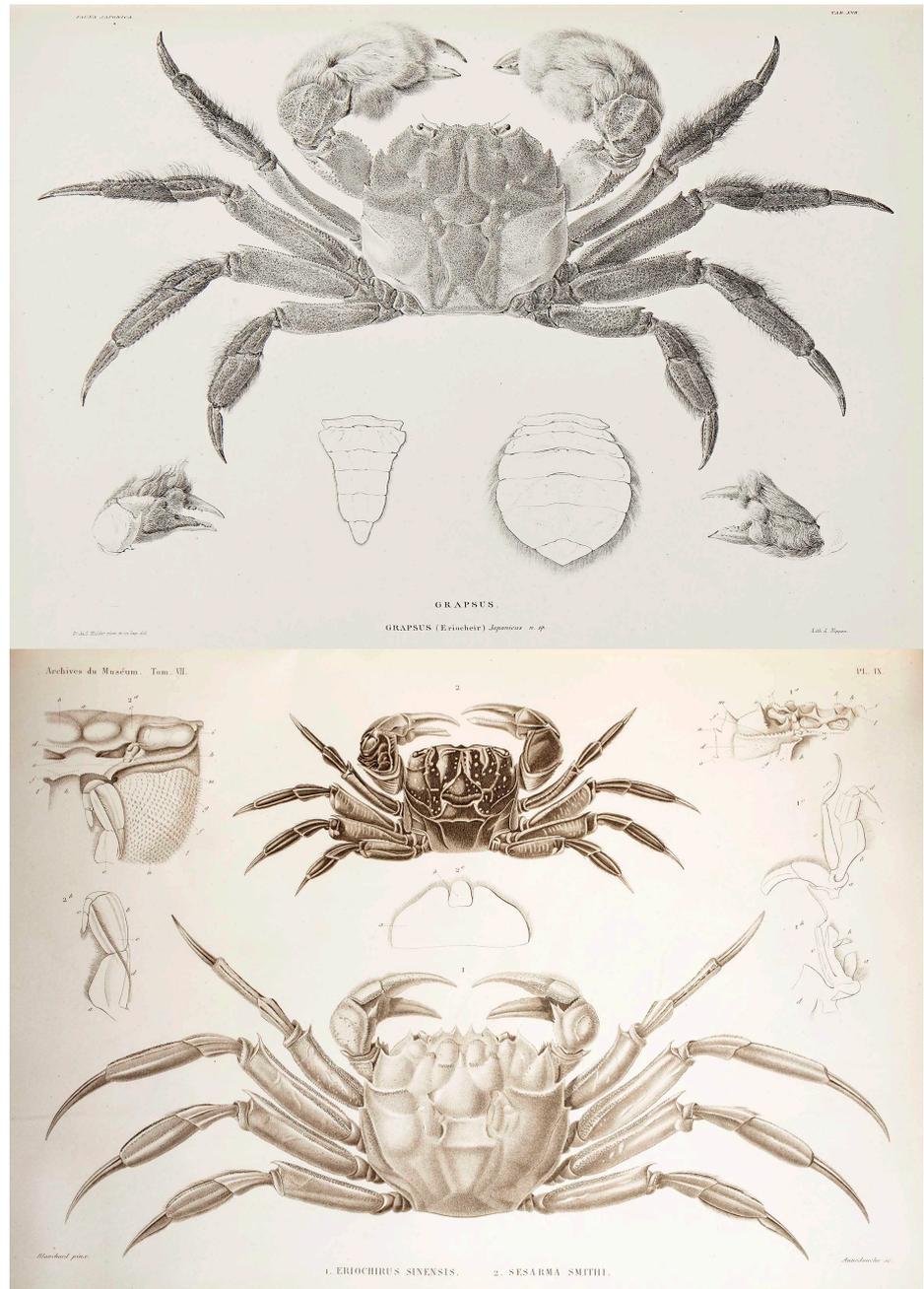


Figure 6. Carapace frontal margin. *Eriocheir japonicus*, De Haan, 1835, plate XVII, bilobed (waved) without teeth; *E. sinensis*, H. Milne Edwards, 1854, plate IX with 4 teeth.

Discussion

Identification of Eriocheir japonica

The Japanese and Chinese mitten crabs can be distinguished by the morphology of their frontal carapace margins. *Eriocheir japonica* can be identified by its bilobed/waved frontal margin without teeth (De Haan 1835, plate XVII; Guo et al. 1997; Figure 6) compared with the 4-teeth frontal margin of *E. sinensis* carapace (H. Milne Edwards 1854, plate IX; Guo et al. 1997; Figure 6). The carapace morphology of mitten crabs from Den Oever, The Netherlands, River Thames, Kent, England and Tsushima,



Figure 7. *Eriocheir* adult male carapace morphology dorsal view and front details. a, d, the undescribed cryptic species, collected from Den Oever, The Netherlands; b, e, *E. sinensis* from River Thames, Kent, England; c, f, *Eriocheir japonica* from Tsushima, Japan, NHM 1907.12.9.1. Taken by Kevin Webb, NHM Photo Unit.

Japan is compared in Figure 7. While the mitten crab from Tsushima, Japan (Figure 7c, f) has a bilobed/waved frontal margin, the mitten crabs from Den Oever and the River Thames (Figure 7a, b, d, e respectively) have a similar 4-teeth frontal margin. Consequently, the Dutch and the Dee Estuary material cannot be assigned to *E. japonica* and could be assigned to an *Eriocheir* species with four teeth (*E. sinensis* and *E. hepuensis*) or to a possible undescribed cryptic species. According to Guo et al. (1997), the hepu mitten crab is a valid species and can be distinguished using the distal morphology of the G1 (Figure 5b) and article proportions of the 5th pereiopod (Figure 8d) among other characters (Guo et al. 1997, Table 1).

Identification of second invasive European Eriocheir

The male G1 distal morphology is regarded as a reliable species diagnostic character in brachyuran crabs (e.g., Balss 1937; Bott 1970; Serène 1984). Guo et al. (1997) noted that, although differences were subtle, G1 morphology was a valid character distinguishing mitten crab species. Although the G1 illustrations by K. Sakai (2013) are beautiful, they are too schematic to be helpful and suggest their morphology is too complex to draw accurately. Consequently, the distal tips of *E. japonica*, *E. sinensis*, *E. hepuensis*, and mitten crabs from The Netherlands were scanned here for comparison using confocal laser microscopy (Figure 5). The CLSM images are congruent with the carapace frontal margins and suggest that the Dutch material (Figure 5d, e) should not be assigned to *E. japonica* (Figure 5c) because their distal tips are different. Furthermore, all three mitten crabs with 4 teeth in the frontal margin, *E. sinensis* (Figure 5a), *E. hepuensis* (Figure 5b) and Dutch material (Figure 5d, e), also have distinct distal G1 morphology. The new evidence from morphology of both frontal margin and G1 suggest that Dutch and Dee Estuary mitten crabs most likely belong to an undescribed cryptic species.

Issues with E. japonica misidentifications

There are previous reports of invasive populations for *E. sinensis* (cf. Lowe et al. 2000), *E. hepuensis* (cf. Naser et al. 2012) and *E. japonica* (cf. Jensen and Armstrong 2004; Hayer et al. 2019). Nevertheless, confirmation of *E. japonica* as an invasive is required and appears to be in doubt. The record of Jensen and Armstrong (2004) from the Columbia River, ca. 3 km west of the Astoria-Megler Bridge, Astoria, Oregon, West coast of USA is now regarded as *E. sinensis* (Low et al. 2010; Benson and Fuller 2019). Similarly, the report of the Japanese mitten crab in Europe by Hayer et al. (2019) is here considered erroneous because, although their figure 6A is of low magnification and poor definition (see further *Eriocheir* images from the same authors at <https://www.boldsystems.org/>), their images show a carapace frontal margin with 4 teeth (vs frontal margin bilobed/waved and without teeth of *E. japonica*, De Haan 1835, plate XVII; Figures 6, 7c, f). Therefore, it is suggested here that the mitten crab images of Hayer et al. (2019) and the BOLD database (<https://www.boldsystems.org/>) belong, in fact, to the cryptic species reported by the present study.

The present study follows Chan et al. (1995) conclusion that the material described by Stimpson (1858, 1907) as *E. rectus* Stimpson, 1858 is in fact a juvenile of *E. japonica*, and agrees with their careful designation of a neotype, so that the species established by Stimpson becomes a subjective junior synonym of *E. japonica* in accordance with the rules of Zoological nomenclature. The *Eriocheir* sp. [= *Eriocheir recta* sensu Stimpson 1858; species inquirenda] described by K. Sakai (2013) as presenting a waved carapace front with a shallow median sinus, should be attributed to *E. japonica*, as originally explained by Chan et al. (1995). Furthermore, the front of *E. japonica* described by K. Sakai (2013: fig. 1A) compares well with plate XVII (De Haan 1835) and Figures 6 and 7 of the present study. Therefore, the statement by K. Sakai (2013) that the establishment of a neotype by Chan et al. (1995) has “nomenclatural consequences and not a taxonomic one” is illogical and invalid under the current zoological Code (ICZN 1999) and it has only created confusion within mitten crab taxonomy.

Problems with DNA-based identification of European Eriocheir invasives

The evolutionary relationships among the seven COI haplotypes analysed here (orange arrows in Figure 4) indicate that a second *Eriocheir* invasive species, different from *E. sinensis*, is present in European catchments. While haplotypes 01–06 are assigned to *E. sinensis*, mitten crab samples from The Netherlands and Wales belong to a different clade, associated with putative *E. japonica* sequences. The new morphological evidence presented here shows that Dutch and Welsh mitten crabs (with COI haplotype 7) have 4 teeth carapace frontal margins and cannot be attributed to *E. japonica*.

The comparison of Figure 4 with results from Xu et al. (2009: fig. 2) suggests a possible origin of the second invasive mitten crab species in Europe. Haplotype 07 of the present study forms a well-supported clade with haplotypes EJJ23-25, corresponding to specimens collected from Vladivostok and South Korea (Xu et al. 2009: figs. 1, 2, Table 1). Four specimens of Vladivostok mitten crab were recently sent to the NHM, London (courtesy of Tin-Yam Chan, National Taiwan Ocean University) and the frontal carapace margins of these mitten crabs have four teeth as do the specimens from The Netherlands and the Dee Estuary (and for that matter Hayer et al. 2019: fig. 6A). Therefore, the identification of the Vladivostok, The Netherlands, the Dee Estuary mitten crabs, and that figured by Hayer et al. (2019: fig. 6A) as *E. japonica* can be excluded and they most likely represent an undescribed cryptic *Eriocheir* species with native distribution around Vladivostok and South Korea. Although the material referred by Hayer et al. (2019) was studied by Cristian Bernardi of the Università degli Studi di Milano, no comparison appears to have been made with true *E. japonica* from its native area. Genbank misidentification probably led Hayer et al. (2019) to assign their Haplotype EJ H1 to *E. japonica*.

European population genetics

The discovery of a second species of *Eriocheir* in Europe might seem unexpected, but previous genetic studies on European mitten crab populations have focused on northern and eastern areas (Hänfling et al. 2002; Hänfling and Weetman 2003; Herborg et al. 2007; Czerniejewski et al. 2012). The Netherlands and Welsh populations were possibly not properly sampled in previous studies. The results presented here confirm that the second *Eriocheir* invasive species, corresponding to haplotype 7, can be found in The Netherlands and North Wales. Sequences available in the BOLD database matching our haplotype 7 were obtained from specimens caught in the catchment of the Rhine River, close to Bonn (MT01737), from Poland (OZIMP066-15), and from a seafood retailer and studied by Cristian Bernardi (CBCC038-11, CBCC039-11, CBCC040-11). Although previous authors acknowledged high pairwise genetic distances (greater than 2.2%) between our haplotype 7 and *E. sinensis*, no morphological differences with other Chinese mitten crabs had been pointed out before. The integrative taxonomic analyses presented here suggests that, given the current state of mitten crab taxonomy (see Naser et al. 2012), the species carrying haplotype 7 should not be assigned to *E. japonica*, but rather to an undescribed *Eriocheir* from the Russian region around Vladivostok.

Previous studies already emphasized the need for a critical assessment of both DNA barcodes and *Eriocheir* taxonomy (Costa et al. 2007). The taxonomy of *Eriocheir* has been intensively debated in the last few decades (Guo et al. 1997; Zhao et al. 2002; Tang et al. 2003; Naser et al. 2012) but remains confusing in parts. The morphological similarities between congeneric

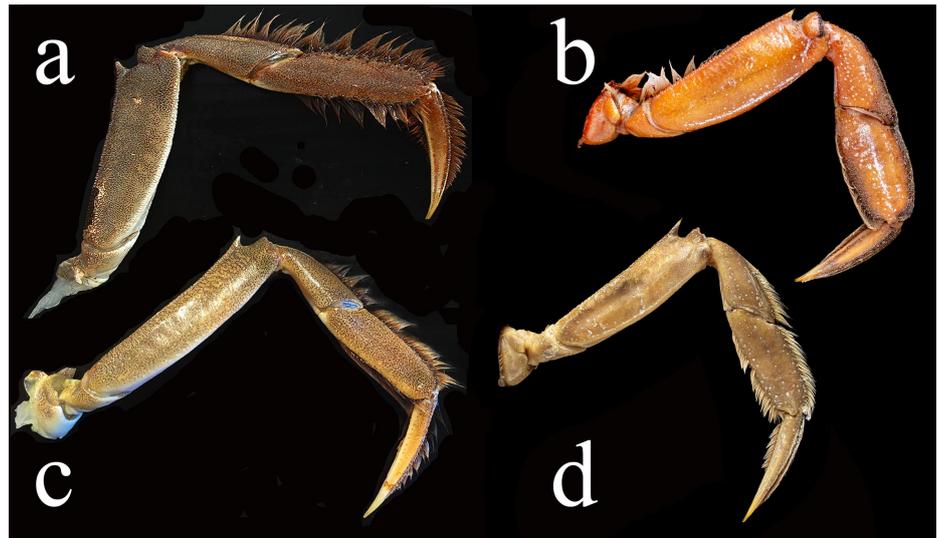


Figure 8. Fifth pereiopod, fourth ambulatory leg. a, undescribed cryptic species from Den Oever, Netherlands; b, *Eriocheir japonica* (De Haan, 1833) from Tsushima, Japan, NHM 1907.12.9.1; c, *Eriocheir sinensis* H. Milne Edwards, 1853 adult male from Thames River, Kent; d, *Eriocheir hepuensis* Dai, 1991, adult female from Shatt Al-Basrah Canal, Iraq, NHM 2011.8035-8037. Photographs by Kevin Webb, NHM Photo Unit.

species due to the recent divergence of the group make it even more arduous to discern among taxa, often leading to misidentifications. Nevertheless, specimens from Hollands Diep and Den Oever are distinct, in terms of haplotype sequences, morphology and taxonomical characters, from mitten crab individuals from the Thames, identified as *E. sinensis*. At least three morphological traits can be established as distinguishing features between the cryptic *Eriocheir* species and *E. sinensis*. Despite differences in the carapace physiognomy between individuals from Den Oever and Thames River might be difficult, especially in the shape of the frontal teeth of the carapace, the Dutch and River Thames material can be distinguished with regard to the distal morphology of the G1 (Figure 5a vs 5d, e) and the fifth pereiopod (Figure 8a vs 8b–d).

Finding a second mitten crab species in The Netherlands and Wales but not in England, suggests that Welsh and English populations originated from independent introductions. The mitten crab specimens from Wales belonging to a cryptic *Eriocheir* species most likely originated from The Netherlands, possibly through dredging activities in the Dee Estuary. Evidence available to Natural Resources Wales (statutory Nature Conservation and Regulatory body in Wales) indicates that several Dutch dredging operators were active in the Dee Estuary during the early 2000s. It is highly plausible that those vessels had home ports from the region in Holland where the cryptic *Eriocheir* species has been found. Controls around the uptake and discharge of ballast water only entered into force globally on 8 September 2017. Prior to this date the International Convention for the Control and Management of Ships' Ballast Water and Sediments, 2004 (IMO 2004) were not widely controlled, including the UK. The provisions of the Ballast Water Management Convention, however, are not applicable

to the water in the hopper area of hopper dredgers (IMO 2011). In Wales, the management of hopper water is now regulated through the Marine Licensing process (Marine and Coastal Access Act 2009) and the provision of vessel biosecurity management plans. Nevertheless, the introduction of mitten crabs is expected to continue, with large populations established across the N.E. Atlantic and specimens found as far south as Cadiz, Spain (Cuesta et al. 2004, 2006; Garcia-de-Lomas et al. 2010; González-Ortegón et al. 2020). The results presented here radically challenge current assessments of *Eriocheir* invasion in Europe and should be considered by stakeholders as a new baseline to implement efficient management strategies against invasive mitten crabs.

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Authors’ contribution

Ferran Palero: project planning, sequencing, document writing, supervision of Inma Ferrer-Mateu Master’s project, figures 1, 3, 4; Inma Ferrer-Mateu: project planning, sequencing, document writing, figures 1, 3, 4; Ben Wray: project planning, document writing, funding, collection of mitten crabs from Dee Estuary; Rhian Hughes: collection of mitten crabs from Dee Estuary; David Morritt: project planning, document writing, collection of mitten crabs from Maidstone, Kent and River Thames; Mario Lepage: project planning, document writing, collection of mitten crabs from Gironde Estuary, France; Michiel Kotterman: project planning, document writing, collection of mitten crabs from The Netherland; Magnus van der Meer: project planning, document writing, collection of mitten crabs from The Netherland; Mary Tate: project planning, collection of mitten crabs from Maidstone, Kent; Seyit Ali Kamanli: project planning, document writing, CLSM scanning, images figures 2, 5; Lisa Smith: project planning, sequencing; Julia Llewellyn-Hughes: planning, sequencing; Paul F. Clark: project planning, co-ordination of project, document writing, collection of mitten crabs from River Thames, supervision of Inma Ferrer-Mateu Master’s project.

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