Org Divers Evol (2014) 14:369–382
DOI 10.1007/s13127-014-0176-4

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

Molecular identification and morphological characteristics of native and invasive Asian brush-clawed crabs (Crustacea: Brachyura) from Japanese and German coasts: *Hemigrapsus penicillatus* (De Haan, 1835) versus *Hemigrapsus takanoi* Asakura & Watanabe 2005

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Received: 16 April 2013 / Accepted: 14 May 2014 / Published online: 5 June 2014

Abstract Since 1994, *Hemigrapsus penicillatus*, an Asian brush-clawed shore crab, spreads along Northeast Atlantic coasts. There is evidence that the most recently described and widely accepted sibling species of *H. penicillatus*, namely *Hemigrapsus takanoi*, represents the Asian brush-clawed brachyuran crab in Europe. Morphological characteristics are considered insufficient for species discrimination, and molecular analyses were recommended but have to date not been performed in Europe. To clarify the identity of the non-indigenous Asian brush-clawed crab species, which has been invading intertidal alien *Crassostrea* reefs in the Wadden Sea, we analysed more than 3.5 kpb of mitochondrial and nuclear genes of individuals from invaded German sites and from native Japanese sites. In addition to molecular analyses, we also document key morphological and morphometric characteristics of the same individuals to provide a comparative description. While morphological identification was not confidently feasible, our molecular results confirm the existence of *Hemigrapsus takanoi* as a closely related species to *H. penicillatus* and have identified *H. takanoi* to be the alien brush-clawed crab species in Germany. Furthermore, most of the analysed specimens from Japan and all additional NCBI-listed brush-clawed crabs from Japan, Korea and China which were traditionally classified as *H. penicillatus* in Asia, are de facto *H. takanoi*.

Keywords Sibling species · DNA barcoding · 16S rDNA · NaK-ATPase · Bioinvasion · North Sea

Introduction

Brachyuran crabs play a major role in marine bioinvasions of estuaries and coasts worldwide (Brockerhoff and McLay 2011). One of the best studied invaders is the European shore or green crab *Carcinus maenas* (Linnaeus, 1758), which was first reported from the Northwest Atlantic in 1817. In the meantime, this crab has established itself along the west coast of North America, as well as in South Africa, Japan, Patagonia and Australia (Cohen et al. 1995; Hidalgo et al. 2005). Furthermore, the infamous Chinese mitten crab *Eriocheir sinensis* (H. Milne Edwards, 1853) has invaded the Northeast Atlantic, the North and Baltic Seas and the Mediterranean and has also established populations along the west, and most recently the east coast of North America (Cohen and Carlton 1997; Wolff 2005; Ruiz et al. 2006; Veilleux and de Lafontaine 2007).

In the same context, the western Pacific Asian shore crab *Hemigrapsus sanguineus* (de Haan, 1853), which established populations in the Northwest Atlantic in the 1990s

Electronic supplementary material The online version of this article (doi:10.1007/s13127-014-0176-4) contains supplementary material, which is available to authorized users.

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(McDermott 1991, 1998; Lohrer and Whitlatch 2002; Kraemer et al. 2007; Griffen et al. 2008), is known to have a high invading potential with rapid range expansion. It was introduced to the Northeast Atlantic (Breton et al. 2002; D'Udekem d'Acoz and Faasse 2002; Dauvin 2009; Dauvin et al. 2009) and the North Sea in 1999 (D'Udekem d'Acoz and Faasse 2002; Campbell and Nijland 2004; Wolff 2005). Contemporaneously, a brush-clawed Asian shore crab species, identified as *Hemigrapsus penicillatus* (De Haan, 1835), was introduced into Northeast Atlantic waters. In 1993, Gollasch (1999) first recognized *H. penicillatus* in Europe when he found several specimens on a ship's hull of a car-carrier in the harbour of Bremerhaven (Weser Estuary, Germany, North Sea). However, a reproductive population failed to become established. Nevertheless, brush-clawed crabs invaded the Atlantic coast near La Rochelle in 1994 (Noël et al. 1997), expanding its range north and south along French and Spanish Atlantic coasts (D'Udekem d'Acoz and Faasse 2002; Noël and Gruet 2008). First records from the French site in the British Channel (Vincent and Breton 1999; Breton et al. 2002; Dauvin et al. 2009; Dauvin and Delhay 2010; Dumoulin 2004; Soors et al. 2010) and in the North Sea (Nijland and Beekman 2000; Nijland 2006; Obert et al. 2007; Gittenberger et al. 2010) document the spread of the species (Fig. 1a). However, first sightings along European coastlines probably reflect multiple independent introductions (Fig. 1a: 1994 French Atlantic, 1997 French British Channel, 2000 The Netherlands).

Several years after the discovery of the Asian brush-clawed crab species in the Northeast Atlantic, two sympatric forms (form I, II) of *H. penicillatus* in the Nanakita River (Japan) were identified based on allozyme gel electrophoresis of 12 enzymes (Takano et al. 1997). In 2005, Asakura and Watanabe (2005) described the newly discovered sibling species (form II) as *Hemigrapsus takanoi*, and a detailed examination of species-specific morphological characteristics of several individuals in Tokyo Bay was carried out by Mingkid et al. (2006). In contrast to these studies, Sakai (2007) re-examined the material from Asakura and Watanabe (2005) and pointed out that the observed variation in pigmentation patterns is not a useful tool for the discrimination of the two species. He also emphasized that male first pleopods showed no fundamental difference in morphology and therefore concluded that *H. takanoi* has to be regarded as a synonym of *H. penicillatus* (Sakai 2007). In their reply, Asakura et al. (2008) discussed the revalidation of *H. takanoi* and pointed out that Sakai (2007) had confused the most reliable criteria separating the two species. Sakai's data clearly show that *H. penicillatus* has dark spots on abdominal somites which were missing in *H. takanoi*. However, Asakura et al. (2008) did not comment on discrepancies mentioned by Sakai (2007) concerning the morphology of male first pleopods.

Most recently, Yamasaki et al. (2011) performed molecular-based determinations using a restriction fragment length polymorphism (RFLP) approach based on a short sequence fragment of the mitochondrial 16S ribosomal RNA (rRNA) gene of several specimens that were collected along the coast of Japan. Their results supported the validity of the sibling species status. Individuals with mixed or unclear morphological characteristics were here attributed to *H. penicillatus*. The first 16S rDNA sequence from a single specimen collected in 1996 in France (Schubart et al. 2001) showed higher similarity to *H. takanoi* than to *H. penicillatus* (Yamasaki et al. 2011). However, the authors conceded that morphological characteristics may not be distinct enough to distinguish between species, especially when small individuals are examined.

In summary, there is increasing evidence that the newly described and widely accepted sibling species *H. takanoi* seems to represent the brush-clawed invader in Europe. Its introduction was presumably caused by transportation through hull fouling or ballast water. Mingkid et al. (2006) found that *H. penicillatus* was predominantly distributed in the outer sections of Tokyo Bay, whereas *H. takanoi* occurred mainly in the bay. As port operations concentrate to the inner sections of bays (Gollasch 1999), the distribution pattern found by Mingkid et al. (2006) supports the assumption of Asakura and Watanabe (2005) that European waters were most likely invaded by *H. takanoi* and not by *H. penicillatus*. On the other hand, both species occur sympatrically in Japan and may thus also coexist in European waters because of their similar ecology (Yamasaki et al. 2011). European scientists refer to *H. takanoi* in more recent records and publications, even though morphological features are considered insufficient for species discrimination and molecular analyses have been recommended but have to date not been performed in Europe.

Study aims

Morphological analyses of Asian brush-clawed crabs from intertidal Pacific oyster (*Crassostrea gigas*) reefs in the Central Wadden Sea (Germany) revealed interindividual similarity but rather mixed or contrasting characteristics when compared with species-specific morphological features described in the existing literature. As a consequence, the invasive brush-clawed crab in our study area has thus not confidently been identified as either *H. penicillatus* or *H. takanoi*.

To clarify the identity of the alien species, two mitochondrial (partial COI, partial 16S rDNA) and two nuclear genes (partial sodium-potassium ATPase α-subunit, complete 18S rDNA) of several German and Japanese specimens were analysed (Fig. 1, Table 1). In addition to molecular analyses, key morphological characteristics were assessed. As such this is the first integrative approach providing a specimen-specific analysis and a comparative description of both native and invasive specimens.

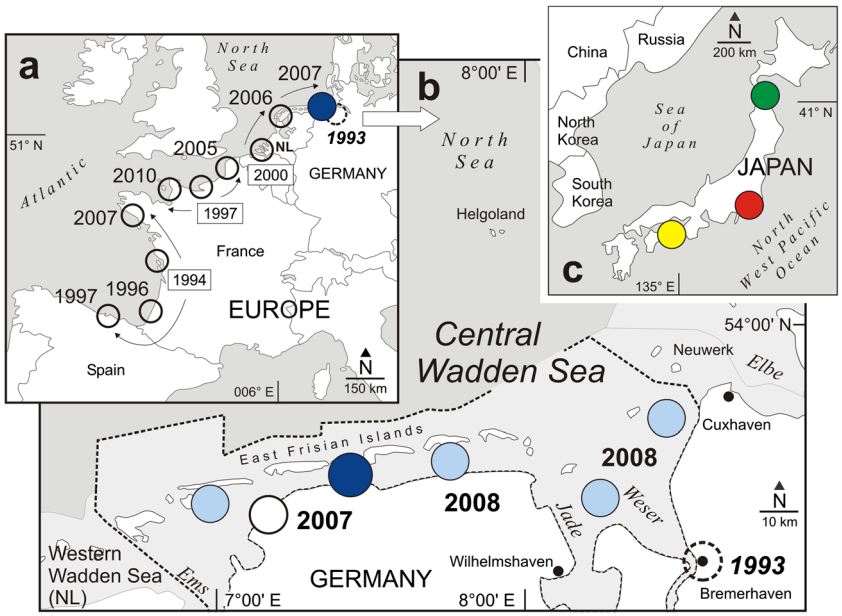


Fig. 1 First records of wild Asian brush-clawed crab populations in the Northeast Atlantic and sampling localities in Germany and Japan. **a** Years of the first record along Northeast Atlantic coasts, record 1993 from a ship hull. Potential independent introductions in *boxes*, *black arrows* indicate potential spread direction. **b** Years of the first records in the Central Wadden Sea, Germany. *Coloured circles* at intertidal *Crassostrea* reef sampling sites (*dark blue Hs*pDN, *light blue Hs*pWS—for details see "Materials and methods"). **c** Sampling localities in Japan (*yellow Hp*S, *green Hp*Y, *red Ht*Y—for details see "Materials and methods")

Material and methods

Morphological and molecular analyses were conducted for a total of 34 specimens originating from three sites in Japan and one site in Germany, resulting in four groups of individuals (Fig. 1a–c; for locality data see Table 1). Each individual was stored separately in 96 % ethanol and tagged with a reference code that combines a species-/origin-specific abbreviation with a group-specific serial number. In addition, each specimen can be identified by a molecular identification number (MT-1462 to MT-1496) and a depository number of the Senckenberg Natural History Museum, Frankfurt am Main, Germany (SMF-43682 to SMF-43715), as listed in Online Resource 1.

Six Japanese specimens were collected at the Yoshinogawa Estuary/Tokushima and identified as *H. penicillatus* by Katsushi Sakai (group *Hp*S, 1–6). In addition, nine Japanese specimens were collected from the shore of Ōma/Aomori (*H. penicillatus* group *Hp*Y, 1–9) and another nine from Haneda/Tokyo Bay (*H. takanoi* group *Ht*Y, 1–9), all of which were identified by Izumi Yamasaki. Ten non-identified German specimens (group *Hs*pDN, 1–10) were collected by the authors from a Pacific oyster (*C. gigas*) reef at the Dornumer Nacken/Central Wadden Sea (low-energy tidal flat with an average salinity 30 PSU). As species identification of small individuals is particularly difficult (Mingkid et al. 2006; Yamasaki et al. 2011), sexually mature but nevertheless small individuals with carapace widths of about 10 mm were collected. To support the evaluation of the morphometric data of males and females, we also collected larger individuals at different *Crassostrea* reefs in the Central Wadden (see Fig. 1b; group *Hs*pWS, light blue symbols, 18 males and 8 females).

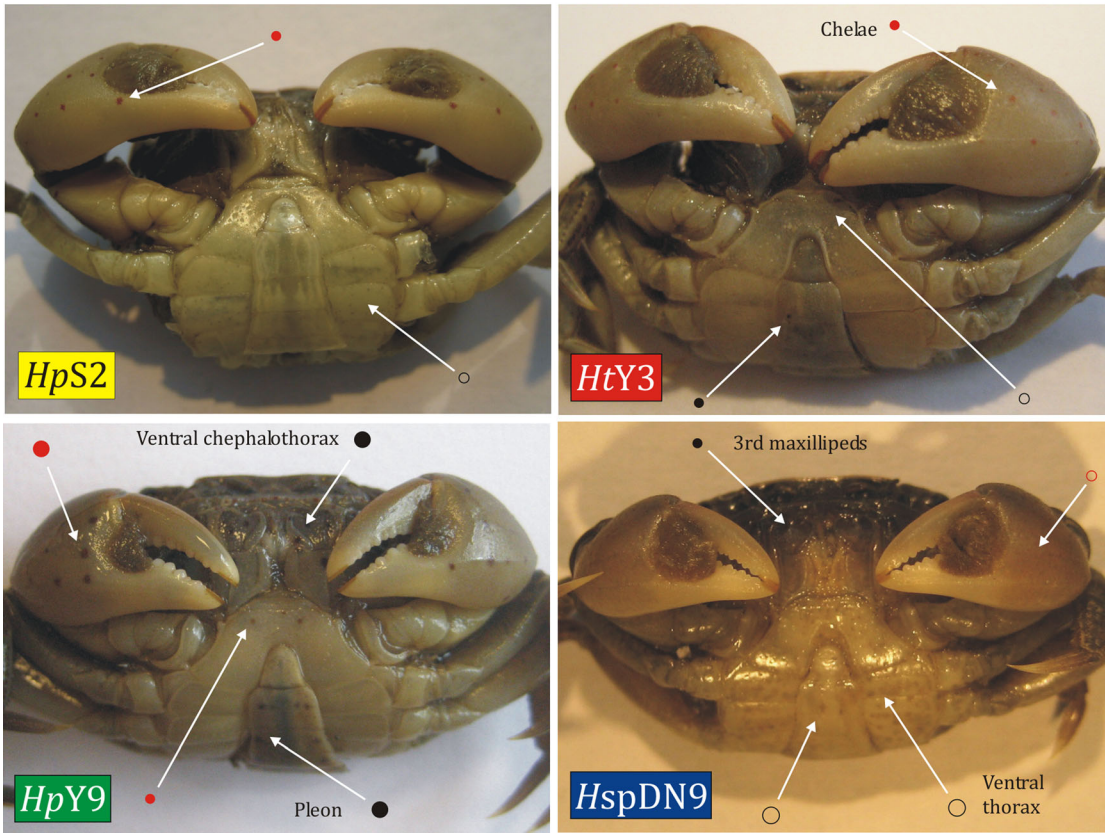
A fifth group was collected between 1823 and 1829 by Philipp Franz von Siebold during his stay in Nagasaki, Japan (32°42′ N, 128°50′ E). The Siebold collection is held by the National Museum of Natural History (Naturalis Biodiversity Centre) in Leiden (The Netherlands) and consists of 16 brush-clawed crabs (*H. penicillatus* (De Haan, 1835) group *Hp*L, white symbols; nine males: six in ethanol, including the lectotype of *H. penicillatus* RMNH D200, and three dry; seven females: five in ethanol and two dry). The museum individuals (*Hp*L) were analysed by morphological measurements only because pigmentation had strongly faded. Molecular analyses of tissue material from this group failed.

Morphological analyses

Morphological analyses followed the methods described in Takano et al. (1997), Asakura and Watanabe (2005) and Mingkid et al. (2006) in order to assess analogues on the basis of comparable data. We focused on significant key characteristics which the above authors considered relevant for the discrimination between species. In these studies, the presence (form I, *H. penicillatus*) or absence (form II, *H. takanoi*) of dark spots on the third maxillipeds, on the ventral cephalothorax and on the abdomen (pleon) of both sexes was used as key characteristic to distinguish the two species, although mixed or unclear pigmentation patterns were also found. Spots are "large" in *H. penicillatus* and "small" in *H. takanoi*. These characters were not delineated or measured. The diameter of the soft hair patch in male chelae (DSH) relative to carapace width (CW) differed significantly between species as the majority of hair patches were small (DSH/CW

Table 1 Listed are 34 specimens used for morphological and molecular analyses: locality data is given for four groups of individuals with different origin, species name according to collector's identification: *H. penicillatus* sensu Sakai (*Hp*S), *H. penicillatus* sensu Yamasaki (*Hp*Y), *H. takanoi* sensu Yamasaki (*Ht*Y) and *Hemigrapsus* sp. from the study site Dornumer Nacken, Germany (*H*spDN). Gender (ovigerous females are indicated in italics), carapace width (CW) and pigmentation pattern is given for each specimen. Pigmentation is plotted in *solid circles* when *dots* were obviously present or in *open circles* when faint, *circles* according to colour (*black* or *red*) and size (*small* or *large*) of *dots* at different zones of the ventral face (pleon, thorax, chephalothorax, third maxillipeds) and on the chelae. For details, see photographic illustra tions

Individual ID	*HpS1*	*HpS2*	*HpS3*	*HpS4*	*HpS5*	*HpS6*	*HpY1*	*HpY2*	*HpY3*	*HpY4*	*HpY5*	*HpY6*	*HpY7*	*HpY8*	*HpY9*	*HtY1*	*HtY2*	*HtY3*	*HtY4*	*HtY5*	*HtY6*	*HtY7*	*HtY8*	*HtY9*	*HspDN1*	*HspDN2*	*HspDN3*	*HspDN4*	*HspDN5*	*HspDN6*	*HspDN7*	*HspDN8*	*HspDN9*	*HspDN10*
Species	*H. penicillatus*						*H. penicillatus*									*H. takanoi*									*H.*sp. (?)									
Collector	Sakai						Yamasaki									Yamasaki									Markert									
Country	Japan						Japan									Japan									Germany									
Site	Yoshinogawa (Tokushima)						Õma (Aomori)									Haneda (Tokyo)									Dornumer Nacken (Central Wadden Sea)									
Coordinates	34°04′ N, 134°33′ E						41°31′ N, 140°55′ E									35°33′ N, 139°47′ E									53°42′ N, 007°28′ E									
Collection date	Nov 2008						Sept 2007									Nov 2007									Dez 2008	June 2008								
Gender	female	male	male	male	male	female	female	male	male	female	female	female	female	male	male	male	female	male	male	male	male	female	female	female	male	male	male	*female*	male	female	*female*	*female*	male	male
CW [mm]	16.15	17.47	12.15	21.89	21.06	11.31	18.32	26.22	11.62	22.67	21.45	19.69	22.73	15.66	18.15	15.81	16.39	19.06	18.21	22.14	19.30	17.30	17.99	16.85	11.61	9.53	13.23	14.89	13.19	9.63	11.09	13.66	10.40	8.95
Pleon	○						●		○	●	●	●	●	●	●		○	●				◯						○	○	○	○		◯	○
Ventral thorax		○	○			○	●	●						●	●			○			◯						○	●	●	○	●		◯	○
Ventral chephalothorax	●	●	●	●	●	●	●	●		●	●	●	●	○	●	●	●	●	●	○	●	○	●	●		○	●	●	●	●		●	○	●
3rd maxillipeds	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	●	●	●	●	○	●	●	●	●			●	●	●	●		●	●	●
Chelae	●	●	●	○	●	●	●	●	○	●	●	●	●	●	●	●	●	●	●	○	●	●	●	●	○	○	●	●	●	○	●	●	○	●



<0.18) in *H. penicillatus* and large (DSH/CW >0.18) in *H. takanoi*. The relative size of soft hair patch of *H. takanoi* increased with carapace width. Females of *H. penicillatus* had on average proportionally higher (chela height CHH/chela length CHL 0.52±0.03), but shorter (CHL/CW 0.45±0.03) chelae than *H. takanoi* (CHH/CHL 0.49±0.03; CHL/CW 0.48±0.04). In addition, two species-specific types of male first pleopods were identified, whereas an intermediate type was found in approximately one third of both species.

Pigmentation

Pigmentation pattern on different zones of the ventral face, on pleon, thorax (around the pleon), cephalothorax, third maxillipeds, respectively, and on both outer chelae was registered as "present" when dots showed an obvious and clear appearance with a well defined outline, or as "faint" when dots were visible without a distinctive appearance. In addition, pigmentation was classified according to dot size into small or large, and according to dot colour into black (dark brown was also noted as black) or red categories. Pigmentation was assessed from to the most prominent dot. Examples of dot classification are presented in Table 1. Changes in the colour of the dots due to short-term fixation and storage in ethanol was not observed when compared with freshly collected crabs. Colouring and pigmentation of the museum material from Leiden (*Hp*L) was faded.

Morphometry

Body dimensions involving carapace width (CW), chela length (CHL), chela height (CHH) and outer diameter of the male soft hair patch (DSH) were measured with a digital calliper to the nearest 0.01 mm. The relative size of the male soft hair patch was calculated as the ratio of DSH/CW. The shape of female chelae was expressed as the ratio of CHL/CW and CHH/CHL. The first pleopods of each individual male of the groups *H*spDN, *Hp*Y, *Ht*Y and *Hp*S were detached and examined in detail by microscope before and after removal of setae. Male first pleopods of the group *Hp*L were examined without detachment or setal removal. In one case, a pleopod of the lectotype RMNH D200 was found detached and with setae already removed. This pleopod was examined under a microscope with pictures taken by means of a digital microscope camera (Leica). Only one morphotype of first pleopods was found in all specimens. To document the morphology, one right pleopod with setae and one without setae were dried and mounted on a stack holder in a ball of mesoporous carbon before being spotter-coated with gold for imaging by scanning electron microscopy (SEM).

Molecular analyses

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from dissected walking legs of 34 specimens also examined by morphometric analysis using the QIAmp© Tissue Kit (Qiagen GmbH, Hilden, Germany) and following the manufacturer's extraction protocol. In the case of the *Hemigrapsus* type specimens of the Siebold collection from Leiden, it was not possible to amplify any amplicon. As part of the molecular studies, the mitochondrial COI barcode fragment for 34 specimens, the partial mitochondrial 16S rDNA for 27 specimens and the complete nuclear 18S rDNA for four specimens were amplified and sequenced (cf. Online Resource 1). In addition, a fragment of the nuclear single copy gene sodium-potassium ATPase α-subunit (NaK) from 15 individuals was amplified (cf. Online Resource 1). A detailed summary of all used PCR and sequencing primers, PCR protocols, amplification reactions and temperature profiles are given in Online Resource 2. Amplification reactions for all markers were carried out on a Mastercycler pro S system (Eppendorf, Hamburg, Germany). PCR products were purified with the QIAquick© PCR Purification Kit (Qiagen GmbH, Hilden) and then cycle sequenced and sequenced in both directions at commercial sequencing facilities (Macrogen, Seoul, Korea, or GATC, Konstanz, Germany). All double-stranded sequences were assembled with the Geneious© 5.3 program package (Drummond et al. 2010). BLAST searches were performed to confirm the identity of all new sequences (Zhang et al. 2000; Morgulis et al. 2008). Sequences of protein-coding genes (COI, NaK) were translated to amino acid sequences to check for the presence of pseudogenes also using the Geneious© 5.3 software. All newly analysed sequences are available in GenBank (cf. Online Resource 1).

Phylogenetic and population structure analyses

Besides the data generated in this study, we also integrated NCBI published sequences of our analysed markers, including five published COI DNA barcodes and seven partial 16S rDNA sequences for *H. takanoi* and *H. penicillatus* (10 Dec 2013). With reference to the COI sequences, a BLAST search revealed a mislabelling of two sequences, HM180610 and HM180611 (Kim et al. 2012), which represent DNA barcodes of *H. sanguineus* instead of *H. penicillatus*. Consequently, only the remaining three sequences (*H. penicillatus*: JX502902, JX502903, JX502904) were added to our COI data set. In terms of the 16S rDNA data, the length of the available seven NCBI sequences differed drastically. Only two sequences of *H. penicillatus* (GU731424, AJ278835) had the full fragment coverage of the Palumbi primer pair for *Hemigrapsus* (about 529 base pairs, bp) (Palumbi et al. 1991). The fragment length of the remaining five sequences,

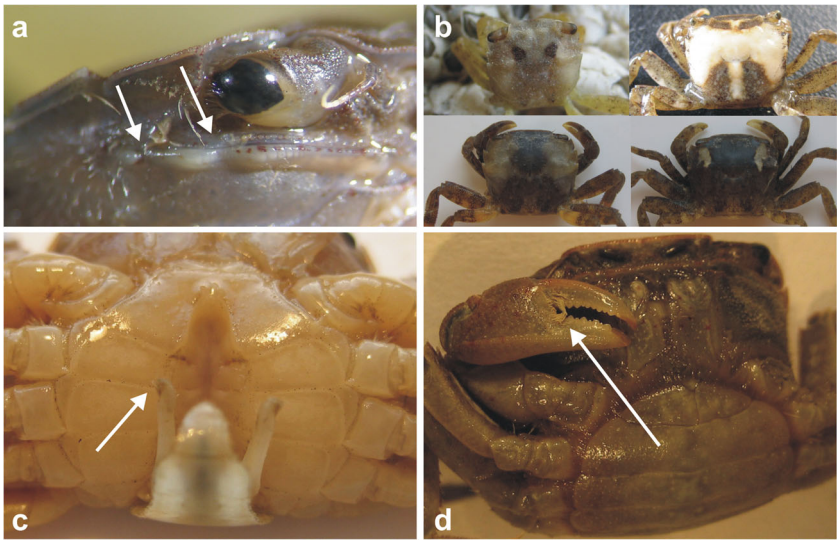


Fig. 2 Morphological characteristics. **a** Double broken infraorbital ridge IOR. **b** Symmetrically arranged spots on the dorsal face. **c** Male pleon fold down, first pleopods facing distal/lateral (setae of the head around a scoop-like nose appears as dark area at the end of the pleopod). **d** Soft hair near the movable finger of a female chela (*Ht*Y2)

nested within the Palumbi fragment, ranged from 211 bp (*H. takanoi*, AB560767) to 480 bp (*H. penicillatus*, AB058628). As a consequence, we analysed two different alignments. The first one included all sequences covering the complete 16S rDNA Palumbi fragment (529 bp, 29 sequences), whereas the second, in the following called "Yamasaki fragment" (Yamasaki et al. 2011), included 34 sequences with a length of 212 bp.

All sequences for each marker were aligned separately using MUSCLE version 3.6 (Edgar 2004), implemented in MEGA 5.2.1 (Tamura et al. 2011) with default settings. Because of nucleotide ambiguities at the 5′ of various sequences, the first five base pairs of the "Yamasaki fragment" were removed from the analysis. Furthermore, sequence AB560768 (*H. takanoi*) were removed from the alignment due to numerous ambiguities in large parts of the 3′ end of the sequence. In summary, the analysed Yamasaki fragment covered 33 sequences and 207 bp, representing position 111–317 of the aligned Palumbi fragment. A detailed listing of all analysed sequences and markers are given in Online Resource 3. All analysed markers were tested for nucleotide bias using a chi-square test of base composition homogeneity across taxa as implemented in PAUP* 4.0b10 (Swofford 2002). We used MEGA 5.2.1 to calculate descriptive statistics of intra- and interspecific variability based on observed distances (*p* distances) as well as TCS 1.21 (Clement et al. 2000) to create maximum parsimony networks for all markers with interspecific *p* distances. Networks were overlaid with the geographic origin and/or taxonomic classification of the haplotypes.

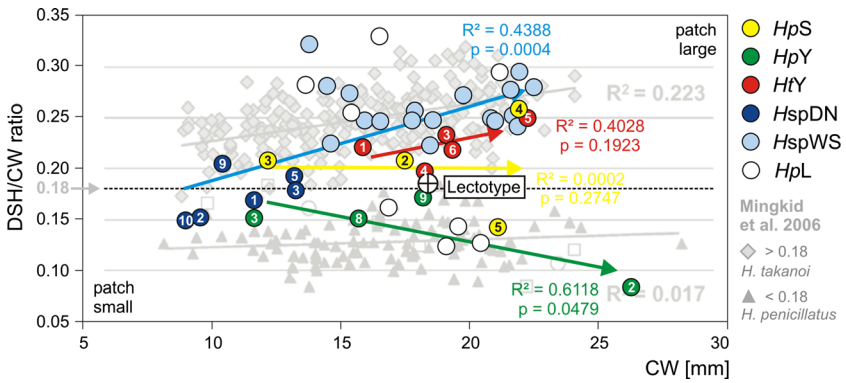


Fig. 3 Relative size of soft hair patch (DSH/CW) plotted against carapace width (CW) for single male crabs out of groups of individuals with different origin and species name according to collector's identification: *H. penicillatus* sensu Sakai (*Hp*S2/3/4/5), *H. penicillatus* sensu Yamasaki (*Hp*Y2/3/8/9), *H. takanoi* sensu Yamasaki (*Ht*Y1/3/4/5/6) and *Hemigrapsus* sp. from the study site Dornumer Nacken, Germany (*H*spDN1/2/3/5/9/10) supplemented by additional ratios of larger individuals from the Central Wadden Sea, Germany (*H*spWS), museum individuals of the Siebold-collection from Leiden (*Hp*L). *Black dotted line* indicates the threshold value after Mingkid et al. (2006) to discriminate between *H. penicillatus* (<0.18) and *H. takanoi* (>0.18), results of their study in *grey*

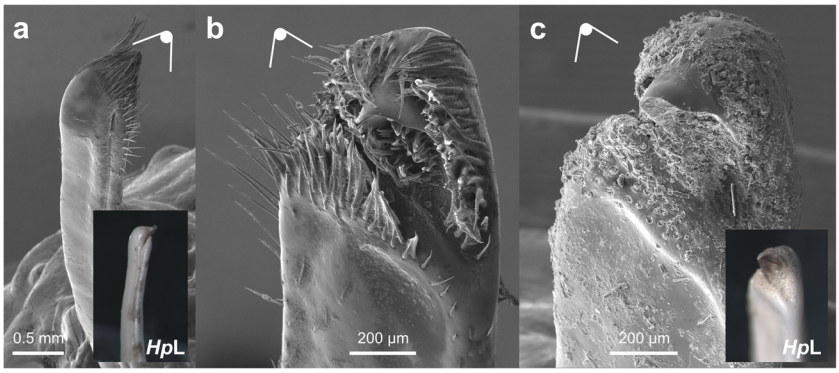


Fig. 4 Morphology (REM) of Japanese and German male right first pleopod of Asian brush-clawed crabs. **a** Total view (*proximal*). **b** Enlarged with setae (*lateral/distal*). **c** Without setae (for orientation of the pleopod see Fig. 2c). Symbol in (**a**) indicates the view direction in (**b**) and (**c**), symbols in (**b**) and (**c**) indicate the view direction in (**a**). *Small photo* (digital microscope camera) displays right the first pleopod of the lectotype *H. penicillatus* RMNH D200 of the Siebold collection from Leiden (*Hp*L)

Results

Morphological analysis

Both sexes are characterized by a subquadrate carapace, three anterolateral teeth on each side and a double broken infraorbital ridge (Fig. 2a). Colouring and pigmentation of the museum individuals (*Hp*L) are faded. In all other groups of the recent individuals, the colouring of the dorsal carapace is divers, varying between brownish, greyish and greenish, while in some German individuals two symmetrically arranged spots occur on the left and right sides of the dorsal carapace (Fig. 2b). Basic colouring of the ventral face and the chelae vary from beige to light brown and dark brown. Mean carapace width of the museum individuals (*Hp*L) is 17.14±2.49 mm (males 17.94±2.48; females 16.12±2.27). Mean carapace width (Table 1) of all recent individuals is 17.15±3.89 mm (males 17.28±4.18; females 16.93±3.45). Mean carapace width of individuals identified as *H. penicillatus* by Sakai (*Hp*S) is 16.67±4.40 mm (males 18.14±4.43; females 13.73±3.42), of *H. penicillatus* from Yamasaki (*Hp*Y) 19.61±4.32 mm (males 17.91±6.16; females 20.97±1.93), of *H. takanoi* from Yamasaki (*Ht*Y) 18.12±1.91 mm (males 18.90±2.28; females 17.13±0.68), of *H.* sp. from the Dornumer Nacken in Germany (*H*spDN) 11.62±2.03 mm (males 11.15±1.83; females 12.32±2.39) and the mean carapace width of the supplemented larger individuals from the Central Wadden Sea region in Germany (*H*spWS) is 18.20±2.69 mm (males 18.54±2.93; females 17.42±1.98). Three ovigerous females were identified in the German group (*H*spDN4, *H*spDN7, *H*spDN8).

Pigmentation

Pigmentation on the different zones of the ventral face (pleon, thorax, cephalothorax, third maxillipeds) and on the chelae is present in all recent groups (*Hp*S, *Hp*Y, *Ht*Y, *H*spDN), except for one German male individual (*H*spDN1) which had a few

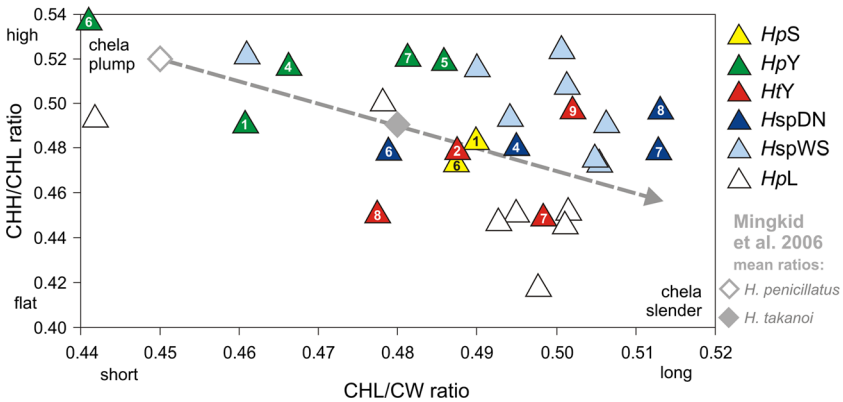


Fig. 5 Shape of female chelae plotted as chelae length in relation to carapace width (CHL/CW) against relative chelae height (CHH/CHL) for single female crabs out of groups of individuals with different origin and species name according to collector's identification: *H. penicillatus* sensu Sakai (*Hp*S1/6), *H. penicillatus* sensu Yamasaki (*Hp*Y1/4/5/6/7), *H. takanoi* sensu Yamasaki (*Ht*Y2/7/8/9) and *Hemigrapsus* sp. from the study site Dornumer Nacken, Germany (*H*spDN4/6/7/8) supplemented by additional ratios of larger individuals from the Central Wadden Sea, Germany (*H*spWS), museum individuals of the Siebold collection from Leiden (*Hp*L). Mean ratios for female *H. penicillatus* and *H. takanoi* after Mingkid et al. (2006) as *grey symbols*; *dotted line* indicates a trend to slender chelae in female *H. takanoi*

small faint red dots on its chelae. Pigmentation pattern on the ventral face was highly variable and no significant pattern within or between groups was identified (Table 1). In the following, trends in pigmentation pattern not appropriate for identifying single individuals are described. The presence of several dots on the cephalothorax and on the third maxillipeds is almost consistently observed in all individuals. Dots on the cephalothorax are small and black in groups *Hp*S and *H*spDN, black in *Hp*Y and red in *Ht*Y. Larger dots are only common in *Hp*Y. Differences in pigmentation on the pleon are not distinct between groups, but are more obvious when compared with the pigmentation pattern of other zones. Dots on the pleon of individuals of group *Hp*Y do not occur in all individuals, but a pigmented pleon is common, while some individuals also have larger dots. By contrast, dots are underrepresented, mainly small and primarily faint in the other groups. Colouring of the dots in these groups is almost consistently black (or dark brown), irrespective of appearance (present/faint) or size (small/large). Dots on the thorax have comparable colouring, but are fewer in number than on the pleon, and a clear presence of pigmentation occurs only in some individuals of groups *Hp*Y and *H*spDN. Dots on the chelae are commonly present and red in all groups. Even though small and faint dots occur on *Hp*Y chelae, the majority of the *Hp*Y dots are larger than in the other groups.

Relative size of male soft hair patch

All male chelae had a soft hair patch near the movable finger (Table 1). The relative size of the patch (DSH/CW) increases with carapax width for all German individuals (individuals of group *H*spDN and *H*spWS) (Fig. 3). Most of the ratios of the smaller German individuals (*H*spDN) fall below the threshold ratio of 0.18, which was identified by Mingkid et al. (2006) as a discrimination criterion between *H. penicillatus* and *H. takanoi*. The additional larger German individuals have ratios that are clearly above the threshold ratio, the ratios being almost exclusively higher (larger relative patch size) than in all other recent individuals investigated. All ratios of individuals of group *Ht*Y are above 0.18 and the relative size of the patch is also related to increasing crab size. Individuals of group *Hp*S show no clear relation to changes in carapax width, but the majority of the individuals have patch ratios above the threshold value, only one individual (*Hp*S5) falling below. By contrast, all relative patch size ratios of *Hp*Y individuals fall below the threshold value and ratios decrease with increasing carapax width. The male museum material (*Hp*L) showed a high variability in patch size. DSH/CW ratios ranged between 0.123 and 0.330, the lectotype RMNH D200 having a ratio of 0.185. Accordingly, the males of the Siebold collection from Leiden (*Hp*L) consist of two groups of individuals: four males with large patches (2 ethanol, 2 dry) and four individuals with small patches (3 ethanol, 1 dry).

Several female chelae have some soft hair near the movable finger (Fig. 2d) (*Hp*S1, *Hp*S6, *Ht*Y2/Fig. 2, *Ht*Y9, *H*spDN6, *H*spDN7, *H*spDN8), some having a smaller pleon compared to other females (*Hp*S1, *Hp*S6, *H*spDN6).

Male first pleopod

We identified one first pleopod morphotype (Fig. 4) in all recent individuals and identified the same type in the six male museum individuals (*Hp*L) which were stored in ethanol, including the lectotype RMNH D200. The erect stem of the first pleopod has a shouldered groove along its proximal side (Fig. 4a). The groove ends at a head region of the pleopod where a setaceous margin extends down to the front side (Fig. 4b, c). The setae on the diagonal frontal margin at the end of the pleopod grows more or less straight (Fig. 4b), whereas the setae of the head is curved, pointing to the outside of the pleon, or rather lateral/distal as the orientation of the pleopod itself (Fig. 4a). The back of the head has no setae (Fig. 4a). The head narrows to a straight top which, observed from the front, appears narrowed compared to the rest of the stem. A large non-setaceous scoop-like nose opens up in the middle of the top (Fig. 4b, c). Curved setae closely surround the frontal top of the head and nose, whereas the top setae are longer (Fig. 4a) and setae below the nose and on the sides are shorter. Some pleopods have one or two small notches at the scoop-like nose, which appear to be mechanical damages.

Shape of female chelae

No clear characteristic shape differences of female chelae were detected between groups (Fig. 5). Shape varies within groups and is most diverse in group *Hp*L and varies stronger within groups *Hp*Y and *H*spDN than in groups *Ht*Y and *Hp*S. The latter are represented by only two individuals. The majority of proportional measures are related to the mean ratio identified by Mingkid et al. (2006) as being characteristic for a chela shape of *H. takanoi* female, which is also true for almost all individuals of group *Hp*Y. However, a trend from a plump (short and high) shape in group *Hp*Y to a more slender (long and flat) shape in the groups *Ht*Y and *Hp*S is apparent. Most museum females (*Hp*L) have a slender (*H. takanoi*) type of chelae. Female chelae of German individuals, by contrast, tend to be “mighty” (long and high), the measurements revealing more combinations of long and high ratios than in other groups. Almost all length and most height values of German female chelae

exceed the mean values identified for *H. takanoi* by Mingkid et al. (2006).

Molecular data

The final five molecular alignments consist of 684 bp (COI), 529 bp (16S rDNA: Palumbi fragment), 127 bp (16S rDNA: Yamasaki fragment), 631 bp (NaK) and 1814 bp (18S rDNA). None of the four markers show significant differences in base composition (chi-square test for all: 1). Both sequenced mitochondrial gene fragments are AT rich (COI: A 0.27, C 0.19, G 0.18, T 0.36; 16S rDNA Palumbi fragment: A 0.36, C 0.11, G 0.16, T 0.37), as is typical for these arthropod genes (e.g. Simon et al. 2006; Raupach et al. 2010; Wesener et al. 2010; Rajaei et al. 2013).

The DNA barcode analyses for the 37 specimens of *H. penicillatus/takanoi* reveal 17 different haplotypes (Fig. 6a). Of 40 polymorphic sites, three (7 %) are at the first and 37 (93 %) at the third codon position. No amino acid replacement substitutions were detected. The maximum parsimony analysis using the default setting of a 95 % connection limit resolved in two unconnected subnetworks (Fig. 6a), separating all nine analysed specimens of *H. penicillatus* sensu Yamasaki from all others. Lowering the connection limit to 90 % reveals a putative connection between these subnetworks via 21 mutational steps. The DNA barcodes of the analysed crabs classified as *H. penicillatus* sensu Yamasaki consist of five different haplotypes, including four single haplotypes (h11–h14; singletons) and one haplotype (h10) with a higher frequency (n=5). By contrast, 12 different haplotypes were found for all other studied crabs (n=28). Here, one dominating haplotype (h1) was detected, representing the most abundant haplotype of the given data set (n=10). However, most of these were scored as singletons (n=8). Three haplotypes (h1, h2, h3) are shared by specimens of different classification. Uncorrected pairwise genetic distances among the COI haplotypes within specimens of *H. penicillatus* sensu Yamasaki range from 0 to 0.6 % and from 0 to 1.2 % for all others; distances between both cluster range from 3.8 to 4.1 %.

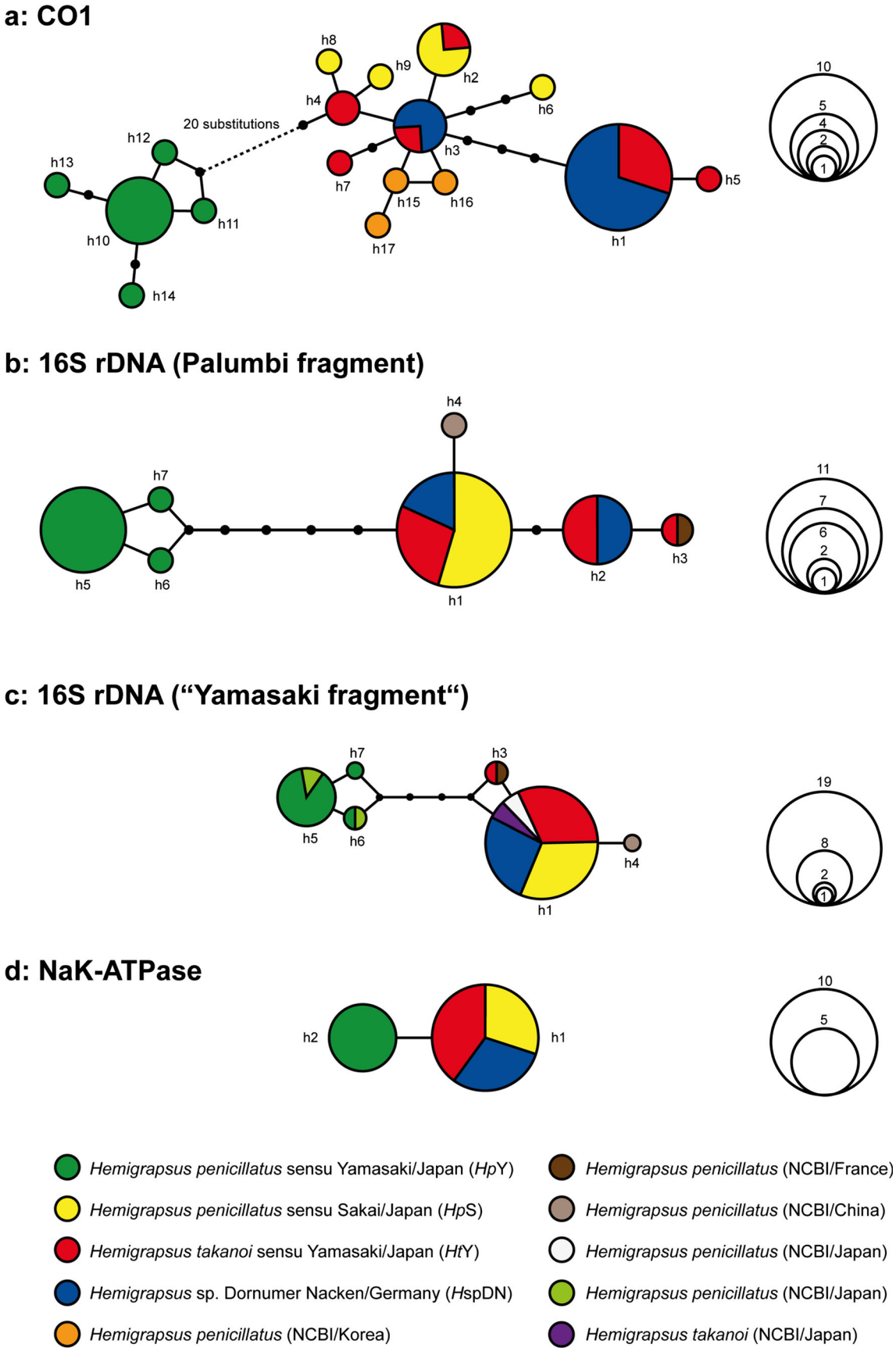
With its seven haplotypes, the 16S rDNA data supports the splitting of the COI network, albeit with a somewhat lower degree of genetic variability and resolution (Fig. 6b, c). Here, p distances among the 16S rDNA haplotypes (Palumbi fragment) within specimens of *H. penicillatus* sensu Yamasaki range from 0 to 0.4 % and from 0 to 0.8 % for all others, whereas distances between both cluster have values between 1.3 to 1.5 %. In this context, both mitochondrial clusters are also found among the analysed subset of nuclear NaK sequences: specimens classified as *H. penicillatus* sensu Yamasaki can be separated by one polymorphic site (third codon position, no amino acid switch) from all others (Fig. 6d). However, there is no variation among the four examined complete 18S rDNA sequences.

Discussion

Over the last few years, the supplementary use of molecular markers for species identification and classification has become increasingly more popular. In this context, DNA barcoding represents a highly valued method in modern biodiversity assessment studies (e.g. Hebert et al. 2003; Pfrender et al. 2010; Nagy et al. 2012), and it is not surprising that many recently published species descriptions include barcode sequence data (for crustaceans, e.g. Komai et al. 2011; Yoshida et al. 2011; Chen et al. 2012; Carrison-Stone et al. 2013; Riehl and Kaiser 2012). In the case of *H. takanoi*, available molecular data were so far restricted to (a) allozyme data (Takano et al. 1997) and (b) some sequence data and RFLP analysis of a short fragment of the mitochondrial 16S rDNA (Yamasaki et al. 2011). As part of the present study, more than 3.5 kbp of mitochondrial and nuclear genes were analysed, including the DNA barcode fragment, to build up a robust molecular framework by which to clarify the identity of the non-indigenous Asian brush-clawed crab species invading German intertidal alien *Crassostrea* reefs. Our molecular analyses reveal two distinct mitochondrial clades without intermediates within the analysed crabs, separating the group of nine *H. penicillatus* sensu Yamasaki from all other analysed crabs. Uncorrected pairwise genetic distances of the barcode fragment among both clades range from 3.8 to 4.1 % (22 mutational steps). These values are similar to the distances that were found between two closely related brachyuran species of the genus *Potamon* (4.4 – 4.6 %, 23 mutational steps; Keikhosravi and Schubart 2013). Both clades are also separated by the analysed nuclear NaK gene fragment, which can be used to discriminate closely related species (Manaffar et al. 2011; Kou et al. 2013). By contrast, no substitutions were found within the complete 18S rDNA, where even single substitutions within the hypervariable regions can indicate closely related but distinct species (e.g. Raupach et al. 2007, 2010).

In summary, our molecular results confirm the proposed existence of two closely related species (Takano et al. 1997; Asakura and Watanabe 2005; Mingkid et al. 2006; Yamasaki et al. 2011): (a) *H. penicillatus*, which in this study is represented by specimens classified as *H. penicillatus* sensu Yamasaki and (b) *H. takanoi*, which includes all specimens classified as *H. takanoi* sensu Yamasaki, *H. penicillatus* sensu Sakai, *Hemigrapsus* sp. from *Crassostrea* reefs in the Central Wadden Sea (Germany), and all specimens with already published sequence data. Hence, our molecular analysis also shows that most crabs that were traditionally classified as *H. penicillatus* in Asia de facto represent *H. takanoi*.

In contrast to our molecular results, it was not possible to clearly separate the two species when using morphological characteristics for identification. Furthermore, we observed high intrapopulation variability. *H. penicillatus* (*H. penicillatus* sensu Yamasaki) and *H. takanoi* (*H. takanoi*


a: CO1
h8
h9
h2
h4
h6
20 substitutions
h12
h3
h13
h7
h15
h16
h5
h11
h10
h17
h1
h14
10
5
4
2
1
b: 16S rDNA (Palumbi fragment)
h4
h7
h5
h6
h1
h2
h3
11
7
6
2
1
c: 16S rDNA ("Yamasaki fragment")
h7
h3
h5
h6
h4
h1
19
8
2
1
d: NaK-ATPase
h2
h1
10
5
Hemigrapsus penicillatus sensu Yamasaki/Japan (HpY)
Hemigrapsus penicillatus sensu Sakai/Japan (HpS)
Hemigrapsus takanoi sensu Yamasaki/Japan (HtY)
Hemigrapsus sp. Dornumer Nacken/Germany (HspDN)
Hemigrapsus penicillatus (NCBI/Korea)
Hemigrapsus penicillatus (NCBI/France)
Hemigrapsus penicillatus (NCBI/China)
Hemigrapsus penicillatus (NCBI/Japan)
Hemigrapsus penicillatus (NCBI/Japan)
Hemigrapsus takanoi (NCBI/Japan)

◀ **Fig. 6** Statistical parsimony networks showing the mutational relationships among the analysed mitochondrial COI (**a**), 16S rDNA (Palumbi fragment (**b**), Yamasaki fragment (**c**)) and nuclear NaK (**d**) haplotypes of *H. takanoi and H. penicillatus*. Each line between haplotypes represents one mutational step, while putative and missing intermediate haplotypes not recovered in our sampling are indicated by *small black dots*. The *colour* of *solid circles* of the network corresponds to the presented classification code

sensu Yamasaki, *H. penicillatus* sensu Sakai, *Hemigrapsus* sp. from *Crassostrea* reefs in the Central Wadden Sea), with the exception of vague trends, do not show any distinct pigmentation pattern or size and shape of selected external diagnostic characteristics previously identified as being significant (Takano et al. 1997; Asakura and Watanabe 2005; Mingkid et al. 2006; Asakura et al. 2008; Yamasaki et al. 2011). Presence or absence of "dark" spots on the pleon of both sexes has been suggested to be the most reliable criterion for separating *H. penicillatus* (presence) from *H. takanoi* (absence) (Mingkid et al. 2006; Asakura et al. 2008). Even though black dots on the pleon of *H. penicillatus* are prevalent, these are not present on all individuals. The same inconsistency is true for *H. takanoi* because at least one individual has a clear black dot on the pleon, faint dots having been recognized in several other *H. takanoi*. The colouring of dots in both sexes, which was not mentioned by Mingkid et al. (2006) as a significant diagnostic character, tended to be red on the ventral face of *H. takanoi* sensu Yamasaki and black within the other populations, whereas dots on the chelae were red in almost all individuals. The conspicuous presence of red dots in Japanese specimens conflicts with the presence of dark dots in descriptions of other authors (D'Udekem d'Acoz and Faasse 2002; Asakura and Watanabe 2005; Mingkid et al. 2006).

According to Mingkid et al. (2006), all dots on the ventral face of *H. penicillatus* are supposed to be larger than the dots of *H. takanoi*. Although pigmentation is heterogeneous within the different populations, the presence of large dots on more than one characteristic feature of the ventral face is prevalent in *H. penicillatus*. The combination of different diagnostic characters occurring in one individual seems to be helpful when discriminating species on the basis of morphological characteristics, but the decision which characteristic should be rated higher remains unclear. The only *H. penicillatus* (*Hp*Y2) with no dots on the pleon and small dots on other parts of the ventral face had the smallest diameter of soft hair. The combination of these characteristics may promote the decision to assign this individual to *H. penicillatus*, but the relative size of hair patch can only be evaluated in males.

For male specimens, the diameter of soft hair patches on their chelae relative to carapace width has been identified by Mingkid et al. (2006) to be the other significant criterion for reliable species discrimination. With reference to the size of the patch, most of the male crabs would have been identified correctly, but several small German specimens and one out of four *H. penicillatus* sensu Sakai had to be assigned to *H. penicillatus*. It should be noted here that, based on our molecular results, individuals of the Sakai group were identified as *H. takanoi*. The measures of patch size for male museum specimens (*Hp*L) correspond to both *H. penicillatus* and *H. takanoi*. Already Asakura and Watanabe (2005) assumed that the type series is a mixed collection because, on the basis of photographic analysis, they identified at least one male with large patches. Asakura and Watanabe (2005), by contrast, identified the lectotype as being clearly conspecific with *H. penicillatus*, although no supporting data were presented. The measurements of the present study do not support the notion of a "clear" identification because the patch size was slightly above the critical value, indicating that the lectotype might be *H. takanoi*. The patch size increases with carapace width of male *H. takanoi*, while it decreased in *H. penicillatus*. The latter observation is in conflict with the findings of Mingkid et al. (2006), although it has to be kept in mind that they investigated a total of 400 males.

In the present study it was not possible to assign females to one of the two species on the basis of their chelae shapes. In fact, intermediate morphometrics of female chelae were common. A trend from a plump to a more slender shape was observed in Japanese specimens. Females from the German Wadden Sea, by contrast, tend to have very large (high and long) chelae when compared with Japanese females. Using morphological features alone, females with unclear pigmentation, as in the case of specimens *H*spDN7, would most likely have been wrongly identified.

Besides morphological and morphometric characteristics, the species status of *H. takanoi* was investigated using molecular markers, including starch gel electrophoresis (Takano et al. 1997) and partial mitochondrial 16S rDNA sequences in combination with a restriction fragment length polymorphism (RFLP) approach (Yamasaki et al. 2011). While the biochemical interpopulation genetic distance fell into the range of subspecies (Takano et al. 1997), the authors of both investigations suggested a high possibility of reproductive isolation. In contrast to Mingkid et al. (2006) and in accordance with Sakai (2007), we did not observe any differences in the morphology of male first pleopods. The same morphotype of pleopod was also found in the lectotype of the Siebold collection from Leiden. At first sight, this does not argue in favour of a reproductive barrier in terms of mechanical isolation and interpopulation mating pairs may thus successfully copulate. However, pre- and post-zygotic physiological processes and behaviour of the two Asian brush-clawed crabs are still unknown, but different ecological preferences for salinity, tidal height or wave exposure seem likely.

Conclusion

The non-indigenous Asian brush-clawed crab species, which invaded intertidal *Crassostrea* reefs in the Central Wadden Sea (Germany), was identified as *H. takanoi*. The study underlines the difficulty in distinguishing *H. takanoi* from *H. penicillatus* on the basis of morphological characteristics alone. We therefore recommend additional molecular identification of the alien species along the coasts of the Northeast Atlantic because several independent introductions may have resulted in regionally displaced invasions of *H. takanoi* and/or *H. penicillatus* (Fig. 1). Besides natural dispersal, range expansion of the crabs via Pacific oyster transportations between aquaculture facilities is assumed to have facilitated the spread of the species in Europe (Wolff 2005; Dauvin and Delhay 2010). Enhanced alertness in identifying the invader is thus required in regions where intensive aquaculture or mussel transfer is practised. Special attention should also be paid in areas where port operations are expected to increase in the future.

We also recommend molecular identification of populations along Asian coasts because all additional NCBI-listed brush-clawed crabs from Korea, China and Japan were found to be *H. takanoi*. In order to minimize further confusion, a comprehensive re-description of this species has to be undertaken as soon as possible. Most probably, the common and frequently occurring brush-clawed crab known as *H. penicillatus* along Asian coasts has to be renamed. Hypothetically, Takano et al. (1997) could have interpreted the presence of pigmentation on the ventral face merely as a "new" feature of the same species. As a consequence, the common brush-clawed crab species in Asia, and hence the European invader, would have remained *H. penicillatus*.

Acknowledgments Japanese specimens were collected, identified and kindly donated by Katsushi Sakai and Izumi Yamasaki. The Japanese museum material of the Siebold collection was placed at our disposal by courtesy of Karen van Dorp and Charles Fransen (Naturalis Biodiversity Centre, Leiden). Torsten Janßen is acknowledged for his assistance in collecting the German specimens. Special thanks are due to Valeska Borges and Rebekka Schüller for processing specimens in the molecular lab. We are indebted to Burghard Flemming for the English language editing. We are grateful to Jason E. Bond and an anonymous reviewer for constructive comments on earlier versions of the manuscript.

References

Asakura, A., & Watanabe, S. (2005). *Hemigrapsus takanoi*, new species, a sibling species of the common Japanese intertidal crab *H. penicillatus* (Decapoda: Brachyura: Grapsoidea). *Journal of Crustacean Biology, 25*, 279–292.

Asakura, A., Mingkid, W., Yamasaki, I., & Watanabe, S. (2008). Revalidation of *Hemigrapsus takanoi* Asakura & Watanabe, 2005: a rebuttal to "Sakai (2007): comments on an invalid nominal species, *Hemigrapsus takanoi* Asakura & Watanabe, 2005, a synonym of *Hemirapsus penicillatus* (De Haan, 1835) (Decapoda, Brachyura, Grapsidae)". *Crustaceana, 81*, 1263–1273.

Breton, G., Faasse, M. A., Noël, P. Y., & Vincent, T. (2002). A new alien crab in Europe: *Hemigrapsus sanguineus* (Decapoda: Brachyura: Grapsidae). *Journal of Crustacean Biology, 22*, 184–189.

Brockerhoff, A., & McLay, C. (2011). Part II, human mediated spread of Alien crabs. In B. S. Galil, P. F. Clark & J. T. Carlton (Eds.), *In the wrong place – alien marine crustaceans: distribution, biology and impacts. Invading nature - Springer series in invasion ecology, 6* (pp. 27–106). Springer Science + Business Media.

Campbell, S., & Nijland, R. (2004). De Blaasjeskrab, *Hemigrapsus sanguineus*, voor het eerst op het Nederlandse strand. *Het Zeepaard, 64*, 40–44 [in Dutch].

Carrison-Stone, D., Van Syoc, R., Williams, G., & Simison, W. B. (2013). Two new species of the gorgonian inhabiting barnacle, *Conopea* (Crustacea, Cirripedia, Thoracica), from the Gulf of Guinea. *ZooKeys, 270*, 1–20.

Chen, Y.-Y., Lin, H.-C., & Chan, B. K. K. (2012). Description of a new species of coral-inhabiting barnacle, *Darwiniella angularis* sp. n. (Cirripedia, Pyrgomatidae) from Taiwan. *ZooKeys, 214*, 43–74.

Clement, M., Posada, D., & Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology, 9*, 1657–1660.

Cohen, A. N., & Carlton, J. T. (1997). Transoceanic transport mechanisms: introduction of the Chinese mitten crab, *Eriocheir sinensis*, to California. *Pacific Science, 51*, 1–11.

Cohen, A. N., Carlton, J. T., & Fountain, M. C. (1995). Introduction, dispersal and potential impacts of the green crab *Carcinus maenas* in San Francisco Bay, California. *Marine Biology, 122*, 225–237.

D'Udekem d'Acoz, C., & Faasse, M. (2002). De huidige status van *Hemigrapsus sanguineus* (de Haan, 1835) en *H. penicillatus* (de Haan, 1835) in de noordelijke Atlantische Oceaan, in het bijzonder in Nederland, met opmerkingen over hun biologie (Crustacea, Decapoda, Brachyura). *Het Zeepaard, 62*, 101–115 [in Dutch].

Dauvin, J.-C. (2009). Establishment of the Asian shore crab *Hemigrapsus sanguineus* (De Haan, 1835) (Crustacea: Brachyura: Grapsoidea) from the Cotentin Peninsula, Normandy, France. *Aquatic Invasions, 4*, 467–472.

Dauvin, J.-C., & Delhay, J.-B. (2010). First record of *Hemigrapsus takanoi* (Crustacea: Brachyura: Grapsoidea) on the western coast of northern Cotentin, Normandy, western English Channel. *Marine Biodiversity Records, 3*, e101. doi:10.1017/S1755267210000928.

Dauvin, J.-C., Rius, A. T., & Ruellet, T. (2009). Recent expansion of two invasive crabs species *Hemigrapsus sanguineus* (de Haan, 1835) and *H. takanoi* Asakura & Watanabe 2005 along the Opal Coast, France. *Aquatic Invasions, 4*, 451–465.

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., & Heled, J., et al. (2010). Geneious v5.3. Auckland, New Zealand, Biomatters.

Dumoulin, E. (2004). Snelle areaaluitbreiding van het penseelkrabbetje *Hemigrapsus penicillatus* (de Haan, 1835) langs de kusten van de Zuidelijke Bocht de Noordzee, status van haar opmars in de Westerschelde en beschouwingen over de ecologie en het gedrag van de sort. *De Strandvlo, 24*, 5–35 [in Dutch].

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research, 32*, 1792–1797.

Gittenberger, A., Rensing, M., Stegenga, H., & Hoeksema, B. (2010). Native and non-native species of hard substrata in the Dutch Wadden Sea. *Nederlandse Faunistische Mededelingen, 33*, 21–75.

Gollasch, S. (1999). The Asian decapod *Hemigrapsus penicillatus* (de Haan, 1835) (Grapsidae, Decapoda) introduced in European waters: status quo and future perspective. *Helgoländer Meeresuntersuchungen, 52*, 359–366.

Griffen, B. D., Guy, T., & Buck, J. C. (2008). Inhibition between invasions: a newly introduced predator moderates the impacts of a previously established invasive predator. *Journal of Animal Ecology, 77*, 32–40.

Hebert, P. D. N., Ratnasingham, S., & de Waard, J. R. (2003). Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 270*, S96–S99.

Hidalgo, F. J., Barón, P. J., & Orensanz, J. M. (2005). A prediction come true: the green crab invades the Patagonian coast. *Biological Invasions, 7*, 547–552.

Keikhosravi, A., & Schubart, C. D. (2013). Revalidation and redescription of *Potamon elbursi* Pretzmann, 1976 (Brachyura, Potamidae) from Iran, based on morphology and genetics. *Central European Journal of Biology, 8*, 1266–1275.

Kim, D. W., Yoo, W. G., Park, H. C., Yoo, H. S., Kang, D. W., Jin, S. D., et al. (2012). DNA barcoding of fish, insects, and shellfish in Korea. *Genomics and Informatics, 10*, 206–211.

Komai, T., Yang, C.-H., Okuno, J., & Chan, T.-Y. (2011). Revisiting *Pagurus pilosipes* (Stimpson, 1858) (Crustacea: Decapoda: Anomura: Paguridae). *Zootaxa, 3096*, 41–52.

Kou, Q., Hinzheng, L., Chan, T.-Y., Chu, K. H., Huang, H., & Gan, Z. (2013). Phylogenetic relationships among genera of the *Periclimenes* complex (Crustacea: Decapoda: Pontoniinae) based on mitochondrial and nuclear DNA. *Molecular Phylogenetics and Evolution, 68*, 14–22.

Kraemer, G. P., Sellberg, M., Gordon, A., & Main, J. (2007). Eight-year record of *Hemigrapsus sanguineus* (Asian shore crab) invasion in Western Long Island Sound Estuary. *Northern Naturalist, 14*, 207–224.

Lohrer, A. M., & Whitlatch, R. B. (2002). Interactions among aliens: apparent replacement of one exotic species by another. *Ecology, 83*, 719–732.

Manaffar, R., Zare, S., Agh, N., Abdolahzadeh, N., Soltanian, S., Sorgeloos, P., et al. (2011). SNP detection in Na/K ATP-ase gene α_1 subunit of the bisexual and parthenogenetic *Artemia* strains by RFLP screening. *Molecular Ecology Resources, 11*, 211–214.

McDermott, J. J. (1991). A breeding population of the Western Pacific Crab *Hemigrapsus sanguineus* (Crustacea: Decapoda: Grapsidae) established on the Atlantic Coast of North America. *Biological Bulletin, 181*, 195–198.

McDermott, J. J. (1998). The western Pacific brachyuran (*Hemigrapsus sanguineus*: Grapsidae), in its new habitat along the Atlantic coast of the United States: geographic distribution and ecology. *ICES Journal of Marine Science, 55*, 289–298.

Mingkid, W. M., Akiwa, S., & Watanabe, S. (2006). Morphological characteristics, pigmentation, and distribution of the sibling penicillate crabs, *Hemigrapsus penicillatus* (De Haan, 1835) and *H. takanoi* Asakura & Watanabe, 2005 (Decapoda, Brachyura, Grapsidae) in Tokyo Bay. *Crustaceana, 79*, 1107–1121.

Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T. L., Agarwala, R., & Schäffer, A. A. (2008). Database indexing for production MegaBLAST searches. *Bioinformatics, 24*, 1757–1764.

Nagy, Z. T., Sonet, G., Glaw, F., & Vences, M. (2012). First large-scale DNA barcoding assessment of reptiles in the biodiversity hotspot of Madagascar, based on newly designed COI primers. *Public Library of Science ONE, 7*, e34506.

Nijland, R. (2006). De Krabben van Nederland. Penseelkrab *Hemigrapsus penicillatus*. http://www.krabben.net/penseelkrab.html. Accessed 14 Feb 2012. [in Dutch].

Nijland, R., & Beekman, J. (2000). *Hemigrapsus penicillatus* De Haan 1835 waargenomen in Nederland. *Het Zeepaard, 60*, 169–171 [in Dutch].

Noël, P. Y., & Gruet, Y. (2008). Progression du crabe introduit *Hemigrapsus takanoi* Asakura & Watanabe 2005 (Crustacé, Décapode) vers le nord du Golfe de Gascogne. *Bulletin de la Société des Sciences Naturelles de l'Ouest de la France, 30*, 141–148 [in French].

Noël, P. Y., Tardy, E., & D'Udekem D'Acoz, C. (1997). Will the crab *Hemigrapsus penicillatus* invade the coasts of Europe? *Comptes Rendus de l'Académie des Sciences, Sciences de la vie/Life Science, 320*, 741–745.

Obert, B., Herlyn, M., & Grotjahn, M. (2007). First records of two crabs from the North West Pacific *Hemigrapsus sanguineus* and *H. takanoi* at the coast of Lower Saxony, Germany. *Wadden Sea Newsletters, 33*, 21–22.

Palumbi, S. R., Martin, A., Romano, S., McMillan, W. O., Stice, L., & Grabowski, G. (1991). *The simple fool's guide to PCR, version 2*. Honolulu: University of Hawaii Press.

Pfrender, M. E., Hawkins, C. P., Bagley, M., Courtney, G. W., Creutzburg, B. R., Epler, J. H., et al. (2010). Assessing marcoinvertebrate biodiversity in freshwater ecosystems: advances and challenges in DNA-based approaches. *The Quarterly Review of Biology, 85*, 319–340.

Rajaei, S. H., Struwe, J.-F., Raupach, M. J., Ahrens, D., & Wägele, J. W. (2013). Integration of cytochrome *c* oxidase I barcodes and geometric morphometrics to delimit species in the genus *Gnopharmia* (Lepidoptera: Geometridae, Ennominae). *Zoological Journal of the Linnean Society, 169*, 70–83.

Raupach, M. J., Malyutina, M., Brandt, A., & Wägele, J. W. (2007). Molecular data reveal a highly divers species flock within the munnopsoid deep-sea isopod *Betamorpha fusiformis* (Barnard, 1920) (Crustacea: Isopoda: Asellota) in the Southern Ocean. *Deep-Sea Research II, 54*, 1820–1830.

Raupach, M. J., Thatje, S., Dambach, J., Rehm, P., Misof, B., & Leese, F. (2010). Genetic homogeneity and circum-Antarctic distribution of two benthic shrimp species of the Southern Ocean, *Chorismus antarcticus* and *Nematocarcinus lanceopes*. *Marine Biology, 157*, 1783–1797.

Riehl, T., & Kaiser, S. (2012). Conquered from the deep sea? A new deep-sea isopod species from the Antarctic shelf shows pattern of recent colonization. *Public Library of Science ONE, 7*, e49354.

Ruiz, G. M., Fegley, L., Fofonoff, P., Cheng, Y., & Lemaitre, R. (2006). First records of *Eriocheir sinensis* H. Milne Edwards, 1835 (Crustacea: Brachyura: Varunidae) for Chesapeake Bay and the mid-Atlantic coast of North America. *Aquatic Invasions, 1*, 137–142.

Sakai, K. (2007). Comments on an invalid nominal species, *Hemigrapsus takanoi* Asakura & Watanabe, 2005, a synonym of *Hemigrapsus penicillatus* (De Haan, 1835) (Decapoda, Brachyura, Grapsidae). *Crustaceana, 80*, 503–508.

Schubart, C., Cuesta, J. A., & Rodríguez, A. (2001). Molecular phylogeny of the crab genus *Brachynotus* (Brachyura: Varunidae) based on the 16S rRNA gene. *Hydrobiologia, 449*, 41–46.

Simon, C., Buckley, T. R., Frati, F., Stewart, J. B., & Beckenbach, A. (2006). Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. *Annual Reviews in Ecology and Evolutionary Systematics, 37*, 545–579.

Soors, J., Faasse, M., Stevens, M., Verbessem, I., de Regge, N., & van den Bergh, E. (2010). New crustacean invaders in the Schelde estuary (Belgium). *Belgian Journal of Zoology, 140*, 3–10.

Swofford, D. L. (2002). *PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10*. Sunderland: Sinauer Associates.

Takano, M., Ikeda, M., & Kijima, A. (1997). Biochemical and morphological evidence of two sympatric forms, interpreted as sibling species, in the estuarine grapsid crab *Hemigrapsus penicillatus* (De Haan). *Benthos Research, 52*, 111–117.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*, 2731–2739.

Veilleux, É., & de Lafontaine, Y. (2007). Biological synopsis of the Chinese mitten crab (*Eriocheir sinensis*). *Canadian Manuscript Report of Fisheries and Aquatic Science, 2812*, 51pp.

Vincent, T., & Breton, G. (1999). Présence du crabe *Hemigrapsus penicillatus* (de Haan, 1835) dans les basins du port du Harvre

(Normandie, France). *Bulletin trimestrielle de la Société géologique de Normandie et des Amis du Muséum du Havre, 86*, 19–23 [in French].

Wesener, T., Raupach, M. J., & Sierwald, P. (2010). The origins of the giant pill-millipedes from Madagascar (Diplopoda: Sphaerotheriida: Arthrosphaeridae). *Molecular Phylogenetics and Evolution, 57*, 1184–1193.

Wolff, W. J. (2005). Non-indigenous marine and estuarine species in The Netherlands. *Zoologische Mededelingen Leiden, 79*, 1–116.

Yamasaki, I., Doi, W., Mingkid, W. M., Yokota, M., Strüssmann, C. A., & Watanabe, S. (2011). Molecular-based Method to distinguish the sibling species *Hemigrapsus penicillatus* and *Hemigrapsus takanoi* (Decapoda: Brachyura: Vanuridae). *Journal of Crustacean Biology, 31*, 577–581.

Yoshida, R., Osawa, M., Hirose, M., & Hirose, E. (2011). A new genus and two new species of Peltogastridae (Crustacea: Cirripedia: Rhizocephala) parasitizing hermit crabs from Okinawa Island (Ryukyu Archipelago, Japan), and their DNA-barcodes. *Zoological Science, 28*, 853–862.

Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology, 7*, 203–214.