

Liocarcinus corrugatus (Pennant, 1777) (Crustacea: Brachyura: Portunidae): a cosmopolitan brachyuran species?

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Abstract. The swimming crab genus *Liocarcinus* is restricted to European marine ecosystems, except *L. corrugatus* (Pennant, 1777), which also occurs in the Indo-West Pacific. To ascertain if the two populations represent a cosmopolitan species or a species complex, we used a series of morphological, morphometric and molecular phylogenetic analyses on European and Asian material of *L. corrugatus*. We further investigated the phylogenetic context of the genus *Liocarcinus* with related portunid taxa to establish its monophyly. *Liocarcinus* is paraphyletic with respect to *Polybius henslowii*. We propose that *P. henslowii* is a valid species, most likely the result of a very recent speciation within *L. holsatus*. The first gonopods of Asian and European *L. corrugatus* are similar in shape, but the carapaces of the Asian specimens are significantly longer. While *L. corrugatus* is monophyletic, the European and Asian populations separate into two distinct clades with high support. Based on the consistent morphological difference and the deep genetic differentiation (p-distance of 4.5% for COX1), the European and Asian populations of *L. corrugatus* should be treated as two distinct species. An old name is available and the Asian population should now be recognised as *Liocarcinus strigilis* Stimpson, 1858.

Key words. *Liocarcinus corrugatus*, *Polybius*, gonopods, morphometry, geometric morphometrics, molecular phylogeny

INTRODUCTION

The geographical distribution of a species can extend from being a range-restricted endemic to wide-ranging and cosmopolitan. In marine environments, species are considered cosmopolitan when they occur in their natural habitats in more than one ocean basin. Thus, a cosmopolitan distribution of a given species depends primarily on the connectivity of ocean basins and reflects its dispersal ability. Wide-ranging dispersal in invertebrates is mostly connected to the presence of a planktonic larval phase (as in most marine brachyuran crabs). In fact, there are only a few absolute physical barriers that would restrict gene flow between oceans (Palumbi, 1994). Natural dispersal between ocean basins in the abyssal deep sea (depth: 1000 m and more) is favoured by its uniform environmental condition (in contrast, e.g., to the ranges of

littoral species that can be strongly determined by latitudinal environmental gradients).

Next to natural dispersal pathways, anthropogenic factors have increasingly contributed to the dispersal of species, which would otherwise not be able to exhibit a cosmopolitan distribution as realised today (e.g., active introduction of non-native species, construction of canals, or the “stowaways” in the ballast water of vessels). Examples of invasive brachyuran crabs that gained a global distribution are *Eriocheir sinensis* H. Milne-Edwards, 1853 (Varunidae) (Herborg et al., 2003) and *Carcinus maenas* (Linnaeus, 1758) (Portunidae) (Bals, 1955; Carlton & Cohen, 2003).

For a species to be cosmopolitan, its populations need to be connected with each other to allow gene flow and prevent incipient speciation, e.g., mediated by larval dispersal (Palumbi, 1994; Spivak & Schubart, 2003). Restriction of gene flow between conspecific populations due to, for example, distance or geo-physical barriers, can cause their genetic divergence and may potentially lead to speciation (Burton et al., 1999). Distinguishing between wide-ranging species that are at first glance cosmopolitan due to similar morphological characters, and those that are “true” cosmopolitan species with ongoing gene flow between their populations, is difficult if based on morphology alone. Often, morphological characters do not differ sufficiently to distinguish cryptic and separately evolving lineages (Jesse et al., 2010), and genetically distinct species have frequently been described as a single cosmopolitan morphospecies (Klautau et al., 1999).

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Twelve recent species of the genus *Liocarcinus* are currently accepted: *L. holsatus* (Fabricius, 1798) (type species), *L. bolivari* (Zariquiey Alvarez, 1948), *L. corrugatus* (Pennant, 1777), *L. depurator* (Linnaeus, 1758), *L. maculatus* (Risso, 1827), *L. marmoreus* (Leach, 1814), *L. navigator* (Herbst, 1794), *L. pusillus* (Leach, 1816), *L. rondeletii* (Risso, 1816), *L. subcorrugatus* (A. Milne-Edwards, 1861), *L. vernalis* (Risso, 1816), and *L. zariquieyi* Gordon, 1968 (Ng et al., 2008; Spiridonov et al., 2014). Whereas nearly all species of *Liocarcinus* are exclusively distributed in at least one of the European waters (North Sea, East Atlantic or Mediterranean Sea), only *L. corrugatus* is also distributed in the Indo-West Pacific, occurring from New Zealand and Australia to Japan and the Red Sea (Stephenson & Campbell, 1960; El-Zayed, 2003; Yaldwyn & Webber, 2011). The records of *L. corrugatus* in the Red Sea mentioned by Stephenson & Campbell (1960) and Yaldwyn & Webber (2011) were based on specimens of *L. subcorrugatus* as they synonymised both species because of their morphologic similarities.

If *L. corrugatus* is indeed a cosmopolitan species, it would represent an interesting case study to assess the variability of morphology and genetics across its range, and to investigate possible reasons for such a wide-ranging distribution, including assessment of the strength of gene flow necessary to ensure genetic coherence. In this study, we perform comparative morphological and genetic analyses to determine whether European and Asian *L. corrugatus* can be considered as one species, applying a combination of morphological, morphometric (both traditional and geometric methods), and molecular analyses on European and Asian *L. corrugatus*. Moreover, we investigated the phylogenetic context of the genus *Liocarcinus* with additional closely related portunid taxa, the phylogenetic placement of *L. corrugatus* within its genus and estimated the divergence time between specimens of *L. corrugatus* from the European and Asian ocean basins.

Taxonomic history of *Liocarcinus corrugatus* (Pennant, 1777). *Liocarcinus corrugatus* (Pennant, 1777) currently has four subjective synonyms: *Cancer pellitus* Forskål, 1775, *Portunus leachii* Risso, 1827, *Portunus strigilis* Stimpson, 1858, and *Portunus borradailei* Bennett, 1930. The species was first validly described as *Cancer corrugatus* from material from Loch Jurn in Scotland. Risso (1827: 3) subsequently established *Portunus leachi* from southern Europe, but his description leaves no doubt that his species is conspecific with *C. corrugatus*. The name *Cancer pellitus* Forskål, 1775, is more problematic as it is the oldest name and had been ignored in all treatments of European Brachyura until Ng et al. (2008: 149) highlighted the problem. Forskål (1775: 92), in his book on Red Sea animals, recognised a species he called *Cancer pellitus* obtained from Marseille (southern France) and Constantinople (present day Istanbul, Turkey). He also referred to the figure of a crab in Rumphius (1705: pl. 6 fig. O). His Mediterranean specimens as well as Rumphius' specimen from an unspecified location are therefore types of *Cancer pellitus*. None are extant. Forskål (1775: 92) describes the species as follows:

“CANCER PELLITUS; *brachyurus*; *thorace hirto, inæquali,*

utrinque quinque dentato: fronte obtuse dentata: plantis posticis membranaceis, ovatis, ciliatis.

Rumph. mus Tab. 6 fig. O *Gall. Crable velouté. Massiliae.*

DESCR. Totus obscure-testaceus. *Frons* in medio obtuse tridentata, & insuper dente minori juxta oculos. *Thorax* oblongus, depressus, rugis transverso-obliquis tuberibusque inequalis; post oculos utrinque serratus dentibus 5. contiguus. *Chelæ* extus quinquangulatae: digiti introrsum dentati tuberculis obtusis. *Carpus* forti spina armatur. *Pedes* quinti paris toti compressi, maxime articuli duo ultimi, pilis dense ciliati: planta ovato-lanceolata, sulco utrinque medi elato, glabro, longitudinali.

b) *Constantinopoli Varietatem* vidi hujus Cancri, minorem multo, glabram, fronte aliis prorsus obtusa & truncata, aliis acute-dentata: chelis sæpe quinquangulatis, interdum vix angulatis, nisi ab utroque margine.”

This description, and Forskål's referral to the species as setose (i.e., “*Crable velouté*”) and from Marseille (i.e., “*Massiliae*”), means that the species referred to is most likely *Liocarcinus corrugatus*. Strangely, this does not match with Rumphius' figure, which is of a much smoother animal and probably, therefore, another species of *Liocarcinus*. *Cancer pellitus* Forskål, 1775, is clearly an older name than the better-known *Cancer corrugatus* Pennant, 1777. Fortunately, Ng et al. (2008: 156) solved this problem when they commented “*Cancer pellitus* Forskål, 1775, is a name not used since its original description. One of the authors (PKLN) has examined the original description with L. B. Holthuis, and we are sure that it is conspecific with *Liocarcinus corrugatus* (Pennant, 1777) (unpublished data). We here invoke Article 23.9.2 of the Code to conserve the better known name. This action makes *Cancer corrugatus* Pennant, 1777 a nomen protectum and *Cancer pellitus* Forskål, 1775, a nomen oblitum.

Stimpson (1858: 38) described *Portunus strigilis* from an unspecified number of juvenile specimens from Kagoshima, Japan (see also Stimpson, 1907: 74–75, pl. 9 fig. 6). Shortly after, Miers (1879: 33–34) obtained specimens from Goto Island, Japan, and synonymized it with *Portunus corrugatus* (Pennant, 1777). Rathbun (1902: 25) first transferred it to *Liocarcinus* and commented that the carapace of *L. strigilis* was proportionately longer and narrower, the anterolateral margin is relatively longer than the posterolateral margin, the median frontal tooth is more triangular with the sides at right angles to each other with the tip acute. Balss (1922: 101) was the first to treat it as a subspecies, *Liocarcinus corrugatus strigilis*, noting that the morphological differences mentioned by Rathbun are not distinct enough to justify two separate species and he therefore considered the Japanese form as a geographic variant of the European *Liocarcinus corrugatus* (see also Yokoya, 1933: 173).

Portunus borradailei (Bennett, 1930), was described from a supposed small dried male from New Zealand, but later, Bennett (1964: 65) showed that it was actually a female specimen. Citing a paper by Palmer (1927) re-describing



Fig. 1. Sampling sites of *Liocarcinus corrugatus* specimens. Asia: black dots, Europe: grey dots, Historical records (Stephenson & Campbell, 1960; El-Zayed, 2003): open circles. The numbers correspond to the first column in Table 1.

European material of *L. corrugatus*, Bennett (1964: 14, 65) considered the slight differences insignificant and synonymized the two species. Stephenson & Campbell (1960: 92–93) summarised the state of knowledge for the species (as *Macropipus corrugatus*) and decided that none of the differences appeared to be significant, and as they had limited material from Australia, felt that it was best only one species was recognised. This has been followed by all workers since; although there remains some debate on its generic assignment.

MATERIAL & METHODS

Examined specimens. In total, we used 53 specimens of *L. corrugatus* for our analyses (Table 1). Eighteen specimens were used for the morphological analyses, 31 specimens for the morphometric and geometric morphometric analyses, and four specimens for the molecular analyses. The specimens are derived from samplings during 1965–2011 and comprise samples from the Mediterranean Sea and the Atlantic (hereafter referred to as European) and Japan, Taiwan and the Philippines (hereafter referred to as Asian; Fig. 1). All but a few specimens used for the morphological analyses are stored either in the collection of the Senckenberg Research Institute and Natural History Museum Frankfurt, Germany (www.sesam.senckenberg.de); or in the collection of the Lee Kong Chian Natural History Museum (formerly Raffles Museum of Biodiversity Research), Singapore. DNA samples of the specimens used for the molecular analyses are deposited in the DNA Bank, Grunelius-Moellgaard-Labor of

Senckenberg Research Institute and Natural History Museum Frankfurt, Germany.

Morphology. For the morphological analyses, we studied the male first gonopods (G1) of *L. corrugatus* from Asia and Europe with a Nikon SMZ-800 optical microscope. The G1 morphology is a standard tool for taxonomic description of brachyurans as they can differ significantly between species and also show relationships better than external adaptive characters (Türkay, 1975). Here, we compared the overall shape of the G1 and in particular, its tip.

Morphometry. In total, eight linear measurements were taken on each individual with sliding calipers to the nearest 0.01 mm. The following distances were measured (see Fig. 2A, B): Carapace width (CW), carapace length (CL), carapace depth (CD), left chela width (ChLW), right chela width (ChRW), left chela length (ChLL), right chela length (ChRL), right 4th pereiopd (Pe). Absent, regenerating or damaged body parts reduced in some cases the total number of variables for individual specimens, and these were treated statistically as missing data. Ratios of CW and CL were analysed separately and together with ratios of the other measurements (CW/CD, CW/Pe, ChLW/ChLL, ChRW/ChRL, CW/ChLL, CL/ChLL, CW/ChRL, CL/ChRL, ChLL/Pe, ChRL/Pe).

Differences in morphometric characters were tested statistically by first standardising all ratios, setting the maximum value of each ratio to one and the remaining

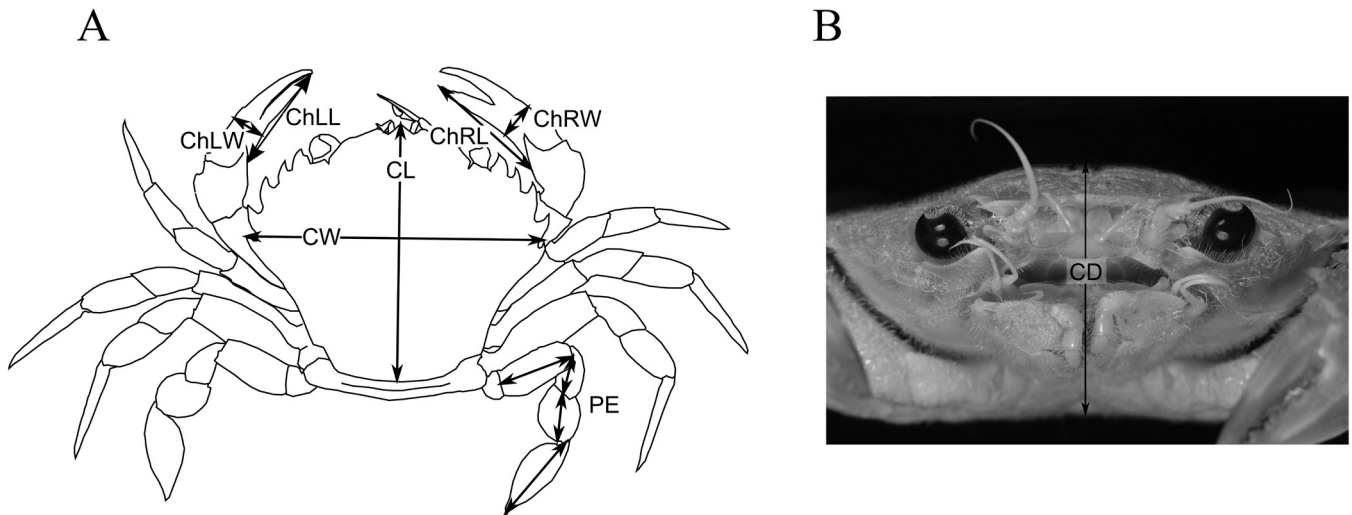


Fig. 2. *Liocarcinus corrugatus*, Locations of the linear distances measured: A, on the dorsal side of the carapace and the chelae; B, from the frontal side. CW = carapace width, CL = carapace length, ChLW = left chela width, ChRW = right chela width, ChLL = left chela length, ChRL = right chela length, Pe = right 4th pereiopod, CD = carapace depth.

values proportional to this value, followed by an arcsine square root transformation. The mean CW/CL ratios were compared using a *t*-test with unequal variances. All distance measurements were log transformed. Linear regressions of the carapace width against the carapace length using the equation $y = b + a \cdot x$ were performed and the regression coefficients were tested using Analysis of Covariance (ANCOVA). Possible differences in ratios between specimens were then tested with a permutation test because of non-normal distribution of the data, while the linear measurements were tested using a two-way Analysis of Variance (ANOVA) and a subsequent Tukey HSD post hoc test with a Bonferroni correction for the *p*-values. A Principal Component Analysis (PCA) was then performed separately on the ratios and measurements to assess which ratios and measurements mainly contribute to the potential separation of Asian and European specimens. As a PCA is sensitive to missing data, missing values (ratios: $n = 74$ out of 341; linear measurements: $n = 48$ out of 248) were predicted using the functions `estim_ncpPCA` and `imputePCA` of the R package `missMDA` (Husson & Josse, 2013). A Multivariate Analysis of Variance (MANOVA) and a subsequent Wilks Test were performed on the ratios and measurements to assess if the variables are different enough to assign specimens to either the Asian or the European group. The statistical analyses were conducted in the R 3.03 environment (R Core Team, 2014) using the packages `reshape2` 1.2.2 (Wickham, 2007), `car` 2.0-19 (Fox & Weisberg, 2011), `missMDA` 1.7.2 (Husson & Josse, 2013), `FactoMineR` 1.25 (Husson et al., 2013), `MASS` 7.3-30 (Venables & Ripley, 2002), `lmPerm` 1.1-2 (Wheeler, 2010), `survival` 2.37-7 (Therneau, 2014) and `ggplot2` 0.9.3.1 (Wickham, 2009).

Geometric Morphometry. To assess shape variation in the carapaces between European and Asian *L. corrugatus*, we applied landmarks based on geometric morphometrics. Images of each specimen were taken using a Canon Power Shot A530 digital camera (Canon Inc.) with a resolution of 6.0 megapixels. No zoom was used and the distance was

kept constant to ensure similar orientation and scaling for all images. A scale was included at the same level as the carapace. A total of 14 landmarks and 13 semi-landmarks (see Fig. 3A for the configuration) were digitised using the program `tpsDig` 2.17 (Rohlf, 2013). Only the left side of the carapace was used to avoid duplication of equivalent landmarks and computation problems (symmetry) (Rufino et al., 2004). To convert semi-landmarks into landmarks we adopted the methods suggested by Zelditch et al. (2012) using the program `tpsUtil` 1.58 (Rohlf, 2013). The landmark configurations were then applied to a generalized Procrustes superimposition (Rohlf & Slice, 1990) using the program `tpsRelw` 1.53 (Rohlf, 2013), in order to remove the variation of size, orientation and position of the landmark configurations of each specimen.

Statistical analyses regarding the geometric morphometry were performed with the R-packages `geomorph` 1.1-5 (Adams & Otárola-Castillo, 2013), `shapes` 1.1-9 (Dryden, 2013) and `Morpho` 2.0 (Schlager, 2014). A principal component analysis was performed revealing that the first 13 principal components explain 95% of the variance, so only these were used for further analyses. Because of the unequal sampling sizes a permutation test with 10,000 permutation rounds was conducted on the principal component scores to compare the distance between the two groups. To test for differences in sex, European and Asian specimens were tested separately using the same procedure mentioned above.

Molecular analyses. DNA was isolated from muscle tissue of walking legs using a modified protocol of the CTAB (cetyltrimethyl ammonium bromide) method (Murray & Thompson, 1980). Four marker genes with a total of 2190 base pairs (bp; alignment length excluding primers) were amplified, which included a part of the mitochondrial large ribosomal subunit 16S rRNA, tRNA^{Leu}, part of the ND1 and the cytochrome b oxidase subunit 1 (COX1) and the nuclear encoded histone 3 (H3) gene. The following conditions for polymerase chain reaction (PCR) were applied: 35 cycles,

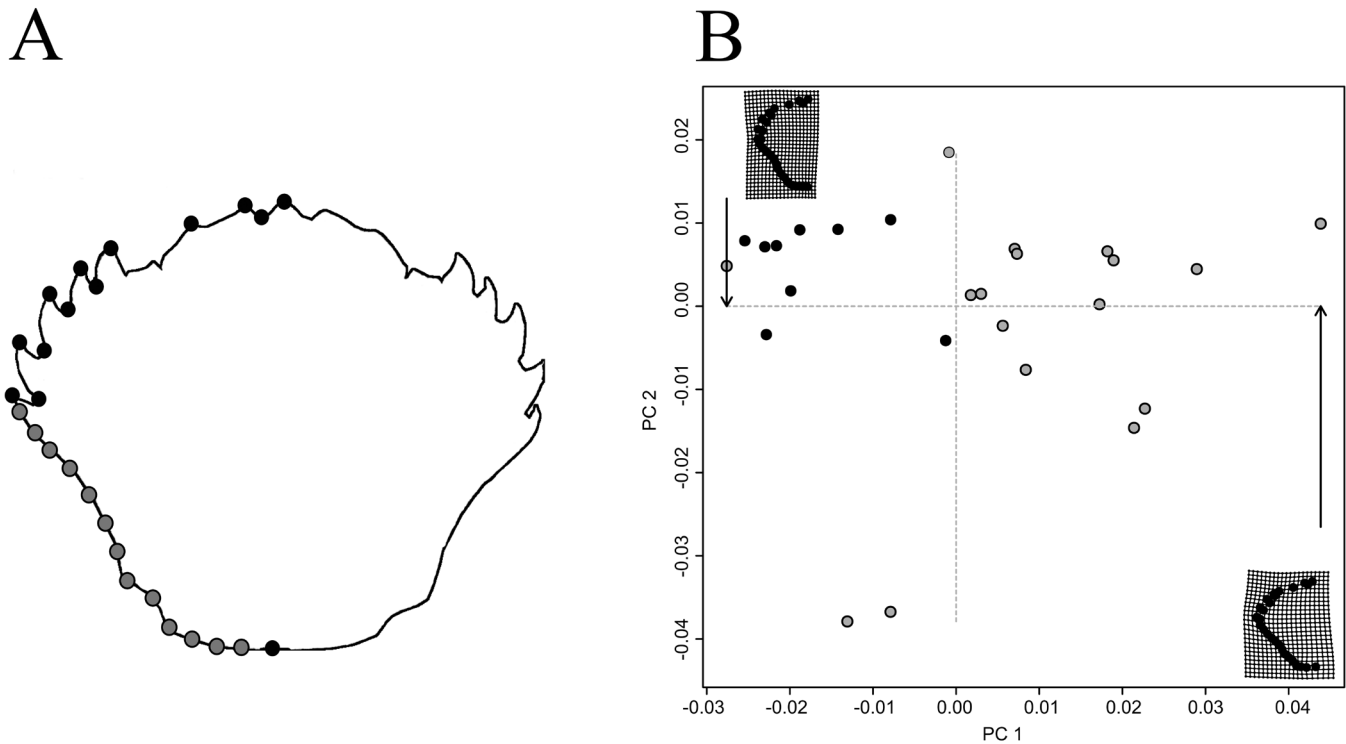


Fig. 3. A, Positions of the landmarks (black dots) and semi-landmarks (grey dots) on the carapace. Only one side of the carapace was used to avoid duplication of equivalent landmarks. B, Principal Component Analysis (PC1 and PC2) of carapace landmark configurations of the Asian (black) and European (grey) specimens. PC 1 explains 36 % of total shape variation, PC2 17 %. In the bottom right and upper left corner deformation grids of carapace shapes shows extremes of shape variation along PC1.

initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 50–55°C for 40 sec, extension at 72°C for 40 sec and a final extension at 72°C for 10 min. For the 16S rRNA towards ND1 partitions we used the Primers 16L29 (as 16L2, Schubart et al., 2001) with an annealing temperature of 50°C, 16L6 and 16L11 (Schubart, 2009) with an annealing temperature of 51.4°C and the two new designed primers NDL1hol (5'- GGAGTCGCCTTTGTTACGTT - 3') and NDL1dep (5'- AGGGGTTGCTTTTGTACTTT - 3'), both with an annealing temperature of 55°C. As reverse primers we used 16H37 (Klaus et al., 2006) with an annealing temperature of 50°C and NDH1 and NDH5 (Schubart, 2009), both with an annealing temperature of 51.4°C. The forward and reverse primer for the COX1 gene were COL6b and COH6 (Schubart & Huber, 2006) with an annealing temperature of 55°C and for the H3 gene H3af and H3rf (Colgan et al., 1998) with an annealing temperature of 55.2°C. PCR products were purified with the Sure Clean Kit (Bioline). Forward and reverse strands were sequenced on an ABI 3730 capillary sequencer (Applied Biosystems). In the phylogenetic analyses supplementary sequences obtained from GenBank were included (see Table 1 for accession numbers).

Sequences were aligned manually with the software BioEdit 7.2.5 (Hall, 1999). The single alignments had the following length: 648 bp for 16S rRNA gene, 144 bp for tRNA^{Leu}, 397 bp for ND1, 667 bp for COX1, and 334 bp for H3. All new sequences have been submitted to GenBank (Accession Numbers in Table 1). *Scylla serrata* (Forskål,

1775) (Portunidae) served as outgroup (FM208779.1 for 16S rRNA, FJ827758.1 for COX1 and FM208793.1 for H3). Bayesian phylogenetic analyses were done in BEAST MC3 v1.7.5 (Drummond et al., 2012). For all partitions a HKY+Gamma model of sequence evolution was used as suggested by jmodeltest 2.1.4 (Darriba et al., 2012). For the 16S rRNA, COX1 and H3 genes an uncorrelated lognormal relaxed clock and a Yule tree prior was used. We assumed a mean rate of substitutions of 0.65% per million years (Myr) for 16S rRNA gene and 0.83 % per Myr for COX1 (normal prior distribution for the uncorrelated lognormal relaxed clock with 10.0% standard deviation; upper cutoff 20.0%; 95% quantile for 16S rRNA 0.54–0.78% per Myr and for COX1 1.39–1.93% per Myr), as based on the divergence of sesarimid crabs at the Isthmus of Panama (Schubart et al., 1998). For the remaining mitochondrial partitions, a very broad normally distributed prior was applied for the substitution rate (mean 2.0% per Myr, SD = 2.0; 5–95% quantile 0.00–3.31% per Myr), that covers the presumed evolutionary rates for mitochondrial genes (Papadopoulou et al., 2010) and is in line with fossil and biogeographically calibrated brachyuran mitochondrial rates (Schubart et al., 1998; Klaus et al., 2010). For the nuclear H3 gene we applied a rate of 0.19% per Myr (SD = 0.04; 5–95% quantile 0.12–0.26% per Myr) estimated based on a fossil calibrated phylogeny of Old World freshwater crabs (Klaus et al., 2010). We performed three independent MCMC (Markov Chain Monte Carlo) chains for 50 million generations, sampling a tree every 10,000 generations (delta = 1.0). The first 500 trees were excluded from the analysis (“burn-in phase”). Convergence of the

Table 1. Specimens used for the morphological (x), morphometric (*) and molecular (#) studies. If available, their museum catalogue number, an additional number for individualization, their locality, and the genetic database (GenBank) accession numbers for the sequences are given. The numbers in the first column corresponds to the numbers in Fig. 1. HTR = Helgoland Trench SMF and ZMG Senckenberg Museum, Frankfurt am Main; ZRC Zoological Reference Collection, Raffles Museum at the National University of Singapore.

Site (see Fig. 1)	Species	Sex	Locality	Catalogue No. (Additional Individualization No.)	GenBank Accession No.		
					16S rRNA- tRNA ^{Leu} -NADHI	COXI	H3
1	<i>Liocarcinus corrugatus</i> (*)	male	Europe, Italy, Naples	SMF 3680	—	—	—
2	<i>Liocarcinus corrugatus</i> (*)	male	Europe, Croatia, Dubrovnik	SMF 4522	—	—	—
3	<i>Liocarcinus corrugatus</i> (x,*)	male	Europe, Greece, Sporades	SMF 6778 (1120)	—	—	—
3	<i>Liocarcinus corrugatus</i> (x,*)	male	Europe, Greece, Sporades	SMF 6778 (1121)	—	—	—
3	<i>Liocarcinus corrugatus</i> (*)	male	Europe, Greece, Sporades	SMF 6778 (1122)	—	—	—
3	<i>Liocarcinus corrugatus</i> (*)	female	Europe, Greece, Sporades	SMF 6778 (1123)	—	—	—
3	<i>Liocarcinus corrugatus</i> (*)	female	Europe, Greece, Sporades	SMF 6778 (1124)	—	—	—
4	<i>Liocarcinus corrugatus</i> (*)	male	Europe, Greece, Kalymnos Island	SMF 7399	—	—	—
5	<i>Liocarcinus corrugatus</i> (*)	male	Europe, Portugal, Madeira	SMF 7513 (179)	—	—	—
5	<i>Liocarcinus corrugatus</i> (*)	female	Europe, Portugal, Madeira	SMF 7513	—	—	—
7	<i>Liocarcinus corrugatus</i> (*)	female	Europe, Italy, Elba	SMF 12762	—	—	—
8	<i>Liocarcinus corrugatus</i> (*)	female	Europe, Portugal, Azores	SMF 20454	—	—	—
9	<i>Liocarcinus corrugatus</i> (*)	female	Atlantic, off Western Sahara, M36-118	SMF 28471 (699)	—	—	—
9	<i>Liocarcinus corrugatus</i> (x,*)	male	Atlantic, off Western Sahara, M36-118	SMF 28471 (698)	—	—	—
9	<i>Liocarcinus corrugatus</i> (x,*)	male	Atlantic, off Western Sahara, M36-118	SMF 28471 (700)	—	—	—
9	<i>Liocarcinus corrugatus</i> (x,*)	male	Atlantic, off Western Sahara, M36-118	SMF 28471 (701)	—	—	—
10	<i>Liocarcinus corrugatus</i> (*)	female	Atlantic, off Western Sahara, M36-118	SMF 28472	—	—	—
11	<i>Liocarcinus corrugatus</i> (*)	female	Europe, Greece, Crete	SMF 28473 (1116)	—	—	—
11	<i>Liocarcinus corrugatus</i> (x,*)	male	Europe, Greece, Crete	SMF 28473 (1117)	—	—	—

Table 1. Continued.

Site (see Fig. 1)	Species	Sex	Locality	Catalogue No. (Additional Individualization No.)	GenBank Accession No.		
					16S rRNA- tRNA ^{Leu} -NADH1	COXI	H3
13	<i>Liocarcinus corrugatus</i> (*,#)	male	Europe, Spain, Ibiza	SMF 32760	KU560470	KP795935	KP965203
—	<i>Liocarcinus corrugatus</i> (*)	male	Europe, Mediterranean Sea	ZMG 1086 (1118)	—	—	—
—	<i>Liocarcinus corrugatus</i> (*)	female	Europe, Mediterranean Sea	ZMG 1086 (1119)	—	—	—
6	<i>Liocarcinus corrugatus</i> (*)	female	Asia, Japan	SMF 7645	—	—	—
12	<i>Liocarcinus corrugatus</i> (*)	female	Asia, Japan	SMF 30812 (721)	—	—	—
12	<i>Liocarcinus corrugatus</i> (*)	male	Asia, Japan	SMF 30812 (722)	—	—	—
12	<i>Liocarcinus corrugatus</i> (*)	male	Asia, Japan	SMF 30812 (723)	—	—	—
12	<i>Liocarcinus corrugatus</i> (*)	male	Asia, Japan	SMF 30812 (724)	—	—	—
14	<i>Liocarcinus corrugatus</i> (x,*,#)	male	Asia, Philippines, Balicasag Island	ZRC 2013.1396	KU560471	—	KP965204
15	<i>Liocarcinus corrugatus</i> (*,#)	female	Asia, Taiwan, Yilan county	ZRC 2013.1397	KU560472	KP795936	KP965205
16	<i>Liocarcinus corrugatus</i> (x,*)	male	Asia, Philippines, Panglao	ZRC 2013.1395	—	—	—
17	<i>Liocarcinus corrugatus</i> (*,#)	female	Asia, Taiwan, Yilan county	ZRC 2013.1398	KU560473	KP795937	KP965206
18	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Japan, Ozuchi	—	—	—	—
18	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Japan, Ozuchi	—	—	—	—
18	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Japan, Ozuchi	—	—	—	—
18	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Japan, Ozuchi	—	—	—	—
16	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Philippines, Panglao	—	—	—	—
16	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Philippines, Panglao	—	—	—	—
14	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Philippines, Balicasag Island	—	—	—	—
14	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Philippines, Balicasag Island	—	—	—	—
14	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Philippines, Balicasag Island	—	—	—	—

Table 1. Continued.

Site (see Fig. 1)	Species	Sex	Locality	Catalogue No. (Additional Individualization No.)	GenBank Accession No.		
					16S rRNA- tRNA ^{Leu} -NADHI	COXI	H3
14	<i>Liocarcinus corrugatus</i> (x)	male	Asia, Philippines, Balicasag Island	—	—	—	—
—	<i>Liocarcinus corrugatus</i> (#)	—	—	—	GQ268542.1	GQ268536.1	—
—	<i>Liocarcinus depurator</i> (#)	female	Europe, Germany, HTR	SMF 44080	KU560480	KP795934	KP965202
—	<i>Liocarcinus depurator</i> (#)	—	—	—	FM208767.1	—	—
—	<i>Liocarcinus holsatus</i> (#)	female	Europe, Germany, Loreley Bank	SMF 44051	KU560486	KP795928	KP965197
—	<i>Liocarcinus holsatus</i> (#)	male	Europe, Germany, Helgoland	SMF 44061	KU560485	KP795931	KP965199
—	<i>Liocarcinus holsatus</i> (#)	—	—	—	FM208766.1 GQ268540.1	GQ268538.1	—
—	<i>Liocarcinus navigator</i> (#)	male	Europe, France, Roscoff	SMF 44087	KU560475	KP795938	KP965207
—	<i>Liocarcinus navigator</i> (#)	female	Europe, France, Roscoff	SMF 44087	KU560476	KP795939	KP965208
—	<i>Liocarcinus navigator</i> (#)	—	—	—	GQ268541.1	GQ268537.1	—
—	<i>Liocarcinus maculatus</i> (#)	—	—	—	FJ174892.1	FJ174949.1	—
—	<i>Liocarcinus marmoreus</i> (#)	female	Europe, Portugal, Azores	SMF 36275	KU560483	KP795933	KP965201
—	<i>Liocarcinus (marmoreus)</i> (#)	male	Europe, France, Roscoff	(1135)	KU560482	KP795929	KP965198
—	<i>Liocarcinus marmoreus</i> (#)	—	—	—	GQ268547.1	GQ268535.1	—
—	<i>Liocarcinus pusillus</i> (#)	female	Europe, North Sea, Dogger Bank	SMF 44040	KU560474	KP795927	KP965196
—	<i>Liocarcinus pusillus</i> (#)	—	—	—	GQ268539.1	GQ268534.1	—
—	<i>Liocarcinus vernalis</i> (#)	male	Europe, Turkey, Istanbul	SMF 2991 (704)	KU560481	KP795930	—
—	<i>Liocarcinus vernalis</i> (#)	—	—	—	FM208768.1	—	—
—	<i>Liocarcinus zariqueiyi</i> (#)	male	Europe, Italy	SMF 36278	KU560477	KP795940	KP965209
—	<i>Liocarcinus zariqueiyi</i> (#)	female	Europe, Italy, Naples	ZMG 1083 (694)	KU560478	KP795941	KP965210

Table 1. Continued.

Site (see Fig. 1)	Species	Sex	Locality	Catalogue No. (Additional Individualization No.)	GenBank Accession No.		
					16S rRNA- tRNA ^{Leu} -NADHI	COXI	H3
—	<i>Liocarcinus zariquieyi</i> (#)	male	Europe, Italy, Naples	ZMG 1083 (692)	KU560479	KP795942	KP965211
—	<i>Macropipus tuberculatus</i> (#)	—	—	—	FM208769.1	GQ268530.1	—
—	<i>Necora puber</i> (#)	—	—	—	FM208771.1	GQ268532.1	—
—	<i>Polybius henslowii</i> (#)	female	Europa: Portugal	SMF 32759 (185)	KU560484	KP795932	KP965200
—	<i>Polybius henslowii</i> (#)	—	—	—	GQ268543.1 FM208765.1	—	—
—	<i>Portunus latipes</i> (#)	—	—	—	FM208764.1	—	—
—	<i>Thia scutellata</i> (#)	—	—	—	FM208782.1	—	—

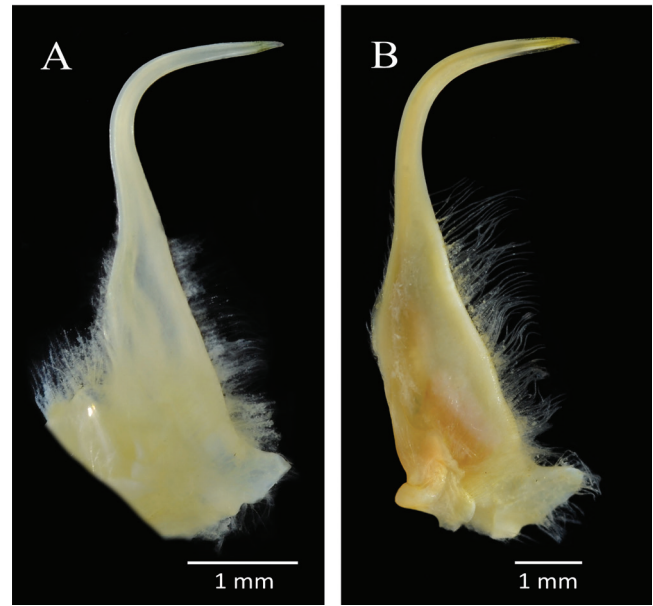


Fig. 4. G1s (left side, ventral view) of A, Asian; B, European *L. corrugatus*.

Markov chain and potential autocorrelation between iterations was investigated with Tracer 1.6 (Rambaut et al., 2013). The maximum credibility tree with median node heights was calculated with TreeAnnotator 1.7.5 (part of the BEAST package). Maximum-Likelihood Analysis was performed on the concatenated alignment with the program RAxML HPC-SSE 3.0.17 (Stamatakis, 2014). We partitioned the data set according to gene and used 1000 bootstrap replicates for estimation of node support. The fast bootstrapping algorithm was used (CAT approximation) while a GTR+Gamma model of sequence evolution was applied for the final tree search. A pairwise comparison of uncorrected sequence divergences (p-distances) was performed for the 16S rRNA, COX1 and the ND1 partitions using the program MEGA 6.06 (Tamura et al., 2013).

RESULTS

Morphology. The overall shapes of the G1s of both European and Asian groups are very similar (Fig. 4). The basal part is broad and stout, becoming narrower until it ends in a pointed tip. After approximately two-thirds of the length, the G1 bends outwards. The curvature is similar in both groups. At the basal part of the G1, long plumose setae can be found up to the beginning of the curvature. In some European specimens, the tip of the G1 appears to be relatively sharper than in the Asian ones, but this character is not stable throughout all examined specimens, and probably represents intraspecific variability.

Morphometry. The carapace width/length (CW/CL) ratio of European individuals was significantly higher than the CW/CL ratio of the Asian specimens (t -test: $t_{df=28,80} = 4.81$, $p < 0.001$, Fig. 5A). To analyse the effect of size on the CW/CL ratio, regression coefficients of the two regions were compared using an ANCOVA. No significant interaction between the two regions and the covariate carapace width

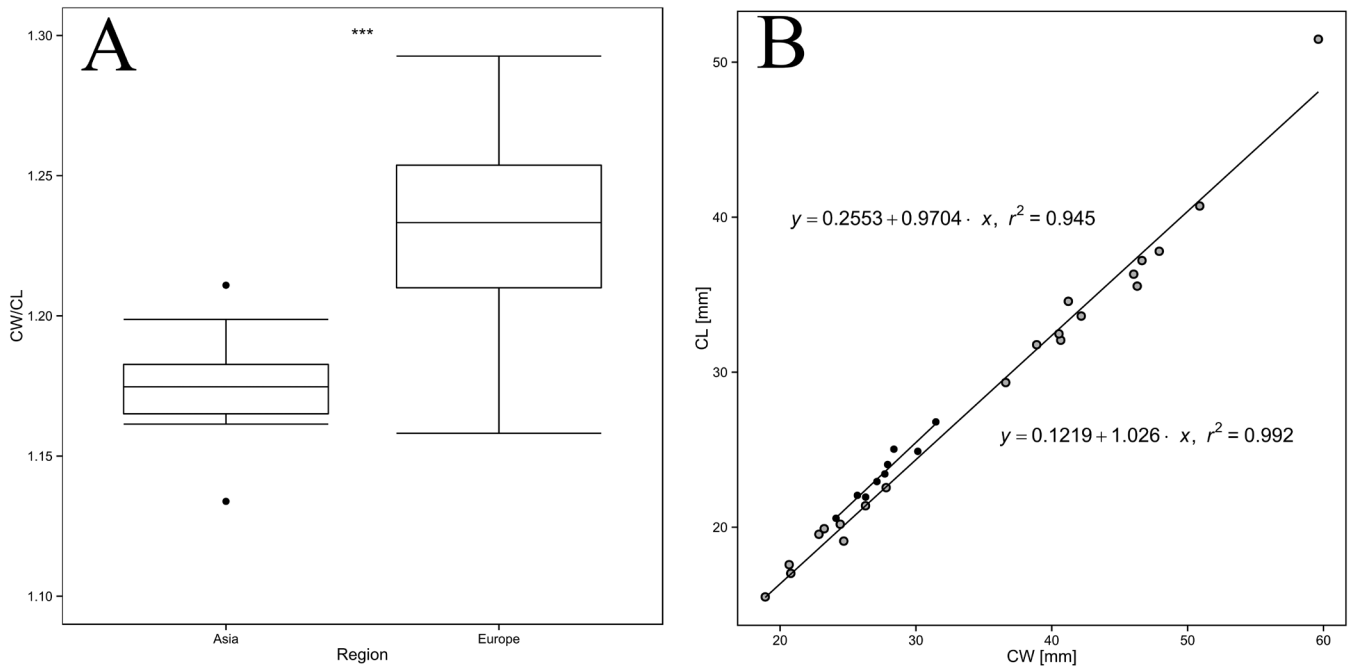


Fig. 5. A, Box and whisker plot of the carapace width/length ratio of Asian and European *L. corrugatus* with median (bold line) and interquartile ranges (1.5xIQR of lower and upper quartile). *** indicates a p-value < 0.001; B, Regressions of carapace length against carapace width of individual crabs from the regions Asia (black) and Europe (grey). Lines shown are best-fit lines by least squares regression with equation and correlation coefficient (r^2).

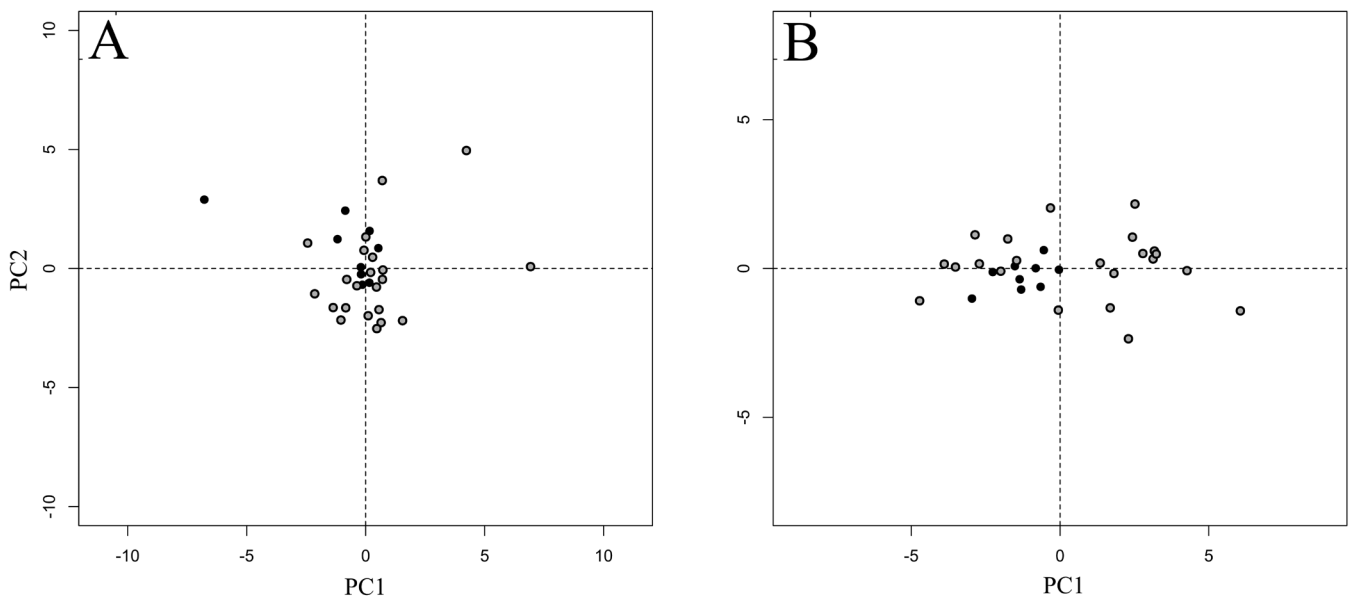


Fig. 6. Principal component analysis of all ratio data measurements and of the: A, linear measurements; B, Asian (black) and European (grey) specimens. The first PC explains 39.48% and 86.40% of the shown variance and is plotted against the 2nd PC which explains 28.71% and 11.40% of the shown variance, respectively.

were observed ($F_{df=1} = 0, p = 1.0$), which indicates that the slopes of both regression lines are similar, and there was thus no significant difference in the growth rate of Asian and European crabs (Fig. 5B). A second, more parsimonious model was then performed without the interaction to test again for a significant difference in the slope. This ANCOVA showed that the factor “region” has a significant effect on the carapace length ($F_{df=1} = 11.33, p = 0.002$). Due to sampling individual sizes starting at around 20 mm carapace width, allometric growth could be excluded; the allometric

coefficient is $\alpha = 0.97$ and $\alpha = 1.02$ for Asia and Europe, respectively (isometry: $\alpha = 1$).

ANCOVAs on the regressions showed no significant differences in sex in Asian ($F_{df=1} = 0.363, p = 0.57$) and European crabs ($F_{df=1} = 0.009, p = 0.93$). As the sample size was unbalanced in number and size, a subset including all Asian specimens and all European specimens with a carapace width to 37 mm was tested as well. The ANCOVA on regression coefficients showed no significant difference

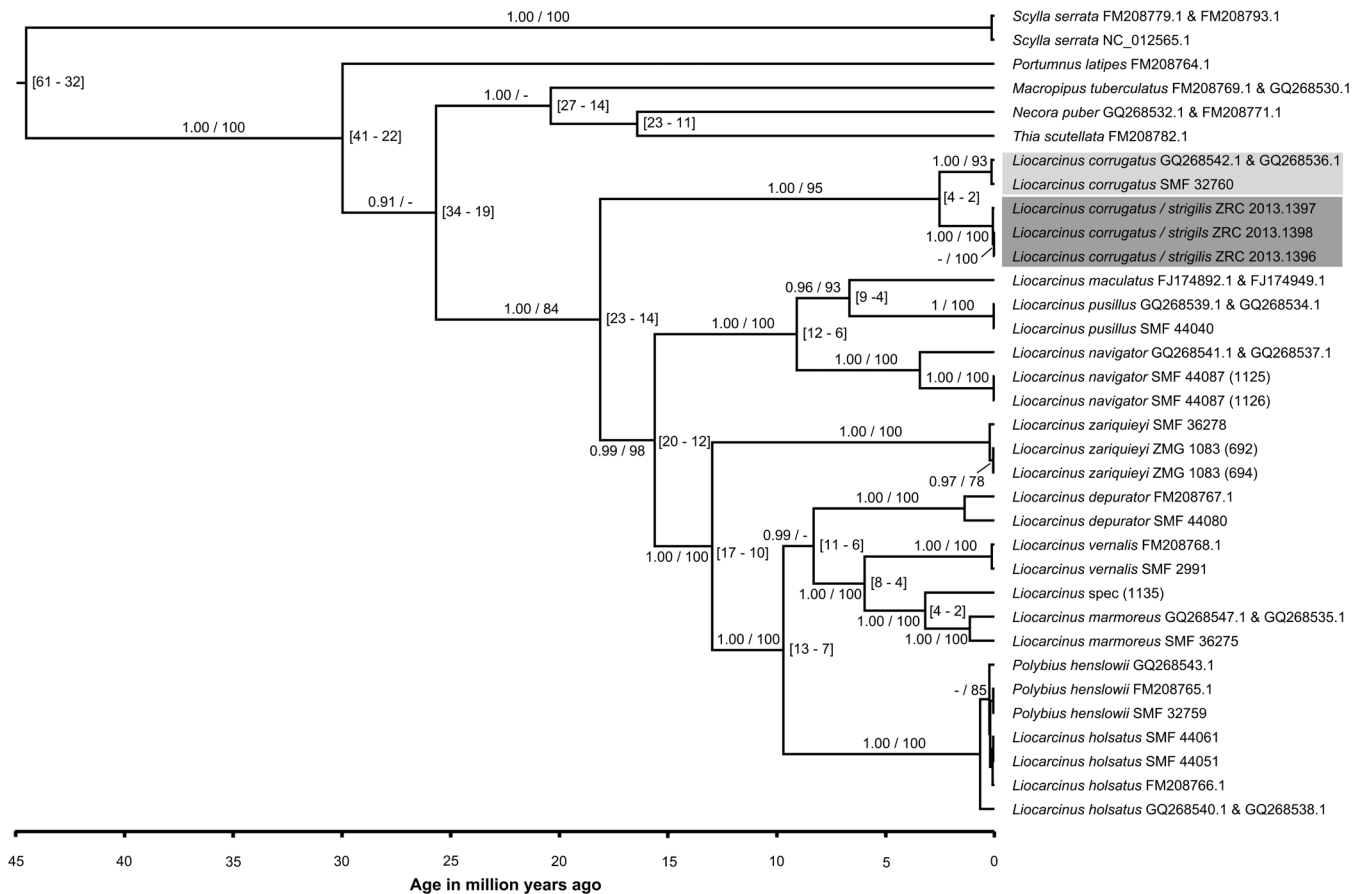


Fig. 7. Maximum credibility tree with median node heights of the Bayesian phylogenetic and divergence time analysis in BEAST MC3 based on the concatenated data set (16S rRNA, ND1, tRNA^{Leu}, COX1, and H3). At the branches the posterior probabilities (> 0.90), the bootstrap values (> 50), and at the nodes the divergence time estimates (95% credibility intervals) are given. Dashes (–) on nodes indicate weak/no support. The Asian *L. corrugatus/strigillis* are shaded in dark grey, the European *L. corrugatus* are shaded in light grey. *Scylla serrata* served as outgroup. For specimens that do not have a museum voucher number (downloaded sequences from previous studies), the GenBank accession nos. are given instead of the SMF, ZMG or ZRC catalogue no. *Liocarcinus spec.* (no 1135) is a juvenile specimen sampled at the *Station Biologique Roscoff*, France that was not identifiable morphologically.

in the interaction between the regions and the covariate carapace width ($F_{df=1} = 0.31, p = 0.59$). The permutation test of all ratios revealed significant effects between the ratios ($F_{df=10} = 37.10, p < 0.001$) but not between interactions with the regions ($F_{df=10} = 0.43, p = 0.21$). The first principal component (PC) explains 39.48% of all variance, the second PC 28.71% (Fig. 6A).

The MANOVA and subsequent Wilks Test revealed that the ratios did not differ enough from each other to assign specimens to a group in a Linear Discriminant Analysis. The ANOVA of the log transformed linear measurements showed no significant effect in the interaction with the Region ($F_{df=7} = 0.85, p = 0.55$). The first two principal components of the PCA based on the linear measurements accounted for 97.80% of the shown variance, with 86.40% on the first PC (Fig. 6B). The Wilks Test revealed that the linear measurements as predictors for either the Asian or European region vary only to a small degree and cannot distinguish the groups.

Geometric morphometry. The first two Principal Components based on the superimposed landmark configurations

accounted for 53% of total shape variation of *L. corrugatus* in the sample (Fig. 3B). Carapace morphologies on the extremes of the first PC (36% of total shape variation) revealed that the carapaces of the European specimens (grey dots) are broader (positive PC1 scores) than the Asian ones (black dots, negative PC1 scores).

The permutation test for comparing the distance between the two group means by random assignments of observations to this group differences using the principal component scores revealed a distance of 0.03 between the group means and a p-value of 0.0004. No significant differences between sexes were detected.

Molecular analyses. In the maximum credibility tree of the Bayesian analysis, posterior probabilities above 0.90 are shown as well as divergence time estimates with their 95% credibility interval (Fig. 7). *Portumnus latipes* (Pennant, 1777) is a sister taxon to *Macropipus tuberculatus* (Roux, 1928–1830), *Necora puber* (Linnaeus, 1776), *Thia scutellata* (Fabricius, 1793) and all species of *Liocarcinus*, including *L. corrugatus* from all populations. *Liocarcinus corrugatus* diverged from the other species approximately 24–14 Mya.

Divergence of *L. holsatus*–*P. henslowii* and *L. depurator*–*L. vernalis*–*L. marmoreus* is well supported and makes the taxon name *Liocarcinus* paraphyletic (see also Schubart & Reuschel, 2009).

Liocarcinus corrugatus of the European and Asian regions are well separated from each other as sister groups; the divergence time estimate ranging between 4–2 Mya. The pairwise comparison of uncorrected sequence divergences (p-distances) showed distances of 2.8–3.1% for the 16S rRNA partition, 4.5% for the COX1 partition and 4.6–5.1% for the ND1 partitions between the European and Asian *L. corrugatus* sequences, respectively.

DISCUSSION

The genetic analyses confirm that the systematics of the genus *Liocarcinus* is in urgent need of revision as posited by earlier studies (e.g., Mantovani et al., 1992; Passamonti et al., 1997; d'Udekem d'Acoz, 1999; Ng et al., 2008; Spiridonov et al., 2014). d'Udekem d'Acoz (1999) proposed a different system for the Polybiinae, consolidating all species of *Liocarcinus*, *Macropipus* Prestandrea, 1833, *Necora* Holthuis, 1987, and *Polybius* Leach, 1820, into one genus, *Polybius*, stating that neither of them represents a monophyletic group. However, this approach has been challenged as the mere merging of genera does not help resolve the confusing taxonomic situation (Türkay, 2001; Ng et al., 2008), and obscures the phylogenetic diversity within Portunidae.

Based on our molecular results we can provisionally divide the genus *Liocarcinus* into the following groups: (1) European and Asian *L. corrugatus* as an early-branching clade; (2) *L. navigator*, *L. maculatus*, *L. pusillus*; (3) *L. zariquieyi*, (4) *L. depurator*, *L. vernalis*, *L. marmoreus* and (5) *L. holsatus* and *Polybius henslowii* Leach, 1820. *Liocarcinus holsatus* and *Polybius henslowii* (both type species of their respective genus) are not distinguishable by the genetic markers used as *P. henslowii* clusters within *L. holsatus*. Our results thereby confirm the earlier findings by Schubart & Reuschel (2009) who stated that *L. holsatus* is “genetically almost identical” to *P. henslowii* in the mitochondrial large ribosomal subunit 16S rRNA and the nuclear encoded histone 3 (H3) gene. Although genetically similar, the biological behaviour of both species is different. Whereas *L. holsatus* is a benthic species which uses its swimming capacity temporarily for capturing prey or fleeing from predators, *P. henslowii* has a highly developed swimming behaviour with a therefore adapted carapace and pereopod morphology (Hartnoll, 1971) and shows periodic pelagic phases in large swarms (Signa et al., 2008). Therefore, we propose that *P. henslowii* is a valid species and most likely the result of very recent speciation within *L. holsatus*. The application of more variable molecular markers might reveal the extent of genetic isolation between both species. This, however, does not change the conclusion that *Polybius* sensu stricto is probably a synonym of *Liocarcinus* sensu stricto. Although *Polybius* is the senior name, we propose to use *Liocarcinus*, for the latter is mentioned more frequent in the literature, also probably due to the many species compiled in the genus. To resolve the issue of synonymy of this two

names, a detailed revision of the genera *Liocarcinus* and *Polybius* is necessary.

As the genus *Liocarcinus* is also a morphologically heterogeneous group of species (Passamonti et al., 1997), it is conceivable to split this genus into several genera that reflect both genetic and morphological characters. Froggia & Manning (1996) suggested the following groups based on morphological similarities: (1) *L. holsatus*, *L. marmoreus* and *L. vernalis*; (2) *L. maculatus*, *L. pusillus* and *L. zariquieyi*; (3) *L. navigator*; (4) *L. depurator* und *L. bolivari*; (5) *L. corrugatus*. This grouping does not match the phylogenetic relationships we identified here or identified by Raupach et al. (2015) based on COI, questioning the morphological characters used for their systematic approach.

In our study, we used several methods to analyse the relationship of European and Asian *Liocarcinus corrugatus*. We showed that while both forms have a similar G1 shape, the shapes of their carapaces differ and they can also be clearly discriminated genetically. The G1s are often very important morphological characters in brachyuran taxonomy, because their shape is usually constant within species and taxon-specific (Türkay, 1975). The shape of the G1s of our analysed European and Asian *L. corrugatus* specimens do not show any consistent characters that could be used for a robust discrimination of the two groups. The morphology of the G1 is, thus, unlikely to pose a barrier for reproduction between specimens from both areas. The differences in the shape of the carapace between Asian and European specimens is more significant and suggests that the species are closely related but different taxa.

Whether the European and Asian *L. corrugatus* are two distinct species has often been discussed (A. Milne-Edwards 1861; Rathbun, 1903; Palmer, 1927; Stephenson & Campbell, 1960; Ng et al., 2008). The main morphological character that separates the two forms is the relatively longer carapace in the Asian forms which also has been confirmed in this study, whereas the shape of the frontal teeth or lobes varies within both regions. We found pairwise sequence differences of 2.8–5.1% in the 16S rRNA, COX1 and ND1 genes of European and Asian *L. corrugatus*, which suggest that Asian and European populations are in fact separate species and not connected by gene flow. Therefore, we recommend the re-validation of *Liocarcinus strigilis* Stimpson (1858) for the Asian “*L. corrugatus*”.

However, inclusion of additional populations for comparative analyses, especially from the Red Sea as a potential linking corridor, and from the southern range of Asian *L. corrugatus*/*L. strigilis* (Australia and New Zealand) will be necessary to ascertain the identity of the Australian populations (for which there exists a name, *Portunus borraidailei* (Bennett, 1930)). From the Red Sea, *Liocarcinus subcorrugatus* was described by A. Milne-Edwards (1861), who suggested this species to be probably conspecific to the *L. corrugatus*/*L. strigilis* from Japan. Palmer (1927), however, stated that there are significant morphological differences (less pronounced front lobes and less strongly corrugated

carapace in *L. subcorrugatus*) between *L. subcorrugatus* and Asian *L. corrugatus*/*L. strigilis*, and regarded them as separate species. We agree, and when material of this Red Sea species becomes available, it might give insight into the morphological and genetic divergence process between *L. corrugatus* and *L. strigilis*.

As European and Asian *L. corrugatus*/*L. strigilis* are deeply separated genetically, and there is a subtle but distinct difference in their carapace shape, they most likely represent two distinct species, and *L. corrugatus* should not be considered a cosmopolitan species. In earlier years, with only limited sampling available and absence of molecular methods, several brachyuran species were described as being cosmopolitan, a status which was revoked after more thorough analyses and identification of cryptic species (e.g., *Ethusina abyssicola* Smith, 1884, Castro (2005)) and *Paractaea rufopunctata* (H. Milne Edwards, 1834) (Guinot, 1976).

Most likely adult and/or larval dispersal capabilities in Brachyura as well as the duration of their larval phase is generally not sufficient to allow for prolonged gene flow between the Atlantic and the Indo-West Pacific, especially since the final closure of the Isthmus of Suez during the Pliocene (Swartz & Arden Jr., 1960; Popov et al., 2004), and with the Southern Ocean acting as a barrier for e.g., *Liocarcinus* and its larvae. This would also be in line with the inferred divergence time of European and Asian *L. corrugatus*/*L. strigilis* of 4–2 Mya, indicating that both species are relicts of a former widespread range throughout the eastern Tethys Ocean. Additionally, several salinity crises in the Pleistocene (Almogi-Labin et al., 1991) killed off most of the marine fauna of the Red Sea so that a recolonisation was only possible from the adjacent Gulf of Aden (for faunistic implications see Türkay, 1986, 1996).

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