

# Reproduction Biology and Population Genetics of the alien Chinese mitten crab (*Eriocheir sinensis*) in Schleswig-Holstein



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## SUMMARY

The Chinese mitten crab *Eriocheir sinensis* (H. Milne-Edwards 1853) (Crustacea, Decapoda, Varunidae) originally inhabits the pacific coast of China and the Korean Peninsula. While this species is regarded as delicacy especially in China, it is an aggressive invasive species in Europe and America, which causes partly serious ecological and economical damages. In the early 20<sup>th</sup> century, the species was likely transferred by ballast water to Continental Europe. Nowadays, the Chinese mitten crab is a common species in German marine and freshwater ecosystems and often occurs in high densities, especially in Schleswig-Holstein (Northern Germany). Schleswig-Holstein is characterized by two ecological different sea basins, the North Sea and the Baltic Sea as the world's largest brackish sea and a net of lakes and rivers draining into these seas. Although established for approximately 100 years, knowledge on the reproduction and population biology had remained scarce. The combination of population genetics and morphological methods in this thesis leads to new and comprehensive insights on the biology of *Eriocheir sinensis*. The central result is that indeed a more or less a homogenous population exists in Schleswig-Holstein, but otherwise clear differences in the reproduction biology between Baltic Sea and North Sea populations were observed. According to the results of this thesis, the Chinese mitten crab has a broad spectrum of reproductive strategies unknown so far, which facilitates the settlement of very different habitat types and thus a successful invasion.

This thesis consists of three central studies: The first study demonstrates for the very first time that the whole reproduction cycle of *Eriocheir sinensis* takes place in the Kiel Bight and presumably also in the whole western Baltic Sea. This finding contradicts the previous assumption that larval development of *Eriocheir sinensis* is only possible at high salinity as occurring in the North Sea. However, besides high mortality rates in late larval stages, larvae could be reared in the laboratory from

ovigerous females collected in Kiel Bight. Furthermore, larvae have been found in the Kiel Bight as well as juvenile crabs in a freshwater lake close to Kiel Bight.

The second study disentangles the population structure and genetic status of *Eriocheir sinensis* in Schleswig-Holstein. Therefore, mitochondrial DNA (COI-Gene) as well as 9 polymorphic microsatellites as molecular markers were used. The genetic variability was partly similar compared to native and higher compared to other European *E.sinensis* populations, which is probably caused by multiple introductions from different and previously separated populations. The genetic data show an intensive gene flow between the populations in Schleswig-Holstein except for one population at the Baltic coast. In the final study, histological analyses of the female reproductive organ show several previously unknown features: juvenile crabs are also migrating in the mating season and are even able to copulate. Females reproducing in the Baltic Sea often copulate more than one time in one mating season and spermatozoa can be stored longer than one mating season. Amongst others, this feature is a hint that females do not inevitably die after one reproduction season as it has been generally assumed previously.

### ZUSAMMENFASSUNG

Die chinesische Wollhandkrabbe *Eriocheir sinensis* H. Milne-Edwards 1853 (Crustacea, Decapoda, Varunidae) kommt ursprünglich an der pazifischen Küste Chinas bis zur koreanischen Halbinsel vor. Während die Art in vor allem in China als Delikatesse gilt, ist sie in Europa und Amerika eine aggressive invasive Art, welche zum Teil erhebliche ökologische und ökonomische Schäden verursacht. Die Art wurde wahrscheinlich zu Beginn des 20. Jahrhunderts über Ballastwasser nach Mitteleuropa eingeschleppt. Mittlerweile ist die Wollhandkrabbe in marinen und limnischen Ökosystemen Norddeutschlands weit verbreitet und tritt vor allem in Schleswig-Holstein vielfach in hohen Populationsdichten auf. Schleswig-Holstein zeichnet sich besonders durch zwei ökologisch unterschiedliche Meere, die Nordsee und die Ostsee als größtes Brackwassermeer der Welt, sowie ein Netz aus Seen und Flüssen aus. Obwohl die Art mittlerweile seit beinahe 100 Jahren etabliert ist, waren die Kenntnisse über die Reproduktions- und Populationsbiologie nur sehr spärlich. Die Kombination populationsgenetischer und morphologischer Methoden in dieser Arbeit führt zu einem völlig neuen und umfassenden Einblick in die Biologie von *Eriocheir sinensis*. Das zentrale Ergebnis ist, dass in Schleswig-Holstein zwar eine homogene Population besteht, aber eindeutige Unterschiede in der Reproduktionsbiologie zwischen Nord- und Ostsee-Populationen festgestellt wurden. Laut den Ergebnissen dieser Arbeit verfügt die Art über ein breites Spektrum an Fortpflanzungsstrategien, die bisher unbekannt waren und ihr die Besiedlung sehr unterschiedlicher Lebensräume und damit eine erfolgreiche Invasion ermöglichen.

Die Arbeit umfasst drei zentrale Studien: Die erste Studie liefert zum ersten Mal den Nachweis, dass der gesamte Reproduktionszyklus in der Kieler Bucht stattfindet und somit vermutlich in der gesamten westlichen Ostsee möglich ist. Dies widerspricht der bisherigen Annahme, dass die Wollhandkrabbe sich aufgrund der erforderlichen hohen Salinität für die Larvalentwicklung nur in der Nordsee fortpflanzen kann.

Neben einer hohen Mortalitätsrate in den späten Larvalstadien konnten Larven von eiertragenden Weibchen aus der Kieler Bucht im Labor gezüchtet werden. Desweiteren wurden sowohl Larven in der Kieler Bucht als auch juvenile Krabben in einem See in der Nähe der Kieler Bucht gefunden.

Die zweite Studie deckt die Populationsstruktur und den genetischen Status von *Eriocheir sinensis* in Schleswig-Holstein auf. Dafür wurden sowohl mitochondriale DNA (COI-Gen) als auch 9 polymorphe Microsatelliten als molekulare Marker verwendet. Die genetische Variabilität ist teilweise ähnlich hoch wie in den Ursprungspopulationen und höher als in den anderen europäischen Populationen, was wahrscheinlich auf wiederholte Einschleppungen aus verschiedenen und bisher getrennten chinesischen Populationen zurückzuführen ist. Die genetischen Daten zeigen einen intensiven Genfluss zwischen den Populationen in Schleswig-Holstein mit Ausnahme einer Population nahe der Ostseeküste.

In der dritten Studie wurden die Reproduktionsorgane sowie die Reproduktionsstrategien von *E. sinensis* mittels histologischer Methoden untersucht. Diese Analysen zeigen bisher unbekannte Besonderheiten: Juvenile Krabben nehmen an der Reproduktionswanderung teil und sind sogar in der Lage zu kopulieren. Weibchen, die sich in der Ostsee fortpflanzen, kopulieren oft mehr als einmal in einer Fortpflanzungssaison und Spermatozoa können länger als eine Fortpflanzungssaison gespeichert werden. Unter anderem wurde damit auch gezeigt, dass die Wollhandkrabbenweibchen nicht zwangsläufig nach einer Fortpflanzungssaison sterben, wie es bisher allgemein angenommen wurde.

## GENERAL INTRODUCTION

### Aquatic Invasions

In the age of globalization, the anthropogenic aquatic species transfer has increased in the last centuries especially due to intensive international ship traffic. Introductions of alien species can lead to profound overall impacts in the ecosystem and to economical losses, mainly in the fishing industry. This can cause substantial control costs (e.g. Fladung 2000, Holland 2000 and references therein, Gollasch 2006, Ricciardi and Kipp 2008). Elton (1958) classified anthropogenic introductions of alien species as one of the most “pervasive, irreversible and devastating impacts of human activity on natural ecosystems“. Detailed studies of coastal invasions started about 30 years ago and direct impacts like reduction or elimination of keystone species and endangered species have been reported (Grosholz 2002 and references therein). Furthermore, direct and indirect impacts on food-web structure and ecosystem function have been detected (Grosholz 2002 and references therein, Leppäkoski 2002, Stepien et al. 2005, Reusch et al. 2010). In 2008, 1016 marine alien species were recorded of which 747 are established in European waters (DAISIE database).

Ballast water and hull fouling are the main factors for the increasing aquatic invasions, but also species introductions for aquaculture become more and more important (Nehring and Klingenstein 2005). Ballast water is carried in bigger ships for stabilisation, for example in cargo ships. In harbour, the ships drain the ballast water, which often contains a lot of non-indigenous species mostly as larval stages. Measurements like ballast water treatment, quarantine options and risk assessment are strongly required and are already partly conducted (Gollasch 2003, Stepien et al. 2005, Stepien and Tumeo 2006, David and Gollasch 2008). For example, in 2004, the International Convention for the Control and Management of Ships' Ballast Water

and Sediments (BWM Convention) was adopted by the International Maritime Organization (IMO). Until 2013, HELCOM countries agreed on the ratification of the BWM Convention. Twelve months after ratification of 30 states in total, the convention will become operative. Currently 28 states, which are about 25% of the world merchant shipping tonnage, have ratified the convention (see <http://www.imo.org>). As an intermediate solution, the convention recommends to exchange ballast water only offshore to reduce the number of transferred alien species. The aim for 2016 is that all ships should be equipped with a ballast water - treatment plant containing special filter layers amongst others, which retains the potential aliens when the ship releases ballast water.

Especially estuaries have a high potential to be invaded by alien species due to many vacant niches (Gollasch 2006, Nehring 2006) and due to a “two-sided invasion pressure” through the ocean as well as through inland waters like canals and rivers (Nehring 2006).

The ongoing introductions enlarge the trend of a homogenized biota not only in the estuaries and therefore cause a worldwide loss of biodiversity (Convention on Biological Diversity (CBD) 2000, Holland 2000, Olden and Poff 2004, Gollasch 2006). Schleswig-Holstein is a very appropriate study area for anthropogenic based introductions due to the intensive international ship traffic at the North Sea and Baltic Sea coast.

In the North Sea and its adjacent rivers, 144 non-indigenous species are established, of which the highest percentage are zoobenthic organisms as mollusks, crustaceans and polychaetes (Nehring 2003, Gollasch 2006). At the German North Sea coast, it comes down to 27 established alien species, which have been registered. The origin of most invertebrate alien species is the Atlantic coast of North America (Nehring and Klingenstein 2005). The amount of alien species decreases from the brackish estuaries to the central North Sea from about 20% to less than 1% as percentage from the total species numbers (Nehring and Klingenstein 2005).

Due to special ecological characteristics and the relative young geological age, the Baltic Sea does not have a stable established fauna-system, but is affected by a relatively high dynamic pattern of regular resettlements and successions. Obviously, this incomplete biocoenosis offers a lot of space for introduced or immigrated species. Currently, 99 alien species were identified as invaders with self-sustaining populations in the Baltic Sea and its adjacent rivers (see: Baltic Sea Alien Species Database, Gollasch 2006).

In the Baltic Sea, no littoral habitat is free of introduced organisms and in many regions of the Baltic Sea alien species are dominating the benthic-pelagic trophic structures (Leppäkoski 2002). The most important countries of origin are North America, the Ponto-Caspian area and the East-Asian coast (Olenin 2005). Macrozoobenthic species are the best documented non-indigenous species group in the Baltic Sea (see: Baltic Sea Alien Species Database). According to Gollasch and Nehring (2006), 82 aquatic alien species are established in German inland waters, where the highest increase of alien species in the last ten years were registered. In some areas, the alien species represent about 18% of the whole fauna. Macrozoobenthos clearly dominates the established alien species, followed by fishes and with only a small percentage amphibians and reptiles.

### Chinese mitten crab as an invasive species

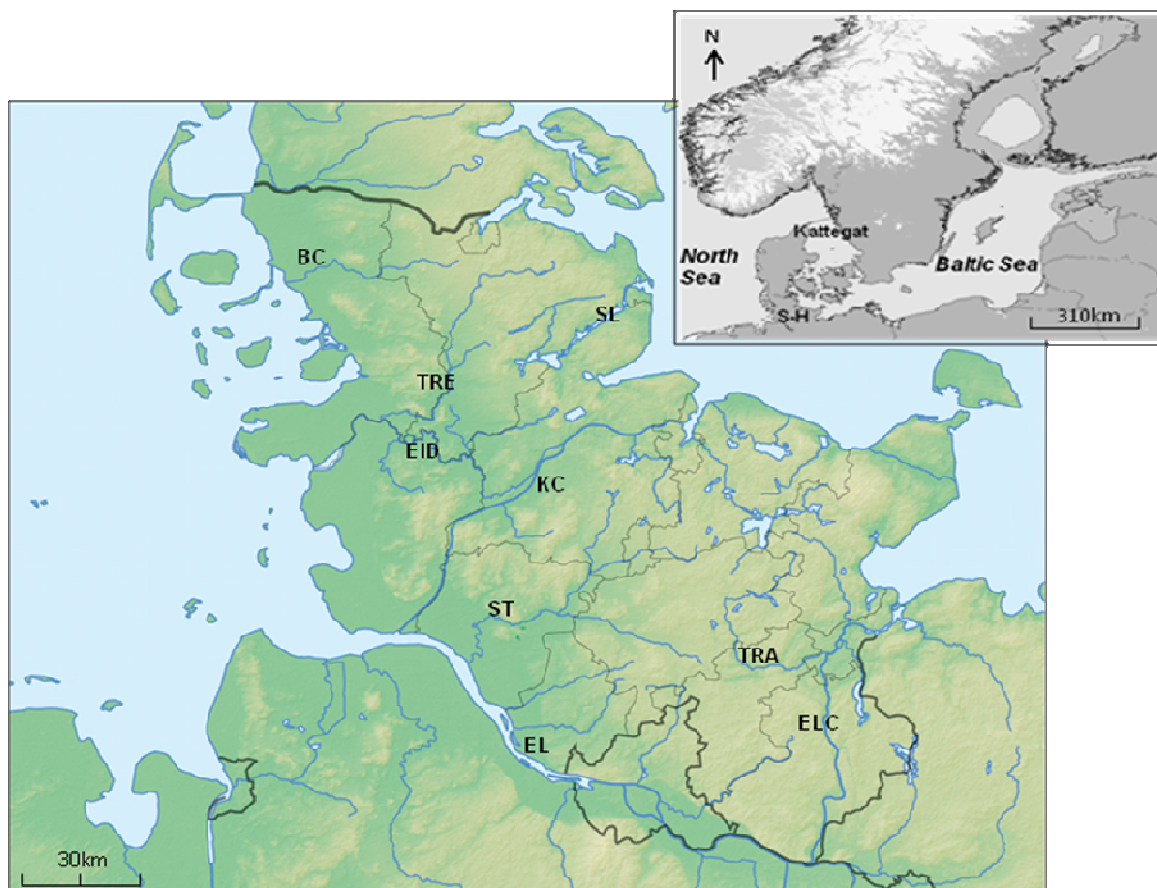
In general, crabs are a suitable group for studying evolutionary processes in aquatic habitats due to the short generation times. Especially cosmopolitan species like Chinese mitten crabs are an interesting species to investigate the parameters, which are important for successful establishment and adaptation in the invaded habitat. *Eriocheir sinensis* is a catadromous species spending most of its life cycle in fresh - or brackish water. For mating, which takes place in autumn, the crabs have to



migrate into estuaries. In its native range along the eastern coast of China and the Korean Peninsula, the Chinese mitten crab is harvested and marketed by an important traditional fishery, since it is regarded as a delicacy. *Eriocheir sinensis* has been introduced to Continental Europe in the early 20<sup>th</sup> century via ballast water and is regarded as a pest due to its serious ecological and economical damages (Peters and Panning 1933). The first specimen was detected in the River Aller in Northern Germany 1912 and in a short period *Eriocheir sinensis* established self-sustaining populations in the Rivers Weser and Elbe (Marquard 1926, Peters and Panning 1933). In the beginning of the 1930s, a massive explosion of the population occurred in those rivers leading to serious ecological damages at the riverbank through burrowing activity and economical losses in fishing industry due to damage of nets and feeding on caught fish (Peters 1936). Since the 1940s, the numbers of crabs have decreased and research and control measurements have been mostly stopped. After several small peaks in species abundance, mitten crab population size varies around early numbers reached in 1930 especially in the Elbe River (Fladung 2000). Nowadays, *Eriocheir sinensis* is a common, established species in Northern Germany. According to Fladung (2000), the absence of serious enemies and competitors as well as the rising water quality in several waters support the successful dispersal in Germany. Especially Schleswig-Holstein offers a very suitable habitat for its catadromous life-cycle.

The species has been reported also along almost the whole Baltic Sea coastline, but in much lower numbers than in the North Sea and its adjacent rivers (Normant et al. 2000, Panov et al. 2003, Ojaveer et al. 2007, Bacevičius and Gasiūnaitė 2008). Currently, the Chinese mitten crab is a serious problem for fishermen in Schleswig-Holstein as this species seasonally occurs in high densities causing the damages like in the 1930s mentioned above (pers.comm. of local fishermen). In Schleswig-Holstein, the Chinese mitten crab occurs in self-sustaining populations in all large rivers like Eider, Elbe and Stör as well as the Bongsieler Canal, which are all leading to the North Sea and in the River Elbe respectively (Figure 1). The Bongsieler Canal

begins at the confluence of Lecker and Soholmer Au (Soholmer Au is a sample location in study 2 and study 3). *Eriocheir sinensis* also inhabits the Kiel Canal in high numbers. Furthermore, the species occurs in the River Treene, River Trave as well as in the Elbe-Lübeck-Canal, which are flowing into the Baltic Sea (see figure 1; Hartmann and Spratte 2005, own observations). *Eriocheir sinensis* also occurs in the Schlei Fjord (own observations, pers.comm. of local fishermen).



**Figure 1:** The study area Schleswig-Holstein with the largest rivers, canals and fjords respectively, where the Chinese mitten crab is established. BC= Bongsieler Canal, EL= Elbe, ELC= Elbe-Lübeck-Canal, EID= Eider, KC= Kiel Canal, SL= Schlei, ST= Stör, TRA= Trave, TRE= Treene. Maps: Baltic Sea map by courtesy of Robert Sommer, Ecology center Kiel; Schleswig-Holstein map: [https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein\\_relief\\_location\\_map.jpg](https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein_relief_location_map.jpg)

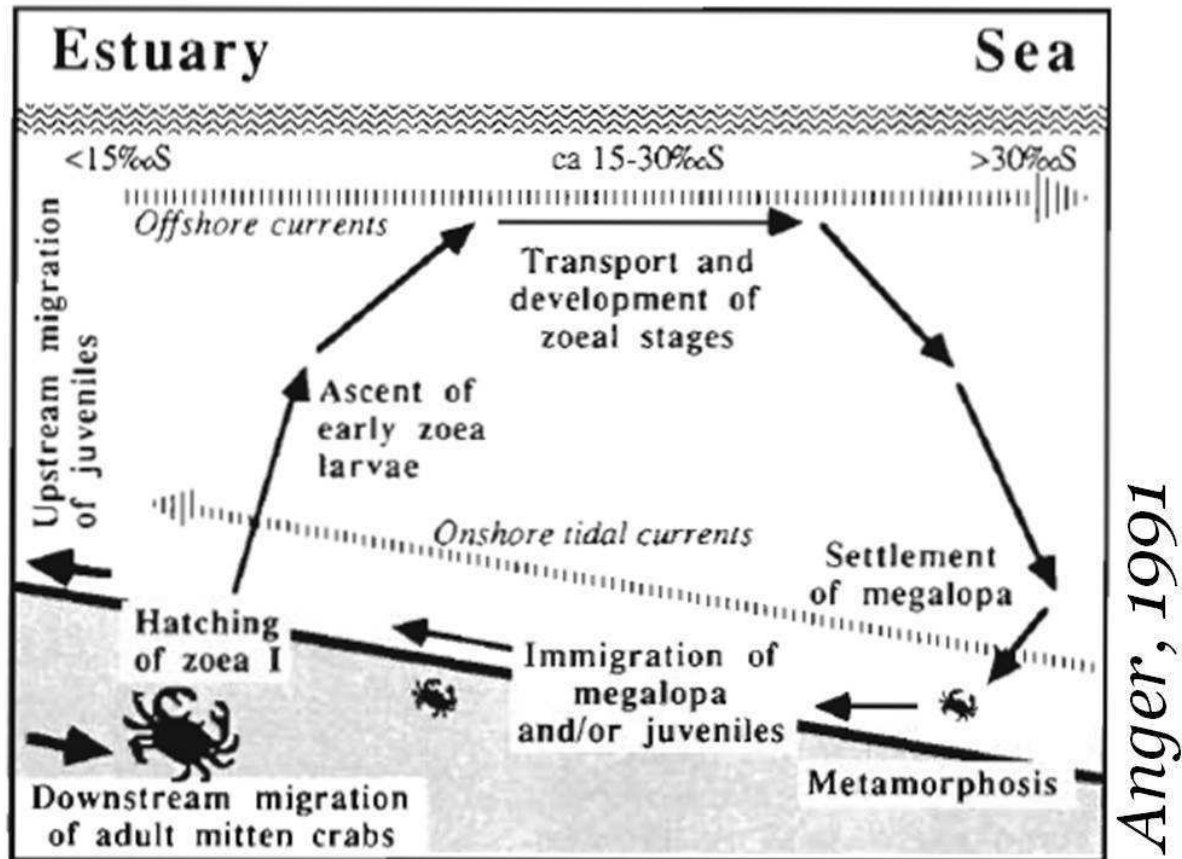
Besides an intensive expansion in the German Elbe and Weser riversystems, at the German North Sea coast and in the Baltic Sea area, *Eriocheir sinensis* also occurs in the following European countries: the Czech Republic (Peters 1938a), the Netherlands (Kamps 1937), France (Hoestlandt 1945), the Mediterranean (Petit and Mizoule 1974) and especially in Great Britain, where the species also spread intensively (Clark et al. 1998, Herborg et al. 2005). In the 1990s, the first Chinese mitten crabs were also detected in the San Francisco Bay in North America (Cohen and Carlton 1997), probably introduced from Europe (Hänfling et al. 2002). In those invaded regions, *Eriocheir sinensis* often causes serious damages as known from Northern Germany (Clark et al. 1998, Rudnick et al. 2000, 2003, Clifford et al. 2005). Occurring in high densities, this species is also considered to have negative impacts on native communities, for example due to competition for space and food with various fish and decapod species, due to feeding on the offspring of rare fish species and finally due to direct predation on decapods (Clark 1998, Veldhuizen and Stanish 1999, Fladung 2000, Rudnick 2000, Gilbey et al. 2008).

Due to its aggressive behaviour, the Chinese mitten crab is currently classified as IAS ‚invasive alien species‘ by the CBD (2000) and is included in the list of the world’s ‘100 worst invasive alien species’ (Lowe 2000).

#### Life cycle and required salinities and temperatures

In Europe, Chinese mitten crabs spend 4-6 years in freshwater or brackish water before they begin their migration downstream to the estuaries in late summer, where they reproduce (Panning 1938, Anger 1991, Fladung 2000). In China, crabs become sexually mature with 1-2 years (Hymanson 1999, Veldhuizen and Stanish 1999, Jin et al. 2002). During this migration, the gonads develop and in the brackish water zones of the estuaries the crabs finally become sexually mature (Hoestland 1948, Veldhuizen and Stanish 1999). Mating and fertilization take place from late autumn until beginning of winter. According to Anger (1991, see figure 2), the mated females move into deeper water and overwinter in the lower estuaries. The eggs are affixed

on the abdomen of the female until the larvae hatch. Females are able to produce 1 to 3 broods in one season with 100.000 to 1.000.000 eggs per brood (Peters 1938b). It is generally assumed that *Eriocheir sinensis* reproduces one time and then die (e.g. Hymanson et al. 1999, Fladung 2000, Rudnick 2003). Larvae hatch in spring and are planktonic for about 1-2 months. The early larval stages are transported into the coastal areas by surface currents. The Chinese mitten crab undergoes five zoeal stages in marine waters before the metamorphosis to the megalopa. The megalopa stage settles down on the bottom of marine coastal areas or embayments. From there it is transported by near-bottom currents toward the mouths of the estuaries, where it develops to a juvenile crab. Juveniles start upstream migration in spring via main river channels, where they enter smaller channels with slower-moving waters. Presumably, tidal currents in estuaries are used for upstream migration. Young juvenile mitten crabs are mainly found in tidal brackish and fresh water areas, older juveniles are located further upstream. The adult Chinese mitten crabs inhabit inland waters, rivers as well as brackish coastal lagoons. This successful dispersal in freshwater as well as brackish water is facilitated through its holo-euryhalinity (Kinne 1971).



**Figure 2:** Life cycle of *Eriocheir sinensis*, taken from Anger 1991.

Parts of the life cycle, i.e., gonadal development, development of the megalopa and the juvenile and adult growth, can take place successfully in freshwater at low as well as high temperatures, whereas the critical stages of the life cycle - the embryonic and larval development - require specific levels of salinity and relatively high temperatures. The higher the temperature, the larger the tolerance towards lower salinity. The detailed salinities and temperatures are shown in table 1 and 2.

GENERAL INTRODUCTION

**Table 1:** Salinity ranges in PSU reported for *Eriocheir sinensis*. (References Jin 1998, Zhao 1999 is a personal communication and presentation respectively, cited in Cohen and Weinstein 2001)

Life stage	Possible range	Optimal range	References
Gonadal development	0-?	-	Hoestland 1948, Veldhuizen and Stanish 1999
Mating and fertilization	- 5-20	10-16 15-17	Hymanson et al.1999 Zhao 1999
Egg adherence	15-20 - ≥15	- 25-30 -	Anger 1991 Buhk 1938 Zhao 1999
Embryonic development	“Pure salt water” ≥25 3 to 27	- - 15 to 20	Panning 1939 Vincent 1996 Zhao 1999
Hatching	-	≈23	Buhk 1938
To zoea I	≥10	15-25	Anger 1991
Subsequent larval stages zoea II-V	15-32	20-32	
Zoea V to megalopa	20-32	25	
Larval development	-	13-26 15	Jin 1998 Zhao 1999
Megalopa to juvenile	10-32	25	Anger 1991
Juvenile growth	0-32	15-25	
Adult growth	0-?		

**Table 2:** Temperature ranges in °C reported for *Eriocheir sinensis*. (References Guo 1996, Jiang 1998, Jin 1998, Zhao 1999 are personal communications and presentations respectively, cited in Cohen and Weinstein 2001)

Life stage	Possible range	Optimal range	References
Mating	18-20	-	Guo 1996
Egg adherence	-	9-12	Jin 1998
Embryonic development	-	15-20	Jin 1998
Hatching	-	15-20	Zhao 1998
Larval development	12-18	15-18 or higher	Anger
		20-25	Jin 1998, Zhao 1999
Juvenile growth	4-30	15-30	Jiang 1998,
Adult growth	4-30	15-30	Hymanson et al.1999, Zhao 1999

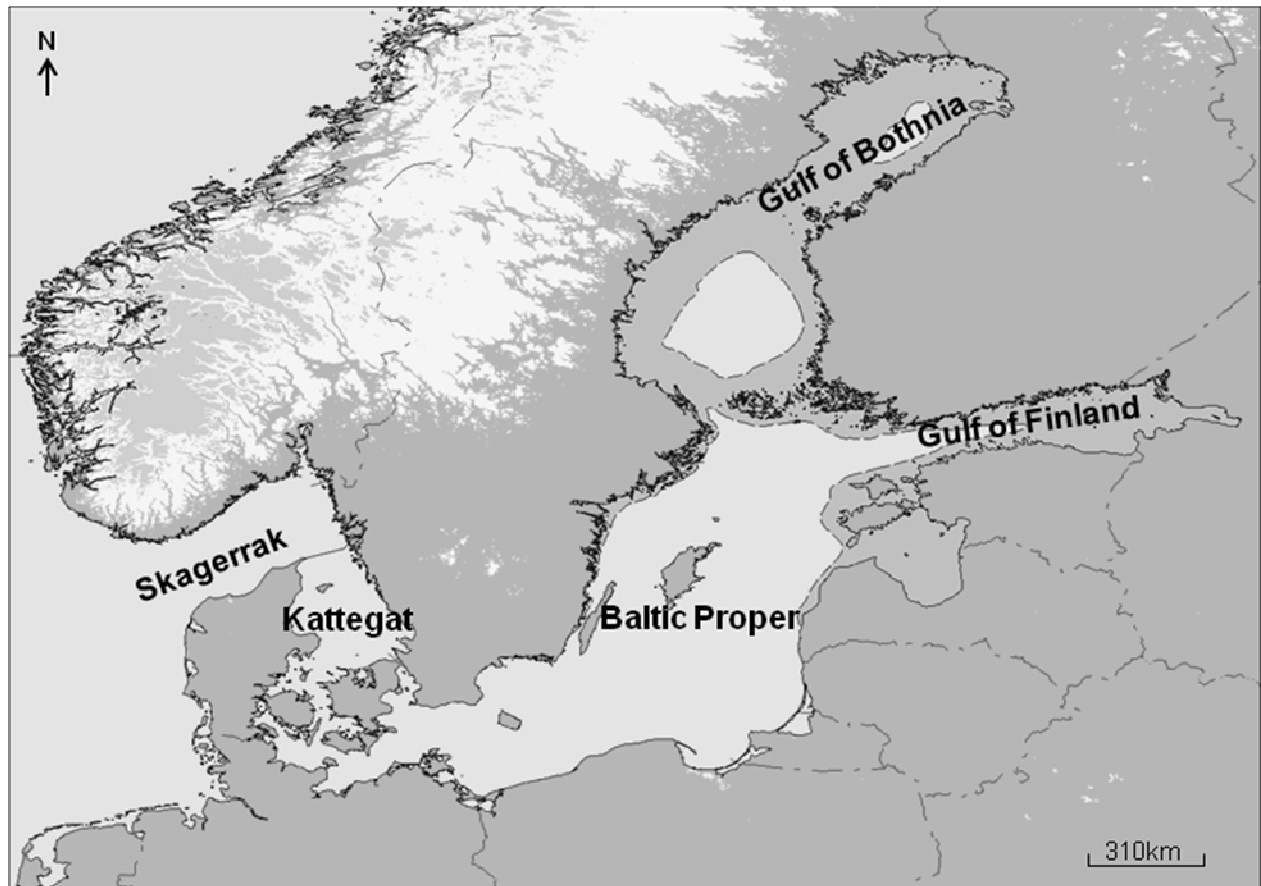
### Occurrence of the Chinese mitten crab in the Baltic Sea

The Chinese mitten crab reached the Baltic Sea via the Kiel Canal in 1927 (Peters 1938a). In 1933 the first individuals were found in the eastern Gulf of Finland and in Vyborg, Russia (see review in Herborg et al. 2003). Reproduction of *Eriocheir sinensis* in the Baltic Sea is an often discussed topic: The unfavourable hydrography for the species might prevent a full reproduction cycle in the Baltic, with an exception of the Skagerrak and Kattegat due to higher salinities (Haathela 1963, Rasmussen 1987, Cohen and Weinstein 2001, Gollasch pers. comm. in Cohen and Weinstein 2001). Due to the freshwater inflow through the rivers as well as the exchange with saltwater of the North Sea, the Baltic Sea is characterized by a vertical and horizontal salinity gradient. The salinity decreases from South and West towards North and

East. The lowest salinity of 2-3PSU is found in the inner parts the Gulf of Bothnia and Gulf of Finland (see figure 3). Surface salinity increases from 6–8PSU in the Baltic Proper to 20–24PSU in the Kattegat. In the western Baltic Sea salinity at the surface is 10PSU, at the bottom 16-18PSU (Sverdrup et al. 1942, Ekman 1953, Leppäskoski et al. 2002 and references therein).

In winter, temperature at the surface is 2-4°C in average. The northern parts of Bothnian Bay are mostly covered by ice 170-190 days, the entrance area of the Gulf of Finland usually 70 days (Leppäskoski et al. 2002 and references therein). Also the fjords of the western Baltic Sea are covered by ice, but not regularly and over a shorter period. In summer, a surface temperature even up to 20°C is quite common in the western Baltic Sea. Mean annual temperature at the surface is 16°C. A distinct thermocline exist in the western Baltic Sea and the Baltic Proper which separates the cold intermediate winter water from the warmer surface layer at about 10-30m. The intensity of this layer turns down towards the Gulf of Bothnia and Gulf of Finland (Leppäkoski 2002 and references therein).





**Figure 3:** The Baltic Sea. Map by courtesy of Robert Sommer, Ecology center Kiel.

The ovigerous females, which have been found at some coasts at the Baltic Sea in the past, did not show a successful reproduction. For example, only three out of 33 ovigerous females, collected between 1927 - 1985 at the Danish Baltic Sea coast, carried eggs. The remaining females were in bad condition and not able to develop (Rasmussen 1987). The eggs of 27 ovigerous females collected in the Kiel Canal in May 1936 did not survive, infected mostly by fungi (Peters 1938b). The Kiel Canal connects the North Sea with the Baltic Sea and shows salinity values from 3 to 13PSU. In the southern Baltic Sea, gravid females are a quite common bycatch in fishing bow nets in the recent years (Ojaveer et al. 2007), but information about success of breeding has not been reported so far.

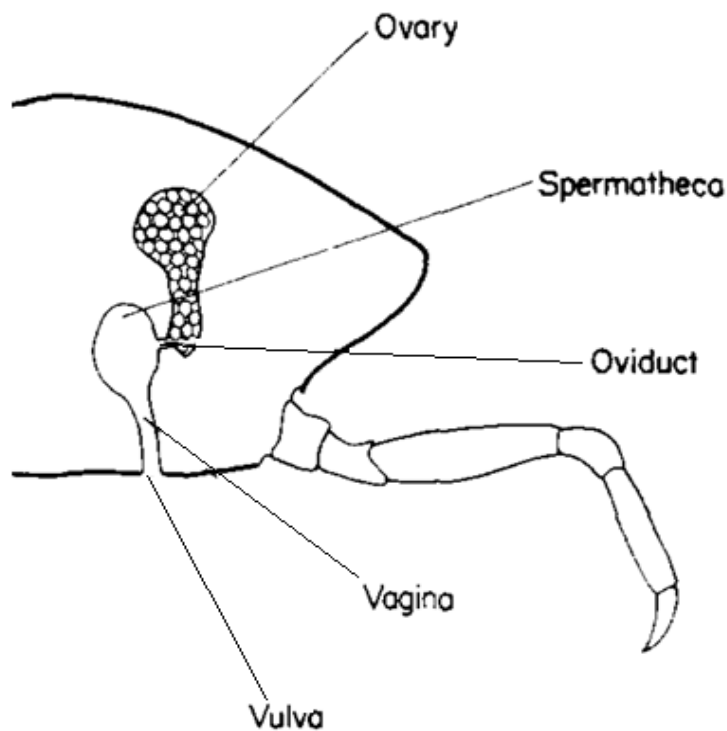
Two ovigerous females at the Swedish east coast south of the Göta Älv River estuary and from Laholmsbukten were collected during springtime 2004 and 2008 (Drotz et al. 2010). Together with the fact of increasing Chinese mitten crabs numbers in the Göta Älv River estuary since the end of 1990s, Drotz et al. (2010) predict the establishment of a self-sustaining population in the Göta Älv estuary in the future due to the more or less appropriate conditions.

Though there are increasing numbers of collected male and female Chinese mitten crabs in the northeastern as well as in the western part, no published data about larvae and juveniles abundances in the Baltic Sea have been available until now. Ojaveer et al. (2007) stated that the occurrence of *Eriocheir sinensis* in the Baltic Sea is regulated by the population dynamics in the southeastern North Sea and in the Elbe estuary. Another possibility for the occurrence in the Baltic Sea is the translocation by ships (Ojaveer et al. 2007). Anger stated that *Eriocheir sinensis* could have constituted a more or less stable population since some decades but without the northern and eastern parts of the Baltic Sea (pers. comm. 2000 in Cohen and Weinstein 2001).

#### Reproduction system of female Chinese mitten crab

According to Hartnoll (1968), the reproductive tract of brachyuran female crabs consists of a left and a right set of the sexual organs. Ovary, spermatheca, oviduct, vagina and vulva are shown in the schematic draw of Hartnoll (1968; see figure 4). The oviduct opens in the spermatheca and the spermatheca in the vagina, described as basal type from Diesel (1991). The vaginae open into the vulvae, which lie at the sixth thoracic sternite. During the copulation, sperms are transported as spermatophores into the spermatheca via the gonophores of the male into the vagina of the female (Hartnoll 1968). Mating of Chinese mitten crabs takes place when both the male and the female are in the intermoult and have a hard carapace, which means that they conduct the so- called hard-shell mating (Peters 1938b). Kobayashi

and Matsuura (1999) also observed this mating behaviour for the congeneric species *Eriocheir japonica*.



**Figure 4:** Cross section of reproductive system of a brachyuran female crab according to Hartnoll 1968.

In the adult and sexually mature female crab, the ovary becomes enormously large and takes almost the whole cavity of the female during mating season. During the yolk accumulation, the so called vitellogenesis, ovaries become dark-yellow or dark-orange and have a Y-shape (Kobayashi et al. 2003). The oocytes increase and show a diameter of about 50-400 $\mu$ m (Jin et al. 2002, Kobayashi et al. 2003). The ripe but unfertilized eggs are transferred through the oviduct into the spermatheca, where the fertilization takes place (Hartnoll 1968, Lee and Yamazaki 1990).

Outside the reproduction season, ovaries are small and white-yellow, H-shaped and oocytes are much smaller (Kobayashi et al. 2003).

### General background information about methods

In the following, short background information is given for the population genetic and histological methods. The complete material and methods are described in the respective studies.

For population genetic analyses, the mitochondrial COI-Gene (cytochrome c oxidase subunit I) and polymorphic microsatellites loci were used as nuclear marker.

The mitochondrial DNA (mtDNA) is a circular, covalently closed, double stranded DNA in the most multicellular organisms and maternally inherited in most cases (Lansman et al. 1983). One to ten identical copies of the mitochondrial genome exist in every cell, facilitating an easy extraction (Rand 2001). In the mtDNA, especially enzymes of the cell energy metabolism as well as proteins of the mitochondrial translation system are coded (Avisé et al. 1987). Cytochrome c oxidase is an enzyme of the mitochondrial respiratory system. Due to its high variability, the COI-gene has been proved as a proper tool for population genetic analyses (e.g. *Eriocheir sinensis* Hänfling et al. 2002, *Carcinus maenas* Roman and Palumbi 2004, *Palaemon elegans* Reuschel et al. 2010). The maternal inheritance of the mtDNA without recombination, haploidy, high mutational rate and shorter coalescence times leads to a greater sensitiveness on demographic processes as fragmentation, founder events or bottlenecks than nuclear markers (Gopurenko et al. 1999, King et al. 1999, Wilke and Davis 2000, Tarjuelo et al. 2001). The results of mtDNA analyses have to be regarded against the background that due to the maternal inheritance neither immigration nor introduction of males has an impact on the genetic variability of the population. Thus, additionally nuclear polymorphic microsatellites were used to gain a comprehensive image of the genetic structure. Microsatellites are short neutral

sequences of the nuclear DNA characterized by short tandem repeat motifs about 2-6 basepairs (for example:  $(CA/GT)_n$  or  $(AAT)_n$ ) (Ashley and Dow 1994, Jarne and Lagoda 1996). In the co-dominant inheritance of the microsatellites, alleles of one locus differ only in the number of the repeated motifs (Ashley and Dow 1994). Hence, variations at one gene locus are characterized as length polymorphisms. Due to their high mutation rate, high polymorphic microsatellite - loci can evolve, which can be even serve as determination of individuals (Halliburton 2004). Together with the neutrality towards selection, they are a very suitable markersystem for population genetic analyses.

Populations of introduced species are mostly small -sized in the beginning, thus the genetic drift plays an important role, influencing the molecular evolution intensively (Wright 1931, Page and Holmes 1998). The genetic drift describes the change of allele and genotype frequencies due to events of chance. Chance events lead to changes in the gene pool of the population, since alleles are either fixed or eliminated, which, in turn, causes a loss of genetic variability. Drastic cases of genetic drift are the bottleneck and founder effect; latter occurs in introduced or immigrated populations, which are separated from the native population. The bottleneck effect occurs after a population was minimized extremely through events like an extreme cold winter, an epidemic plague or extreme environmental pollution.

For histological analyses of the female reproduction organs, the tissues have to be fixed, embedded into paraffin, cut into thin cross or longitudinal sections and stained for light microscopy.

The organs are mostly fixed in Formaldehyde or SUSA (Sublimatsalt/ Mercury(II) chloride) (Heidenhain 1917). Since Formalin binds to enzymes, they are inhibited in their biological activity. Thus, the autolysis and putrefication of the tissue samples is stopped and the organ - structure is preserved. SUSA precipitates all proteins and thus also prevents the autolysis of the tissue by enzymes. This fixation solution

enters the tissues very rapidly. SUSA-fixed preparations yield very good staining-results.

To gain the required contrast for analysing under the microscope the tissues have to be stained with different dyes finally. A common used stain method in histology is the Masson - trichrome stain (Iron hematoxylin-azophloxin- Light Green SF yellowish stain), a three-colour staining method, which facilitates the differentiation of cytoplasm, collagen and cell nuclei in one section (Masson 1929, Goldner 1938).

## **AIM OF THE THESIS**

The presented research aims to elucidate and evaluate the genetic status and reproduction biology of the established alien species *Eriocheir sinensis* in the marine and freshwater ecosystems of Schleswig-Holstein after its introduction 100 years ago. Almost nothing is known about the reproduction biology of this species, e.g. reproduction in the Baltic Sea, duration of sperm storage or copulation frequency. The ecological differences between the North Sea and the Baltic Sea could have led to an independent and local establishment of genetically separated populations. The genetic variability of the introduced Chinese mitten crab is presumably influenced by founder effects but also by ongoing introductions. This thesis is divided in three main chapters, which deal with different aspects of reproduction biology and population genetics. In general, the methods presented here can work as basic approach for evaluation of other invasive aquatic alien species, which will probably establish in Northern Germany in the future.

## STUDY 1

### **First evidence of *Eriocheir sinensis* reproduction from Schleswig—Holstein, Northern Germany, western Baltic Sea**

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#### **Abstract**

*Eriocheir sinensis* (H. Milne-Edwards 1853) (Crustacea, Decapoda, Varunidae) invaded the Baltic Sea over 80 years ago, but there is still little known about mitten crab reproduction in this region. According to present knowledge, reproduction does not occur in the central, eastern and northern parts of the Baltic Sea due to the low salinity present. Sufficient salinity levels for reproduction of the Chinese mitten crab may exist in the western part and in the Kattegat, but published evidence of reproduction is still lacking from the region. Recently however a total of 62 ovigerous females were collected in the eastern part of the Kiel Canal (connecting the North Sea with the Baltic Sea) close to the Kiel Fjord as well as in the Kiel Fjord itself (Schleswig-Holstein, German Baltic Sea coast). The eggs from some of these females were hatched in the laboratory under 'Kiel Fjord salinity conditions' and reared to third zoeal stage. Furthermore, larvae were



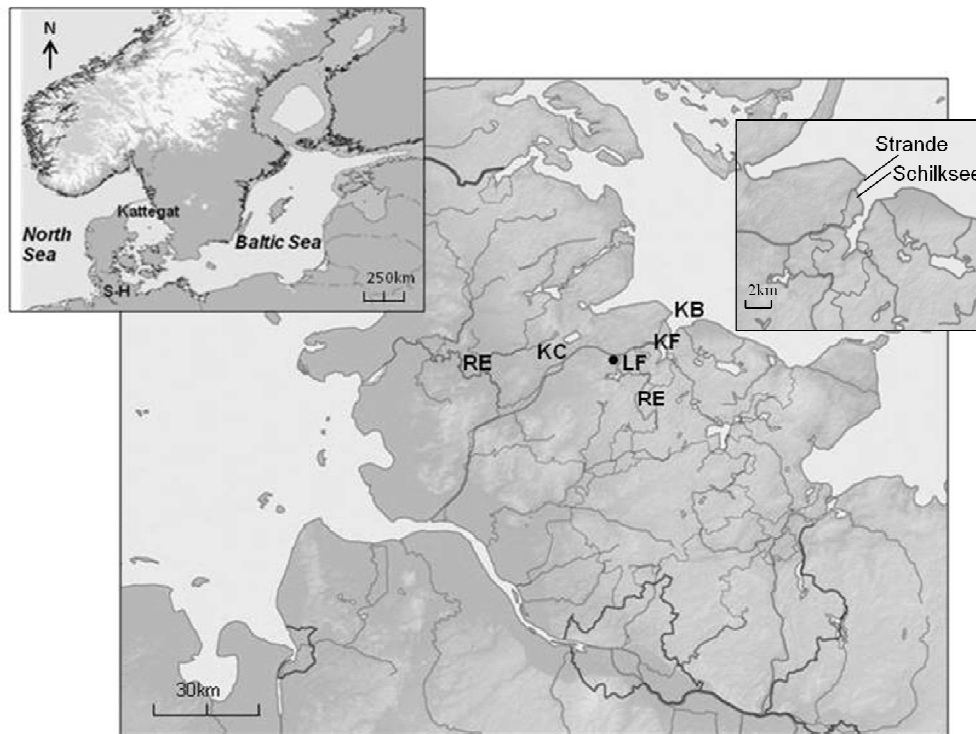
found in plankton samples from the Kiel Fjord and juvenile crabs were present in a lake close to this watershed.

**Key words:** invasive alien species; catadromous life cycle; Kiel Fjord; Kiel Canal; salinity

## Introduction

The native range of Chinese mitten crab *Eriocheir sinensis* reaches from the eastern pacific coast of China to the Korean Peninsula. The species was likely introduced to northern Germany in the early 20th century with ballast water, the first evidence of this invasive species was a male specimen found in the River Aller during 1912 (Peters and Panning 1933). In a relatively short period of time, the Chinese mitten crab established self-sustaining populations mainly in the Elbe and Weser river systems (Boettger 1933; Marquard 1926; Peters and Panning 1933; Peters 1938a). From there the species spread out across a large part of northern Europe including almost the entire coastal Baltic Sea and its adjacent rivers (Bacevičius and Gasiūnaitė 2008; Herborg et al. 2003; Normant et al. 2000; Ojaveer et al. 2007; Panning 1938; Panov et al 2003; Peters 1938a), where it is now a common by-catch, albeit in much lower numbers than in adjacent rivers of countries bordering the North Sea. The Chinese mitten crab has a catadromous life cycle and presently it is generally agreed, that the German population of *Eriocheir sinensis* can only reproduce successfully in the North Sea due to the appropriate salinities - between 15 close to the river mouths and 32 offshore (Cohen and Weinstein 2001 and references therein). Anger (1991) and later also Montú (1996) reared larvae from ovigerous *Eriocheir sinensis* females dredged in the southeastern North Sea and described 1 praezoea, 5 zoea stages and 1 megalopa. Anger (1991) showed that every stage had a different tolerance in terms of temperature and salinity. From the first until the last stage, the larvae become increasingly stenohaline with an optimum

in marine salinity. The survival rate of the larval stages was the highest at temperatures of  $\geq 12^{\circ}\text{C}$ , whereas the possible salinity range for a successful development extended at increasing temperatures. With decreasing temperature the tolerance to lower salinities also decreased and developmental time increased. In summary, the critical stages in the life cycle - embryonal and larval - require



**Figure 1.** The present study was conducted in Schleswig-Holstein (S-H), Northern Germany, at the Baltic Sea coast (Baltic Sea map by courtesy of R.Sommer, Ecology Center Kiel). In the map of Schleswig-Holstein and in the enlargement the places of *Eriocheir sinensis*-catches are shown. KB=Kiel Bight, KC=Kiel Canal, KF=Kiel Fjord, LF=Lake Flehmude, RE= River Eider.

relatively high salinity levels. In contrast, gonadal development, development of the megalopa, juveniles and adult growth occur in freshwater (Cohen and Weinstein 2001 and references therein). With regard to mitten crab reproduction in the Baltic Sea a number of studies have reported this as unlikely (Cohen and Weinstein 2001; Gollasch pers.comm. cited in Cohen and Weinstein 2001; Haathela 1963; Rasmussen 1987) as the salinity in

this sea ranges between 1–7 in the east and north and 9 - 20 in the western part (Rönnberg and Bonsdorff 2004). In contrast, salinity in the Kattegat (Figure 1) is about 12–30 at the surface and 32–34 at the bottom (Rönnberg and Bonsdorff 2004). However, reported numbers of collected male and female Chinese mitten crabs have increased from the northeastern part as well as the western part of the Baltic Sea during the last decade (e.g. Ojaveer et al. 2007 and references therein), but there have been no published data with regard to larvae and juveniles. The purpose of this study is to present the first evidence of *Eriocheir sinensis* successfully reproducing off the German Baltic Sea coast.

## Methods

The study area was the Kiel Fjord and the Kiel Bight in Schleswig-Holstein, Northern Germany (Figure 1). From April to August 2009 marine plankton sampling was vessel-based. Plankton nets of 25µm and 55µm were used for vertical net tows at different depths. The towage time was 5 minutes with a speed of 1 knot. In total, 110 samples were taken. In addition, a total of 40 plankton samples from the Institute for Marine Science, Kiel University (IFM-GEOMAR) were analysed. These were sampled weekly (spring and summer 2009) with a 25 µm-plankton net in the Kiel Fjord and in the eastern Kiel Canal. The plankton samples were fixed with 4% seawater-formaldehyde in 200ml containers. From September to December 2007 - 2009 Chinese mitten crabs were collected by a fisherman as by-catch in standard fish traps in the Kiel Canal close to the Fjord. From the end of April until the end of June 2009 fish traps set in the Kiel Fjord by IFM-GEOMAR were checked 1-2 time per week for crabs. These traps were set at the footbridge of the institute to catch fishes and crabs for the aquarium, for courses and public events. During the study period, a total of 62 ovigerous females were collected as by-catch in the Kiel Canal and 9 gravid females from the traps in the Kiel Fjord. The ovigerous female caught at the end of April 2009 has been deposited at the Zoological Museum Kiel under accession number ZMK

CR2911. The obtained gravid females in May and June were kept in aquaria in the laboratory of the Zoological Museum Kiel. The aquaria were filled with seawater from the Kiel Fjord with a salinity of about 14. The water temperature was kept between 7–11 °C to simulate the conditions found in the Kiel Fjord during spring and subsequently at 12–15 °C as during early summer. The process of hatching was observed and the larvae were photographed. After the larvae had hatched, an initial number of 70 specimens were transferred into 100ml glass vials with wide-bored pipettes. The rearing was conducted as described in the study of Anger (1991), but using the sea water of the Kiel Fjord (see above). The larvae were fed with freshly hatched *Artemia* sp. nauplii and the water was changed every day. In addition to marine sampling, juvenile crabs were collected at a lakeside ca. 11 km from the Kiel Fjord, connected with the Kiel Canal (Lake Flemhude, Figure 1) from spring until summer 2008-2009.

## Results

From the eggs of the ovigerous females collected in spring, 3 different larval stages were reared in the laboratory. On average, about 50% of larvae hatched moulted to zoea II, about one-third moulted to zoea III (Table 2). None of the zoea III survived. The eggs on females caught in early summer were mostly damaged or infected by fungi and none of the few hatched larvae survived. Seven zoea of *Eriocheir sinensis* were found in the plankton samples caught at two sample places in the Kiel Fjord where it merges into the Kiel Bight: 4 zoea were collected on 25 May 2009 at Strande and 3 zoea were found in a sample from Schilksee taken on 11 June 2009 (Figure 1, Table 1). The detailed morphological comparison with the reared larvae and the comparison with the study of Montú et al. (1996) confirmed, that the larvae caught in the Kiel Fjord were zoea II *Eriocheir sinensis* larvae. In search for juvenile crabs close to the Kiel Fjord, exuviae of juvenile crabs were found in June 2008 and May 2009 at the lakeside of Lake Flemhude (Table 1). All these findings represent the first registered records of ovigerous females and larvae in the Kiel Fjord and juveniles close to the Kiel Fjord. Together with the reared larvae in the laboratory the sampled specimens provide evidence that the full life cycle of

*Eriocheir sinensis* may take place in the eastern Kiel Canal and the Kiel Fjord and therefore suggest that *Eriocheir sinensis* is successfully able to reproduce in the western Baltic Sea.

## **Discussion**

The high numbers of ovigerous females in the eastern Kiel Canal during autumn provides evidence for a migration into the Kiel Fjord followed by mating. Furthermore, the periodical presence of ovigerous females in the Kiel Fjord during spring demonstrates active migration behaviour into the Kiel Fjord for hatching. The presence of larvae in plankton samples as well as the reared larvae in the laboratory appears to indicate successful hatching and development processes in spite of the low salinity of the Baltic Sea. Finally, the occurrence of small juvenile crabs in Lake Flehmude close to the Kiel Fjord in May and June provides further evidence to support the hypothesis for successful development of *Eriocheir sinensis* in the Kiel Fjord. Though larvae could be reared only until the third stage, all relevant findings and observations concurrently indicate that successful reproduction of the species in the western Baltic Sea is likely.

In general, salinity is the most limiting factor for a successful egg hatching and larval development of marine brachyuran crabs. According to Buhk (1938) a salinity of about 23 for hatching of *Eriocheir sinensis* larvae is required. Observations in these laboratory experiments included the hatching of eggs in Baltic Sea water with only 14–16 and furthermore a significant amount of prezoeas developing successfully to the zoea I stage. This contradicts the results of Buhk (1938) and shows that the possible salinity range for hatching appears to be larger than assumed so far. In contrast to the estuaries of the North Sea influenced by tidally fluctuating salinities, the Baltic Sea has quite constant salinity conditions. This can be an advantage even when the salinity is suboptimal. The laboratory experiments in Kiel Fjord seawater with salinity close to the lower limit (14–16) together with low temperatures ( $< 12^{\circ}\text{C}$  in the fjord during spring) may explain the high mortality rates of larvae and the lack of success at development beyond zoea III. Cieluch

et al. (2007) showed that the early larval stages of *Eriocheir sinensis* females dredged in the North Sea have a strong regulatory

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**Table 1:** Relevant data for the specimens sampled. Sex and Museum accession number could not be given for every specimen.

Stage	Location	Coordinates	Record dates	number	sex	Museum number	
Adult	Kiel Canal	54°20'36"N, 09°58'10"E	Sept.- Dec.2007- 2009	62	♀	—	
Adult	Kiel Inner Fjord	54°19'46"N, 10°08'58"E	April-June 2009	9	♀	ZMK 2911	CR
Larvae	Kiel Outer Fjord/Strande	54°26'20"N, 10°11'51"E  (starting point of towage)	May 2009	4	—	ZMK 4000	CR
Larvae	Kiel Outer Fjord/Schilksee	54°25'45"N, 10°11'19"E  (starting point of towage)	June 2009	3	—	ZMK 4001	CR
Juvenile	Lake Flemhude	54°20'26"N, 09°57'52"E	June 2008, May 2009	2	—	ZMK 2912, 2913	CR

**Table 2:** Relevant data for the larvae reared in the laboratory. The larvae were reared from ovigerous females caught in the Kiel Fjord in spring 2009. The eggs of the females caught in early summer were all infected by fungi.

Sampling date of ovigerous females	Initial Number of hatched larvae	Number of zoea1	Number of zoea2	Number of zoea3
29.4.2009	70	67	45	32
09.5.2009	70	64	39	24
09.5.2009	70	65	34	30
20.5.2009	70	61	33	22
22.5.2009	70	58	23	9
04.6.2009	—			
11.6.2009	—			
19.6.2009	—			



capacity. Hence, these stages are able to cope with the low salinities in estuaries, where the females are hatching (Cieluch et al. 2007; Rudnick et al. 2003). The subsequent larval stages, which are presumably transported to areas with higher salinities in the North Sea, keep the ability to hyper-osmoregulate, but on a lower level (Cieluch et al. 2007). Therefore they also could cope with low salinities but with a higher mortality rate. Ecological flexibility of *Eriocheir sinensis* larvae is further supported by findings of Anger (1991) and Montú (1996). Both observed that *Eriocheir sinensis* larvae exhibit a unique adaptation to produce an additional stage under unfavourable salinity conditions (zoea stage VI).

The present study suggests that the whole reproduction cycle of *Eriocheir sinensis* may take place in the western part of the Baltic Sea. However, against the background of the physiological investigations of Anger (1991) and Cieluch et al. (2007), these findings may indicate that *Eriocheir sinensis* reproduces in the western Baltic Sea, on an extreme physiological margin of the developmental range, resulting in subsequent high larval mortality.

### **Acknowledgements**

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Photos of reared larval stages are shown in the Annex.

## Study 2

### **Small-scale genetic structure and genetic variability of an established alien species, *Eriocheir sinensis*, in Northern Germany with a special focus on the Baltic Sea**

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## **Abstract**

The Chinese mitten crab was introduced to Europe about 100 years ago by ballast water and is an established alien species in Northern German fresh- and brackish water systems nowadays. The species is often caught in high numbers, especially in Schleswig-Holstein (part of Northern Germany), which is a serious problem for local fishermen. In Schleswig-Holstein, intensive international ship traffic at two coasts and in the Kiel Canal occurs. Due to this fact and due to the two ecological different seas, the brackish Baltic Sea and the North Sea with high salinity, Schleswig-Holstein is a very suitable area for a genetic study about such a successful alien species. Hardly any genetic data of Northern Germany especially of the Baltic Sea area have been available so far. To gain comprehensive information about the genetic status of the mitten crab 100 years after its introduction, sequences of the mitochondrial COI-gene (589 base pairs long) as well as 9 polymorphic nuclear microsatellite loci were analysed in 232 individuals. Nine COI haplotypes were identified. Both marker systems showed partly similar genetic variability compared to native populations and higher variability compared to other European populations, analysed in previous studies. Although our results indicate an intensive gene flow in Northern Germany,

one sampling site close to the Baltic Sea showed a private haplotype, private alleles and significant differentiation to several other sampling sites.

## Introduction

Nowadays, marine invasions are a central factor of global environmental changes, which can lead to serious ecological and economical losses (Nehring 2003). The main factors for anthropogenic introductions are ballast water, tank sediments, hull fouling, aquaculture and the construction of canals (Leppäkoski et al. 2002, Gollasch 2006). Schleswig-Holstein with its two coasts - the North Sea coast and the Baltic Sea coast - is strongly affected by intensive international ship traffic. The seas are connected through the Kiel Canal, which is one of the most ship - frequented canal in the world. Hence, Schleswig-Holstein is an area with a high potential of introductions of alien species and a particularly suitable area for studies about mechanisms of successful invasions.

The Chinese mitten crab *Eriocheir sinensis*, H. Milne-Edwards 1853 (Crustacea, Decapoda, Varunidae) was most likely introduced by ballast waters from the Pacific coast of China to Continental Europe. The first detection was a male in the German River Aller in 1912 (Peters and Panning 1933). In a relatively short period of time, *E. sinensis* could establish self-sustaining populations mainly in the Elbe and Weser riversystems (Peters and Panning 1933, Peters 1938a). Meanwhile, the species has spread out in Schleswig-Holstein and is also found at almost the entire Baltic Sea coast and in its adjacent rivers (e.g. Fladung 2000, Normant et al. 2000, Herborg et al. 2003, Panov et al. 2003, Ojaveer et al. 2007). The Chinese mitten crab is a catadromous species and reproduces in the North Sea (e.g. Anger 1991), but also in parts of the western Baltic Sea (Otto and Brandis 2011).

Populations of introduced species are often influenced by founder- and bottleneck effects. Hence, genetic variation is reduced in most cases, but also can be unchanged or even increased through the mixture of separated native gene pools in the invaded habitat as well as through multiple introductions (Lee 2002, Roman and Darling 2007, Brown and Stepien 2010). New combinations of genotypes may occur in the founder population through multiple introductions from originally separated populations (Hänfling 2007 and references therein). Hence, evolutionary change in

the new habitat can occur quite rapidly. This was shown for several alien species populations: the soapberry bug *Leptocoris tagalicus* (Carroll et al. 2005), the fruit fly *Drosophila subobscura* and salmon *Oncorhynchus spp* (Huey et al. 2005) have evolved genetically based changes within 30-40 years.

General studies about the population structure of Central European Chinese mitten crabs exist, but the situation especially in Schleswig-Holstein has been unknown so far. Northern German mitten crab populations have been only genetically analysed in the context of global overview studies. Hänfling et al. (2002) investigated the invasion history of the Chinese mitten crab in Europe and America using the mitochondrial COI gene. Herborg et al. (2007) analysed a selection of samples from several European countries to investigate population structure and migration among these invasive populations. However, Northern Germany, and Schleswig-Holstein respectively, has not been considered until now, although the mitten crab has been occurred in high numbers in this area since several decades. Regarding the genetic variability, no substantiated statement can be made, since the mitten crab populations in Northern Germany were exposed to massive repeated population decreases (e.g. Fladung 2000, Nehring and Leuchs 2000). Otherwise, repeated introductions of *E. sinensis* by ballast water are possible and plausible due to the intensive international ship traffic at both coasts of Schleswig-Holstein. In a study about aquatic invasive species in Europe, Gollasch et al. (2002) found adult mitten crabs in the sediment of ballast tanks. Above all, the possibility of an independent and local establishment of also genetically separated populations is increased due to the two ecologically very different seas, where the reproduction takes place.

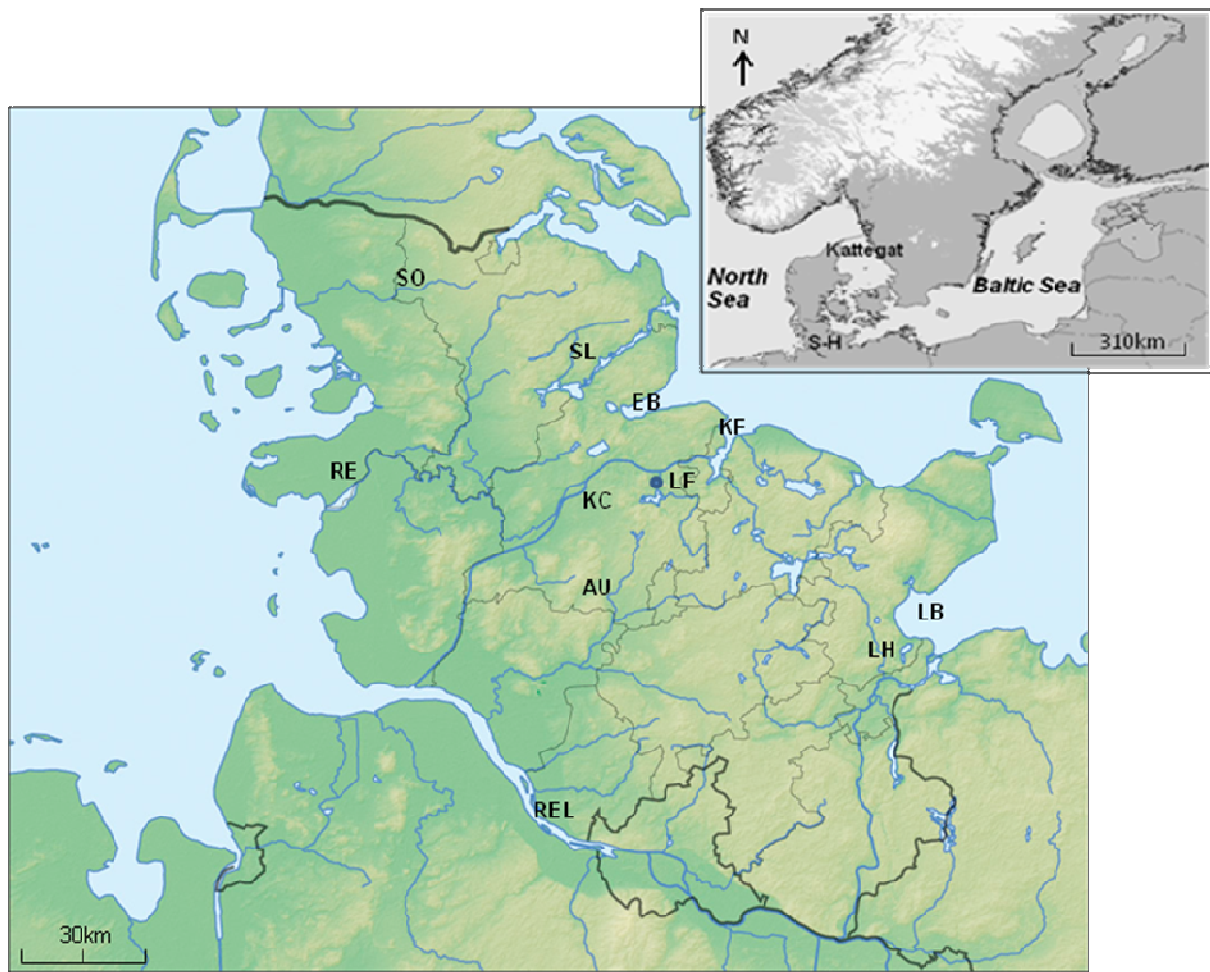
We used the mitochondrial COI-gene and 9 polymorphic microsatellites to gain comprehensive information about the genetic variability and structure of the Chinese mitten crab populations in Schleswig-Holstein. Except the here presented data, no other published genetic data from the German western Baltic Sea area with the adjacent rivers and lakes exist. Concerning the remaining Baltic Sea area, Czerniejewski et al. (2012) analysed mitten crabs in Sweden and Poland, which have not established self-sustainable populations yet.

The specific aims of this study are as follows: (1) to investigate the genetic variability on a regional level of a successful established alien aquatic species about 100 years after its introduction; (2) to compare the genetic variability of Schleswig-Holstein populations to native and other European populations analysed in previous studies; (3) to test for genetic structure, especially if differences between North Sea localities and Baltic Sea localities can be detected; (4) to provide genetic data from western Baltic Sea region, which has not been considered so far.

## **Materials and methods**

### Sampling

In the summer of 2008, 2009 and 2010, 232 mitten crabs were sampled in different localities (Figure 1, table 1) as by-catch in fish traps of local fishermen or were caught by hand by the authors. For all DNA-analyses, tissues of *Eriocheir sinensis* were stored in 99% ethanol or were frozen at -20° C.



**Figure 1:** Sample sites of *Eriocheir sinensis* and all other sites, mentioned in the text. Baltic Sea map by courtesy of Robert Sommer, Ecology Center Kiel. AU= Aukrug, E= Eckernförde, KC=Kiel Canal, KF=Kiel Fjord, LB = Luebeck Bight, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Soholmer Au. Maps: Baltic Sea map by courtesy of Robert Sommer, Ecology center Kiel; Schleswig-Holstein map: [https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein\\_relief\\_location\\_map.jpg](https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein_relief_location_map.jpg)

**Table 1:** Sampling sites of *Eriocheir sinensis* in Northern Germany with coordinates as far as available.

Sampling site	coordinates	Number of individuals
Schlei	-	11
Eckernförde	-	18
Lake Hemmelsdorf	53° 58'N, 10° 48'E	16
Lake Flemhude	54° 20'N, 09° 57'E	50
Soholmer Au	54° 42'N, 08° 52'E	36
Eider	54° 19'N, 09° 08'E	45
Aukrug	54° 05'N, 09° 49'E	16
Elbe	-	40
		<b><math>\Sigma = 232</math></b>

### Laboratory methods

#### *Mitochondrial DNA amplification and screening*

The extraction of total genomic DNA was conducted with the Qiagen DNeasy® Blood and Tissue Kit with the protocol for animal tissues. For the amplification of a fragment of the COI-gene the universal primers LCO1490 and HCO2198 were used (Folmer 1994):

LCO1490 (5'-GGTCAACAAATCATAAGATATTGG-3')

HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3').

A fragment of 589 base pairs (bp) was gained.

The PCR was conducted for each sample like follows: 7.5µl Buffer, 10µl dNTP, 0.75µl of each primer, 2.25µl MgCl, 0.15µl Taq-Polymerase, 2µl DNA-isolate and VE-water filled up to 37.5µl. Amplification was conducted with following temperatures and durations. Initial denaturation: 95°C for 5min, 40 cycles with 1'30min at 95°C, 1'15min at 53°C (annealing temperature according to Hänfling et al. 2002) and

1'30min at 72°C; afterwards a final step of 72°C for 10min. The PCR products were sequenced using the sequencer 3730 XL DNA Analyser, Applied Biosystems. Both strands were sequenced, when ambiguous sites occurred. Sequences were aligned by eye using the BioEdit Sequence Alignment Editor (Hall 1999).

#### *Microsatellite amplification and screening*

For microsatellite analysis 9 di-nucleotide microsatellites Esin06, Esin18, Esin26, Esin36, Esin38, Esin42, Esin55, Esin 67 and Esin 87 were used (extracted and characterized by Hänfling and Weetman (2003), table 2). PCR was conducted for each sample using 3.75µl MgCl-Buffer, 0.75µl dNTP, 1µl of each primer, 0.075µl Taq-Polymerase, 2µl DNA-isolate and VE-water filled up to 18.75µl. PCR amplification was conducted with the thermal profile used for mtDNA except of the annealing temperatures: 50°- 58°C (according to Hänfling and Weetman 2003). The genotyping was done with the capillary sequencer MegaBACE™ 1000. The size of the alleles was determined with the program MegaBACE™ Genetic Profiler ver. 2.2 from Amersham Biosciences.



**Table 2:** Characteristics of the 9 microsatellite loci analysed in *Eriocheir sinensis* (Hänfling and Weetman 2003).

Locus	Primer sequence (5' - 3')	Allele length	Annealing temp. (°C)
<b><i>Esin06</i></b>	F: CCCTTCCATTATCTTAACCTG R: CTGTGCTTCGTCTGTGTATG	107–181	58
<b><i>Esin18</i></b>	F: CACCGTAAGGTTCCGTAA R: AAGCACCCATAAGTCAATGTA	154–196	55
<b><i>Esin26</i></b>	F: AATGGAAAATGAATATGGAA R: ACCCTTGTTCTAACTTTACTCTT	149–259	52
<b><i>Esin36</i></b>	F: GAGCGAGTATGCAAATGAGTAAT R: TTCATTCACGAACAAAACACTAA	212–336	50
<b><i>Esin38</i></b>	F: CTCATCAGTGTTTATGCAACA R: TGGAAAACATTCAACTTATCAC	100–172	50
<b><i>Esin42</i></b>	F: GCACCGCAGTGATAATGTAGTGG R: GATCCTCGTGTGGGCGTGCTTAC	223–253	55
<b><i>Esin55</i></b>	F: GCAGCAGACGAAGAGGTG R: CCAGTTTCACTTAAATTAGCC	82–160	55
<b><i>Esin67</i></b>	F: TTTGGGATTACCTTGTCAACTT R: CGACGCACGACAGAGGAGAGG	139–165	50
<b><i>Esin87</i></b>	F: GCCATAAGGAGGGACAATGATG R: GCATGTAATGGGCAAGACTCTA	123–149	55

## Data analysis

### *Mitochondrial DNA*

The calculations for haplotype diversity and nucleotide diversity in the populations were conducted with the program DnaSP 4.0 (Librado and Rozas 2009). The differences between populations were given as net nucleotide diversities (DnaSP 4.0) and as  $\Phi_{st}$  – values calculated with the program Arlequin ver. 3.5 (Excoffier et al. 2009). Hierarchical AMOVA based on the distribution of the haplotypes in the populations was also conducted with Arlequin. The haplotypes were compared with the Kimura-2-Parameter-Method (Kimura 1980, Jin and Nei 1990; this model was determined for our data set with Findmodel, which is not implemented in Arlequin <http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>). A Median Joining Network on the basis of the pairwise differences using the program Network ver. 4.5.1.0 (Bandelt et al. 1999) was constructed to display haplotype relationships.

To obtain an evaluation of demographic expansion, we conducted a mismatch distribution analysis with Arlequin. The program computes the number of differences between pairs of haplotypes. With these results we constructed a histogram with the Microsoft Office Excel. The shape of the mismatch distribution graph allows conclusions about demographic history. A unimodal distribution (one peak) of the pairwise differences is typical for populations developed from one colonization event and confirms the sudden expansion model. This model is taken as null hypothesis and is tested by Arlequin. A deviation from this model is shown by further peaks of the graph (bimodal and multimodal distributions) as well as by significant values for the Harpending's Raggedness index (Harpending 1994) and the sum of the squared deviations. This is typical for populations, which have developed from several immigration events. Furthermore, also survived bottlenecks with a re-expansion afterwards can lead to a multimodal distribution. Furthermore, we conducted Tajima's test and Fu's  $F_s$ -test for selective neutrality (Tajima 1989a, b; Fu 1997) based on the infinite-site model without recombination to detect possible impact of selection or population change like an expansion. For both tests we used Arlequin. Tajima's test detects sequences which do not fit the neutral theory model at equilibrium. A negative Tajima's  $D$  indicates a population size expansion (e.g. after a bottleneck). When Tajima's  $D$  is positive, a decrease in population size and/or balancing selection

has occurred. We compared the listed confidence intervals in Tajima (1989a, table 2) to the calculated D-value to evaluate the significance. Fu's  $F_s$ -test calculates the probability of observing  $k$  or more alleles in a sample of a given size, which depends on the observed average number of pairwise differences (Fu 1997). A large, significant negative value of  $F_s$  rejects the neutrality of mutations and is indicative for recent expansions, whereas a large positive value is a hint for a bottleneck. Fu's  $F_s$ -test has proved to be especially sensitive to deviation from population equilibrium as it occurs in case of a population expansion (Fu 1997).

### *Microsatellites*

For further calculations with the microsatellite data, the occurrence of null alleles was checked first to avoid an underestimation of heterozygosity. This probability was calculated with the program Microchecker ver. 2.2.3 (Van Oostershout et al. 2004). Also large allele dropout and scoring errors as further reasons for a deficit of heterozygotes was checked with the program Micro-Checker. With the programs Arlequin and Genepop ver. 4.0.10 (Rousset 2008) all 9 loci were tested for pairwise linkage disequilibrium. Deviation from the Hardy-Weinberg-equilibrium was checked also with the program Arlequin. The observed and expected heterozygosity  $H_O$  and  $H_E$  of every locus and population was calculated with the program Genepop. With the program FSTAT ver. 2.9.3.2 (Goudet 1995), the allelic richness of every locus was calculated. This parameter is independent of sample size. In the calculation of the allelic richness all populations are minimized artificially to the lowest presented sample size so that values are comparable among different populations.

The differences between the populations, given as  $F_{ST}$  -Values, as well as an AMOVA were calculated with the program Arlequin. The program Structure 2.3 (Pritchard et al. 2000) using a Bayesian method was run to check for occurring substructures. The data set was tested for the presence of  $K = 1-8$  clusters without a priori information of the origin of individuals. Running conditions included a Burn-in of 100.000 and then 500.000 MCMC runs. For each  $K$ , the data set was analysed 10 times. Every replicate gives the logarithmic probability ( $\ln P(D) = L(K)$ ). The higher the value the more likely it is that this  $K$ -value represents the real number of subpopulations. Since Structure gives only simple likelihood values, we also used the ad hoc statistic  $\Delta K$  (Evanno et al. 2005) to have a better valuation of the true number

of the genetic clusters.  $\Delta K$  assumed to be the most likely  $K$  which has the highest value relatively to its neighbouring values. It is calculated as  $\Delta K = (|mL(K+1) - 2mL(K) + mL(K-1)|) / sdL(K)$ .  $L(K)$  is the natural logarithm of the probability that  $K$  is the correct number of clusters,  $m$  is the mean and  $sd$  is the standard deviation of the replicate runs for the same  $K$  value.

## **Results**

### **Mitochondrial DNA**

Sequences with 589 bp and 9 Haplotypes were achieved. The most frequent haplotypes occurred 64 and 61 times, respectively. The haplotypes differed from each other through 11 polymorphic sites, 7 transitions and 4 transversions.

The haplotypes are numbered serially from ES8 to ES16 according to the study of Hänfling et al. (2002), who have found seven haplotypes numbered serially from ES1 to ES7 (GenBank Accession numbers: AF435113 to 9). ES5 and ES1 in their study (GenBank Acc.nr: AF435113, AF435117) are identical with ES13 and ES14 respectively found in the present study, hence these haplotypes are also termed as ES 5 and ES1 respectively in the following. The seven remaining sequences were all submitted to GenBank, Accession numbers JQ956425 – 31.

**Table 3:** Distribution of the 9 found haplotypes across the 8 sampling sites. AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Soholmer Au, n=sample size. All sequences for the haplotypes except ES13 and ES14 have been submitted to GenBank (Accession numbers: JQ956425 – 31)

site	ES8	ES9	ES10	ES11	ES12	ES13/ES5	ES14 /ES1	ES15	ES16	n
LF			2		9	3	20	13	3	50
SO					10			12	14	36
SL			3		6				2	11
AU				2				14		16
E					5		13			18
RE		5			10		12	9	9	45
LH	6						10			16
REL				4		14	9	13		40
<b>Σ</b>	<b>6</b>	<b>5</b>	<b>5</b>	<b>6</b>	<b>40</b>	<b>17</b>	<b>64</b>	<b>61</b>	<b>28</b>	<b>232</b>

The most frequent haplotypes ES1, ES15 and ES12 (64, 61 and 40 individuals) occurred in 5 of 8 locations. Four haplotypes were found in 2 and 4 locations. In the River Eider and in the Lake Hemmelsdorf, 1 “private haplotype” was found.

The haplotype diversity (HD) in the 8 sampling sites lay in the range from 0.429-0.877 (table 4). The lowest diversity was found in Aukrug, the highest in the River Eider. The nucleotide diversities (ND) show a similar picture, the lowest value were found in Aukrug with 0.364% and the highest again in the Eider with 0.814%, see table 4. The mean value of HD over all populations was 0.7053; ND over all populations was 0.616%.

**Table 4:** Variability within the populations, given parameters based on mitochondrial DNA: haplotype diversity (HD), nucleotide diversity (ND in per cent), standard deviation in brackets. AU= Aukrug, E= Eckernfoerde, LF = Lake Flemhude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Soholmer Au

Population	HD	ND (%)
<b>SO</b>	0.784 (0.032)	0.633 (0.101)
<b>RE</b>	0.877 (0.033)	0.814 (0.110)
<b>SL</b>	0.697 (0.024)	0.622 (0.018)
<b>E</b>	0.688 (0.072)	0.577 (0.098)
<b>LF</b>	0.801 (0.054)	0.782 (0.112)
<b>LH</b>	0.545 (0.159)	0.489 (0.027)
<b>AU</b>	0.429 (0.166)	0.364 (0.032)
<b>REL</b>	0.821 (0.031)	0.651 (0.114)
<b>Ø</b>	0.7053	0.616

Pairwise  $\Phi_{st}$  -values (Distance-method Kimura 2P; table 5) ranged between 0.0092 (between Eckernfoerde and Lake Flemhude) and 0.6996 (between Lake Hemmelsdorf and Soholmer Au). A total of 19 of 28 pairwise differences were significant after Bonferroni-correction (Bonferroni-corrected significance-level  $p = 0.0018$ ), including the comparison Lake Hemmelsdorf and Soholmer Au. Pairwise net nucleotide diversities (table 5) show a similar picture with values between 0.0077

(Eckernförde and Lake Flehmude) and 0.3812 (Lake Hemmelsdorf and Soholmer Au).

**Table 5:** Pairwise values of net nucleotide diversity of the analysed populations (in %) above the diagonal. Pair wise  $\Phi_{st}$  -Values (Distance-method Kimura 2P) of the analysed populations below the diagonal. Both parameters based on mitochondrial DNA-data. Normal= not significant ( $p > 0.05$ ) before Bonferroni - Correction, **bold** and *italic*= significant after Bonferroni-Correction (Significance-Level  $p = 0.05$ , Bonferroni-corrected Significance-Level  $p = 0.0018$ ).

AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Soholmer Au

population	AU	E	REL	LH	LF	RE	SO	SL
AU	--	0.0983	0.0441	0.2285	0.0798	0.0399	0.0374	0.0944
E	<b>0.0779</b>	--	0.1018	0.1887	0.0077	0.0248	0.0447	0.0081
REL	<b>0.0253</b>	0.0941	--	0.1912	0.0804	0.0253	0.0199	0.1147
LH	<b>0.2116</b>	0.1852	<b>0.1902</b>	--	0.2924	0.2541	0.3812	0.2994
LF	0.0733	<b>0.0092</b>	0.0547	0.0971	--	0.0062	0.1173	0.0996
RE	<b>0.0475</b>	0.0118	0.0175	<b>0.5042</b>	<b>0.0054</b>	--	0.0035	0.1784
SO	<b>0.0119</b>	<b>0.0183</b>	<b>0.0218</b>	<b>0.6996</b>	0.0975	<b>0.0047</b>	--	0.1544
SL	<b>0.3084</b>	<b>0.0994</b>	0.0345	<b>0.3735</b>	<b>0.2021</b>	<b>0.1943</b>	<b>0.2798</b>	--

The AMOVA for all eight populations showed that 19.45% ( $p = 0.0014$ ) of the total variance lay on the level among populations and thus 80.55% ( $p = 0.0077$ ) on the level within the populations (Fixation-Index  $\Phi_{st} = 0.19452$ ).

The analysis of the Mismatch distribution led to a distribution of the frequencies of pairwise differences with a high maximum at two differences followed by a peak a little smaller at three differences and a further, very small peak at 5 differences (not shown). The sum of squared deviations (SSD) is 0.056 ( $p$ -value: 0.1000). The *Raggedness*-Index according to Harpending (1994) has a value of 0.20187 ( $p$ -value: 0.0000). The calculations of Tajima's  $D$  and Fu's  $F_s$  yielded negative and significant values (Tajima's  $D$  -0.35580,  $p$  ( $D_{simul} < D_{obs}$ ): 0.0424; Fu's  $F_s$  -6.46729  $p$  ( $sim\_F_s \leq obs\_F_s$ ): 0.02000). The Median Joining Network was constructed with the 9 haplotypes found in Northern Germany (ES8-ES16) and with the 7 haplotypes found in the study of Hänfling et al. (2002). The Median Joining Network (not shown) based

on the differences between sequences, illustrates their close relationship. Two and three mutations occurred most frequently (32 and 31 times). Haplotype ES16 and ES3 (GenBank Acc.nr: JQ956431, AF 435115) were separated by 8 mutations.

### Microsatellites

In no sample null alleles, scoring errors and large allele dropout were found by Microchecker. Furthermore no significant linkage disequilibrium of any two loci was found with the programs Arlequin and Genepop after Bonferroni-correction. All 9 microsatellites used in this study were polymorphic, the number of alleles ranged between 11 (Esin87) and 32 (Esin38), see table 6.

**Table 6:** Characterization of the 9 Microsatellite loci. bp: base pairs, A: number of different alleles.

<b>Microsatellite</b>	<b>Allele length [bp]</b>	<b>A</b>
Esin06	107–181	25
Esin18	154–196	22
Esin26	149–259	24
Esin36	212–336	33
Esin38	100–172	21
Esin42	223–253	17
Esin55	82–160	25
Esin67	139–165	14
Esin87	123–149	11

To evaluate the variability within populations,  $H_O$ ,  $H_E$  and allelic richness (AR) were calculated. The mean value over all loci of the observed heterozygosity ( $H_O$ ) lay between 0.6377 (Aukrug) and 0.8154 (Lake Flehmude), on average 0.75533 over all populations. The mean value of the expected heterozygosity ( $H_E$ ) showed values between 0.7528 (Schlei) and 0.8591 (River Eider). The mean value was 0.8237 over all populations. The mean value of the allelic richness lay between 7.7213 (Aukrug)



and 10.6206 (River Eider), the average value over all 8 populations was 9.2575. In Lake Hemmelsdorf 3 private alleles were detected (table 7).

**Table7:** Mean values of  $H_O$  (observed heterozygosity),  $H_E$  (expected heterozygosity),  $AR$  (allelic richness), mean number of alleles ( $A$ ) and number of private alleles ( $PA$ ) of the 9 analysed microsatellites for every population analysed in Northern Germany. AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Sohlmer Au.

Population	$\bar{H}_O$	$\bar{H}_E$	$\bar{AR}$	$\bar{A}$	PA
SO	0.80671	0.82265	9.228	9.11	-
SL	0.6612	0.7528	8.1806	10	-
E	0.7153	0.8384	9.6347	10.11	-
LF	0.8154	0.8504	10.065	14.5	-
AU	0.6377	0.7982	7.7213	6	-
RE	0.8042	0.8591	10.6206	10.5	-
LH	0.78105	0.8223	8.3796	8.22	3
REL	0.8075	0.8455	10.2298	13	-

**$\bar{H}$  over all**

**populations      0.75533   0.8237   9.2575   10.18**

The calculation of pairwise  $F_{ST}$  showed values between 0.0022 (Eckernförde and Lake Flehmude) and 0.0544 (Lake Hemmelsdorf and River Eider). A total of 16 out of 28 pairwise comparisons were significant after Bonferroni-correction, including the latter one (table 8). For AMOVA among all the populations based on microsatellite data no significant values could be calculated ( $p > 0.05$ ).

**Table 8:** Pairwise  $F_{ST}$  values for *Eriocheir sinensis* sample sites. Significant  $F_{ST}$  -values after Bonferroni-Correction (Bonferroni-corrected Significance-Level  $p = 0,0018$ ) in bold and italic letters.

AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, Soholmer Au

population	AU	E	REL	LH	LF	RE	SO	SL
AU	--							
E	<b><i>0.0241</i></b>	--						
REL	0.0099	<b><i>0.0407</i></b>	--					
LH	<b><i>0.0325</i></b>	<b><i>0.0467</i></b>	0.0361	--				
LF	0.0072	<b><i>0.0022</i></b>	0.0208	0.0442	--			
RE	<b><i>0.0203</i></b>	0.0087	0.0093	<b><i>0.0508</i></b>	0.0023	--		
SO	<b><i>0.0198</i></b>	0.0191	<b><i>0.0104</i></b>	<b><i>0.0544</i></b>	0.0199	<b><i>0.0026</i></b>	--	
SL	<b><i>0.0344</i></b>	<b><i>0.0204</i></b>	<b><i>0.0349</i></b>	<b><i>0.0481</i></b>	<b><i>0.0218</i></b>	0.0322	0.0404	--

The structure analysis yielded no hints for any substructure. All individuals of the 8 sample locations showed the same probability to belong to the assumed clusters. The same result was obtained for higher K values (equal proportion of ancestry of all individuals from all K clusters). Furthermore, none of the  $\Delta K$ -values showed a clear peak ( $\Delta K$  from 0.03 - 1.95, but this statistic is not applicable to  $K = 1$ ) and  $K=1$  had the lowest standard deviation of 0.22. Thus, the individuals most likely seem to belong to one cluster.

## Discussion

Using mtDNA as molecular marker, mitochondrial pseudogenes should be shortly discussed. These mtDNA fragments, localized in the nucleus, are the relatives of known genes but are not able to code proteins any more (Vanin 1985). They were detected in several species, also in *Eriocheir sinensis* (Tang et al. 2003, GenBank Acc.Nr. AF317334, see Buhay 2009 and references therein) and are mainly problematic for phylogenetic and DNA Barcoding studies due to possible overestimation of diversity of species and wrong taxonomic classification. The Numts (nuclear mitochondrial pseudogenes) often typically show indels, double peaks in

forward as well as in reverse sequence and/or stop codons (TAA,TAG). Since none of these features have been detected and furthermore no taxonomic analyses were conducted, the pseudogene-problem is secondary for this study. Furthermore, microsatellites were used to gain a comprehensive picture and hedge against such mitochondrial phenomena.

The chosen 9 microsatellite loci with 192 different alleles coincide with the detection of 204 Alleles in the study of Hänfling and Weetman (2003), who found these microsatellite loci. Thus, the here used loci have the potential to detect variation within population and differentiation between populations.

#### Variability within populations

Based on mtDNA data, the Schleswig-Holstein - populations showed a similar high variability compared to the Chinese populations and a higher variability compared to European populations, analysed by Hänfling et al. (2002; Chinese populations:  $H_D = 0.733-0.778$ ; European populations:  $H_D = 0.00-0.725$ ). These authors found a total of 6 haplotypes in the populations Tagus, Thames, Weser, Elbe and Lake Laascher See, which was surprising yet. In the present study, even 9 haplotypes only in the Northern part of Germany were found. However, in this comparison the different sample sizes must be considered, which were only about half as high as in our study.

To compare the results of our microsatellite analyses with the study of Herborg et al. (2007) and Sui et al. (2009), we calculated the mean values of  $H_O$ ,  $H_E$  and AR of the 4 microsatellite loci which were used in all studies (Esin 42, Esin 55, Esin67 and Esin87). The allelic richness based on a similar smallest sample size of 11, 14 and 18 respectively (see methods), thus the studies can be compared to each other. In the study of Sui et al. (2009), 352 individuals from 9 locations were analysed. Compared to these Chinese populations, similar  $H_E$  was found in our study,  $H_O$  was lower in most cases and allelic richness was mostly lower in our sampling locations. In the study of Herborg et al. (2007), 410 adult individuals from 7 populations all over Europe were analysed compared to one Chinese population, Liaohoe.  $H_E$  and  $H_O$  of almost all populations in our study were higher than in those analysed European

populations. River Eider showed the highest AR in Europe, closely followed by River Elbe. Tagus has the lowest AR of all analysed European populations.

A possible explanation for the relatively high values found in several of our sample locations might be ongoing introductions through the international ship traffic in the River Elbe as well as in the Kiel Canal during the last decades. Also the 7 haplotypes not reported before, can be a hint for that. Multiple introductions as possible reason for high genetic variability in invasive species were also analysed in the study with *Cyclope neritea* (Bachelet et al. 2004), for example. If introduced specimens derive from genetically differentiated populations, genetic variability in the founder population is equal or even higher than in native ones. Together with the intensive gene flow, which is caused by the migration behaviour and pelagic zoea larvae, genetic diversity can be increased. Some other studies found a similar or even higher genetic variability in introduced species compared to native populations, too; e.g. quagga mussels, neogobiin fishes and zebra mussels (Stepien et al. 2005, Stepien and Tumeo 2006, Brown and Stepien 2010).

The amount of genetic variation seems to be of particular importance for invasion success, since this is the basic material for adaptation to the new environment with similar or different conditions compared to the native habitat (Bossdorf et al. 2005). But also low genetic variability seems to be no disadvantage for a successful invasion, which was detected in the study of Tepolt et al. 2009, who analysed the successful invasive crab *Carcinus maenas* in western North America. Several parameters like pre-adaptation, broad ecological tolerances, free ecological niches, no enemies and effective life cycles also seem to play an important role for a successful establishment and expansion (Sax and Brown 2000 and references therein, Tepolt et al. 2009).

### Differentiation among populations

The low values in the pairwise comparisons of the populations as well as no hints for a clear substructure indicate that a homogeneous population exists in Schleswig-Holstein. Regarding the 8 analysed Schleswig-Holstein-populations as one homogenous population, a similar and higher genetic variability, respectively, in comparison to Chinese and European populations was confirmed (mean HD over all

populations 0.7053, see table 4). Concerning the microsatellite data, also a similar  $H_0$  compared to Chinese populations occurred in this case. According to the intensive gene flow in Schleswig-Holstein, the definition “populations” have rather the meaning of sample locations in this case.

The low differences between populations are consistent with the migration behaviour of the mitten crab and were also detected for native populations located north of the River Nanlijiang in China by Sui et al (2009). The study of Hänfling et al. (2002) indicated a high level of gene flow among mitten crab populations within Europe across drainages. Due to the life cycle - frequently long distance migration downstream for reproduction, pelagic larval stages and often long upstream migration of juveniles - the populations of different areas often admix. Thus, it is somewhat surprising that the Lake Hemmelsdorf and River Eider showed a private haplotype and Lake Hemmelsdorf additionally private alleles. Of course, this could be an artifact of sample sizes, hence more crabs from the other sample sites have to be investigated in the future. But perhaps they are a hint for recently introduced specimens from Asia by the international ship traffic at Northern German coasts. In the case of Lake Hemmelsdorf, the partly significant values in the pairwise comparisons also argue for a low gene exchange with this sample site. One explanation could be the geographical layer of this lake (see figure 1). Exchange perhaps rather occurs with populations, which inhabit waters more in the southwest of the Baltic Sea area. Since the lake is directly connected to the Baltic Sea, adult crabs most likely migrate exclusively into this Sea to reproduce. Perhaps most larvae remain in Luebeck Bight and develop to megalopae and the juveniles finally migrate into the Lake Hemmelsdorf. These genetic data could be a hint for a beginning development of a local separated population, which have to be investigated after some more generations with more individuals. In this context, specimens from Mecklenburg-Vorpommern and also Poland have to be included in the comparisons.

### Demographic history

The calculation of Tajima's  $D$  and Fu's  $F_s$  argues for a sudden expansion of the mitten crab population in Northern Germany. The result of the Mismatch distribution analysis is not as easy to interpret. At first view, the three peaks – two clear and one small- seem to support the hypothesis of multiple, perhaps ongoing introductions: a bimodal or multimodal distribution is typical of this scenario. Furthermore, also a bottleneck can cause more peaks. It is known that the mitten crab population decreased heavily in the 1940s but overcame this decline and recovered after some decades (Fladung 2000). Probably both, bottleneck and multiple introductions, have contributed to this mismatch distribution. Also the deviation from the simulated distribution argues for an aberrance to the sudden expansion model. However, only the Raggedness Index is significant; hence this conclusion must be judged with caution. Soon after the introduction of the mitten crab in the 1920s, a population explosion occurred, thus rather a clear confirmation of the sudden expansion model would be expected. An explanation for this unclear result could be the different factors in the invasion history of the Chinese mitten crab that occurred in a relatively short period of time: population explosion soon after introduction, then a massive population decline, after that again increasing population size in combination with ongoing introductions.

### **Conclusions**

This study shows that small scale analysis is important to get a detailed picture of the population structure and population dynamics of a successful invaded species. The genetic analyses implied that the interplay of parameters, like specific amount of genetic variability, gene flow, and multiple introductions plays an important role for invasive species to establish successfully. Due to the intensive ship traffic in combination with a highly frequented canal, Northern Germany must be prepared for aquatic invasions also in the future. Furthermore, Northern Germany probably plays an important role as a source for further expansion and introductions into other regions of the Baltic Sea. Combining the genetic data of this study with further genetic data of *Eriocheir sinensis* inhabiting the Baltic Sea area, detection of invasion

pathways as well as a risk analysis of further expansions of this notorious invasive species will be possible. Thus, in turn, is important for control efforts and management implications also for other invasive aquatic species.

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The network, the table with all  $H_O$ ,  $H_E$  and allelic richness-values and the bar graph of structure analyses are shown in the Annex.

### Study 3

#### **Reproduction biology of the alien Chinese mitten crab *Eriocheir sinensis* in Northern Germany with special focus on the Baltic Sea**

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#### **Abstract**

The Chinese mitten crab *Eriocheir sinensis* (H. Milne-Edwards 1853) is a common species especially in Northern German limnetic and marine water systems and often occurs in high densities nowadays.

About 90 years ago, this alien species has invaded the Baltic Sea and although salinity is not optimal for reproduction, ovigerous and juvenile crabs are collected regularly at the western Baltic Sea coast. Reproduction cycle and structure of female reproduction system are well known in principle, but only little is known about some aspects concerning copulation frequency and sperm storage, especially outside the native range of the mitten crab in the Baltic Sea. The histological analysis of the spermathecae of adult crabs collected before reproduction season evidences that spermatozoa can be stored over longer time and that females do not inevitably die after one mating season as it was generally assumed so far. Furthermore, the analyses showed that female juvenile crabs at the Baltic Sea coast are able to copulate before sexually maturity. Ovigerous females mating at the Baltic Sea coast had spermatophores in the spermatheca, hence they have copulated once again. These features were not found in any analyzed North Sea ovigerous female in this study.



## Introduction

The Chinese mitten crab *Eriocheir sinensis* (H. Milne-Edwards 1853) (Crustacea, Decapoda, Varunidae) originally inhabits the eastern pacific coast of China and the Korean Peninsula. The species was likely introduced to Northern Germany about 100 years ago with ballast water. Since the first observation in 1912, the mitten crab has spread very fast in Northern Germany. The first specimen in the Baltic Sea was caught in 1927. In the following decades the species showed a massive population increase mainly in the Elbe- and Wesersystem. After a decrease in the mid of the 20th century, the species occurs again in high densities especially in Northern Germany nowadays. Meanwhile, *Eriocheir sinensis* has been collected also along the almost entire Baltic Sea coast but in much lower numbers than in the North Sea and its adjacent rivers (for example: Normant et al. 2000, Panov et al. 2003, Ojaveer et al. 2007, Bacevičius and Gasiūnaitė 2008).

*Eriocheir sinensis* has a catadromous life cycle and spends most of its life in freshwater. Migration for reproduction starts in late summer and mating takes place until beginning of winter in the estuaries of the North Sea and Baltic Sea (Peters and Panning 1933, Panning 1938, Anger 1991, Otto and Brandis 2011). In Europe, the Chinese mitten crab becomes sexually mature with an average age of about 4-6 years, in San Francisco bay with 2-4 years and in China with 1–2 years (Panning 1938, Ingle 1986, Anger 1991, Fladung 2000, Jin et al. 2002, Rudnick et al. 2003, Dittel and Epifiano 2009 and references therein). During the downstream migration to the estuaries, the gonads begin to develop and are finally matured at a salinity of minimum 6ppt (Peters 1938b). Mating and oviposition take place in brackish waters of the estuaries finally. *Eriocheir sinensis* as well as the closely related *Eriocheir japonica* conducts a hard-shell-mating that means both sexes have finished moulting and thus have a hard carapace when they are copulating (Peters 1938b, Kobayashi and Matsuura 1999). Females can produce 1-3 batches in one reproduction season each with up to 1.000.000 eggs (Peters 1938b, Herborg et al. 2006). The batches are carried on the abdomen affixed to the setae of the pleopods. It is generally assumed, that mitten crabs die after one mating season (Panning 1939, Hymanson 1999, Zhao

1999, Jin et al. 2002). In Northern Germany, Peters (1938b) presumed an upstream migration of a little percentage of adult crabs in spring after hatching of larvae due to laboratory experiments, but it is not known if they survive and copulate once again in the next reproduction season.

The reproductive system of *Eriocheir sinensis* females consists of ovary, spermatheca, oviduct, vagina and vulva; the spermatheca opens in the vagina and the oviduct in turn opens into the spermatheca (Hartnoll 1968, Diesel 1991, Lee and Yamazaki 1990). During copulation, spermatozoa are packed into spermatophores, and transported into the spermatheca where they are retained or resolved into single spermatozoa and small accumulations of spermatozoa (Beninger 1988, Lee and Yamazaki 1990). Morphology and function of the female spermatheca of *E. sinensis* was studied by Lee and Yamazaki (1990). According to these authors, the spermatheca has an ovoid structure and its wall consists of a single layer of a crumpled columnar epithelium. Lee and Yamazaki described (1990) a special tissue at the mouth of the oviduct leading into the spermatheca. According to the authors, the so-called valve-like tissue makes sure that unfertilized ripe eggs stay in the ovary and prevents that spermatozoa reach the oviduct during egg-laying. The authors documented the egg-laying and fertilization process in detail. The female maturity of the congeneric species *Eriocheir japonica* was described by Kobayashi (2003) as followed: the juvenile phase with immature carapace is followed by the puberty moult to the adult phase. In the juvenile phase, previtellogenesis and primary vitellogenesis in the ovary take place. The secondary vitellogenesis starts after puberty moult and the oocytes are growing. This development is more or less general for brachyuran crabs (Kobayashi 2003 and references therein). For female *Eriocheir japonica*, Kobayashi supposed that copulation is only possible when the vitellogenesis is fully completed (Kobayashi 2003).

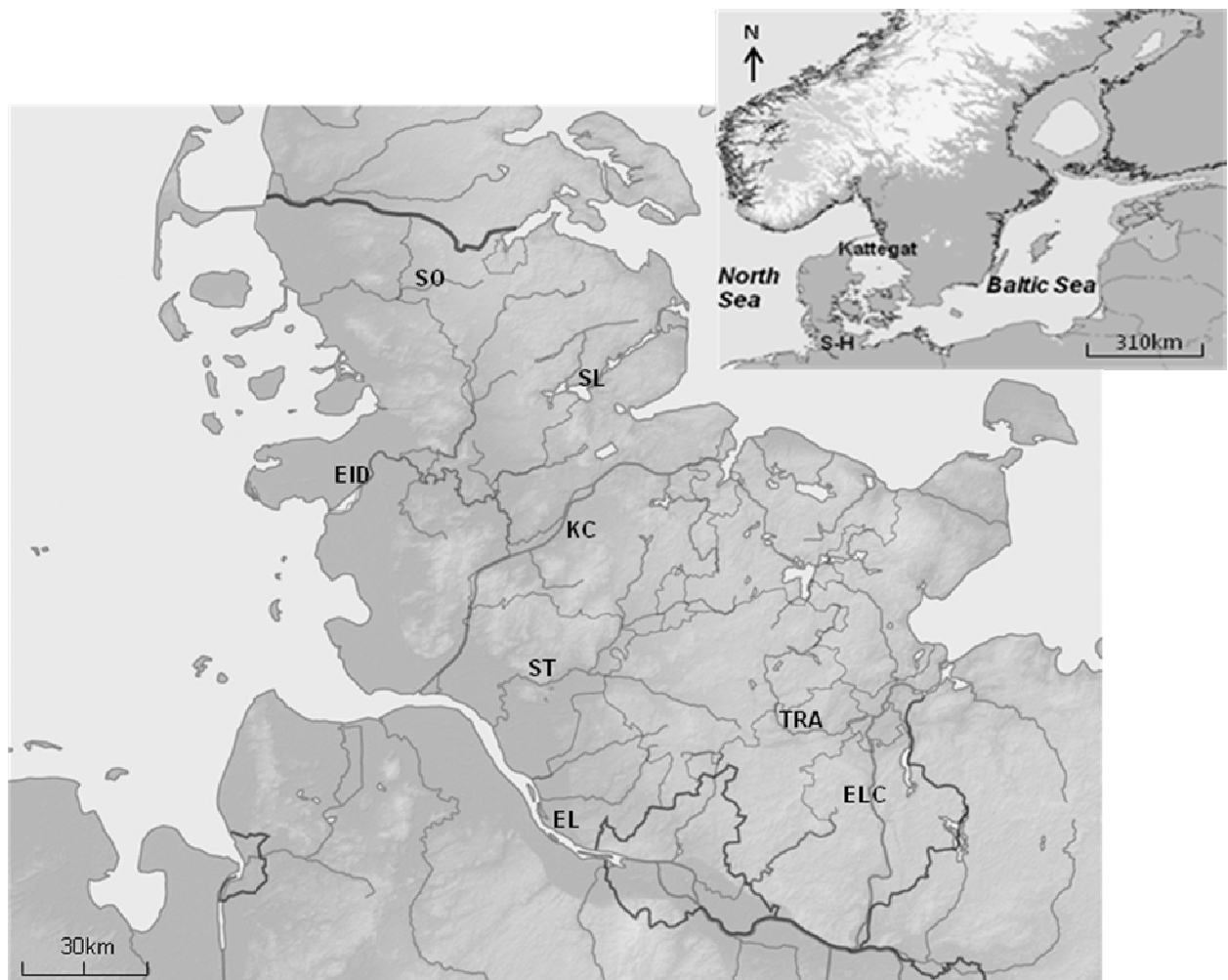
Whereas the general reproductive behaviour of the Chinese mitten crab is well known, information about the situation in the Baltic Sea is still lacking (Anger 1991, Jin et al. 2002, Herborg et al. 2006). The Baltic Sea shows very different ecological conditions than other marine waters inhabited by the mitten crab especially concerning the low salinity values. Otto and Brandis (2011) showed for the first time, that *E. sinensis* can obviously breed successfully in the brackish Baltic Sea at least in the western part besides a high mortality rate during the larval development.

The main aims of the project presented were as followed (1) to study the features in the reproductive system of juvenile females, non-migrating females, migrating and egg-bearing females in Northern Germany; (2) to investigate, whether the populations reproducing in the western Baltic Sea show special adaptations or differences compared to the North Sea populations.

## **Methods**

The sampling and analyses were conducted from July 2008 until December 2010 in the Zoological Museum Kiel and are part of a study to evaluate the population structure and dynamics of this alien species in Northern Germany.

40 ovigerous and 35 juvenile *Eriocheir sinensis* females from the eastern Kiel Canal close to the Kiel Fjord and 40 ovigerous females from the River Eider and Treene close to the North Sea (Figure 1) were collected in the migration time in September-December 2008, 2009 and 2010. These females were caught as by-catch in fish traps by local fishermen. In summer 2008 and 2009, 19 adult females were collected in the Lake Westensee (located close to the Baltic Sea; figure 1) as by-catch and 11 adult females in the Soholmer Au (close to the North Sea; figure 1). These females were collected from the authors partly with dip nets in the flat regions of the riverbank.



**Figure 1:** Schleswig-Holstein, Northern Germany with the sampling places. KC= Kiel Canal, KF= Kiel Fjord, LW= Lake Westensee, RE= River Eider, RT= River Treene. SO=Soholmer Au. Maps: Baltic Sea map by courtesy of Robert Sommer, Ecology center Kiel; Schleswig-Holstein map: [https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein\\_relief\\_location\\_map.jpg](https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein_relief_location_map.jpg)

The juvenile and adult female crabs were distinguished by the shape of the abdomen according to Jin et al. (2002) and Kobayashi and Matsuura (1992): The juvenile females are characterized by an almost triangular or slightly round abdomen with sparse setae (Fig 2). Adult female crabs have a round abdomen with dense setae and the abdomen covers almost the complete thoracic sternum (Fig 2).



**Figure 2:** Adult and juvenile female of *Eriocheir sinensis*

The specimen from 2008 and 2009 were fixed in 15% Formol-Sea/Lakewater and after minimum 4 days transferred into 75% Ethanol. Then the female reproductive system was resected. The females from 2010 were anesthetized with chloroform and dissected immediately after collection. The reproductive organs were fixed in SUSA (Sublimatsalt/ Mercury(II) chloride  $\text{HgCl}_2$ ) (Heidenhain 1917) and then transferred directly into 90% Ethanol. Before embedding, preparations had to be dehydrated (Romeis 1989). This was carried out in an ascending Ethanol sequence - 80%, 90%, 96% and 99% in the case of the Formol-fixed samples, the SUSA-fixed specimen were put into a sequence starting with 96% Ethanol. The samples remained in every solution for 2 x 30 minutes. After that procedure, the tissue was completely dehydrated and preparations were transferred into a lipophilic intermedium (Romeis 1989). Therefore, Methyl benzoate was used. For a complete exchange of ethanol with Methyl benzoate in the tissue and cells, preparations were transferred twice in fresh Methyl benzoate. The next day, the samples were transferred into paraffin and then finally embedded in paraffin. The embedment was conducted with the machine W2110, PSI Ltd., Basel/Switzerland.

Sections of 12 to 16  $\mu\text{m}$  (knife grade d, company Jung, Heidelberg) were produced with rotation microtome Biocut 1130 (Reichert-Jung, Heidelberg). The sections were dried at ca. 50 °C over night in a thermo cubicle (Memmert GmbH, Schwabach) and then stained with Masson-Trichrome stain (Romeis 1989, see table 1). Before

staining, the paraffin has to be removed from the histological sections with orange terpene, isopropyl alcohol and ethanol solutions with different concentrations. To remove sublimate precipitates, the samples have to lie in Lugol's solution and thereafter in sodium thiosulfate 0.25 %. Finally, the samples were transferred into distilled water.

After this preparing procedure, the Masson-Trichrome stain was conducted, solutions and durations are shown in table 1.

**Table 1:** Masson-Trichrome stain protocol, stain steps with duration. (after Romeis 1989).

<b>solution</b>	<b>duration</b>
Iron hematoxylin	1 x 2 min
Tap water	1 x 10 min
Ponceau-Acidfuchsin-Azophloxin	1 x 4 min
Acetic acid 1 %	1 x shortly
Phosphomolybdic acid -Orange-G	1 x 6 min
Acetic acid 1 %	1 x 5 min
Light Green	1 x 5 min
Acetic acid 1 %	1 x shortly
Isopropanol 99.9 %	1 x 5 min
Orange terpene	2 x 10 min

The histological sections were analysed with a light microscope Leica DM RBE and photographed using a Tucsen TCA 5 MP Digital camera.

## Results

### Migration observations

During migration time in autumn, a number of juvenile crabs with carapace width from 22.6 – 36.7mm was observed in the eastern Kiel Canal close to the Kiel Fjord. In 3 years of sampling, a total of 35 migrating juveniles were collected in the eastern Kiel Canal close to the Kiel Fjord (Specimen deposited in the Zoological Museum Kiel).

### Histology observations

#### *General morphology of the spermatheca*

The spermatheca of the adult ovigerous *Eriocheir sinensis* females has a sack-like structure and is about 17mm high and 7mm wide. The lumen of the spermatheca contains masses of free spermatozoa, which are often accumulated (Figure 3D). A seminal fluid staining green fills the spermatheca and surrounds the spermatozoa. Spermatozoa are stained pale purple/brown. The spermatheca consists of two regions with different types of epithelia: the dorsal region with a line of folded simple glandular epithelium, surrounding almost 2/3 of the spermatheca, inter alia at the level of the ovary mouth (Figure 3 schematic draw). In higher magnification, this epithelium is characterized by strongly elongated large bipolar cells, which are covered by microvilli (Figure 3A). The nuclei vary in their size, but are all located basally (Figure 3A). In the ventral region, the spermathecal wall consists of a single layered epithelium with flat cells lined by cuticle (Figure 3B, C).

The vagina consists of an epithelium formed by columnar cells and lined with a cuticle. The vagina belongs to the concave type according to Hartnoll (1968): one side of the vaginal wall is concave and invaginates into the opposite wall; muscles are attached exclusively at the invaginated wall running to the sternal cuticle. This flexible part of the cuticle of the inner vagina wall is stained orange-red, the unflexible part of cuticle at the outer vagina wall has a turquoise colour (sensu Diesel 1989) (Figure 3I).

The valve-like tissue, described in the introduction is characterized by a red-brownish staining and black oval nuclei and extends into the lumen of the spermatheca (Figure

3 schematic draw, Figure 3G). No clear membranes in the magnification are visible as it was also described by Lee and Yamazaki (1990). Towards the lumen of the spermatheca, orange-red stained secretions are visible attached directly to the valve-like tissue (Figure 3H). The spermatheca is attached to the ovary by a connective tissue coloured green-brownish (Figure 3E), except where the oviduct lays between them (Figure 3F).

The ovary was enormously enlarged coloured dark yellow or dark orange and filled almost the whole females inside. The histology analyses show a large amount of developed oocytes with increased yolk accumulation stained orange-brownish (secondary vitellogenesis) and with a diameter of 50-100µm (Figure 3E, 4A). Furthermore, several smaller previtellogene oocytes with a nucleus and nucleolus in the centre are visible (Figure 4B).

#### *Ovigerous female Baltic Sea*

In the reproduction system of 14 ovigerous females collected in the eastern Kiel Canal close to the Kiel Fjord, free spermatozoa as well as numerous oval and elongated spermatophores were observed in the spermatheca and are stained light purple (Figure 5A-C).

The spermatophores are filled with masses of spermatozoa, which are arranged more or less randomly, surrounded by an light-purple fluid. In the oval spermatophores, the spermatozoa are enclosed by a clearly visible wall (Figure 5B), supposed to be produced by the cells lining the anterior vas deferens, proximal to the testis (Beninger et al. 1988, Hinsch 1991).

The elongated spermatophores has no clearly visible wall and thus obviously are dissolving and releasing the spermatozoa (Figure 5A).

The spermatozoa found in the spermatheca of *Eriocheir sinensis* are typical for brachyuran crabs and show a nucleus with the acrosome inside (according to Jamieson 1991, 1995 and references therein) (Figure 5C). In figure 5D oocytes filled with clearly visible yolk granules are shown.



*Ovigerous female North Sea*

In the spermatheca of 40 dissected ovigerous females collected close to the North Sea, no spermatophores were detected.

*Juvenile female Baltic Sea*

In total, 20 subadult females were dissected. The abdomina had a triangular or oval shape respectively and the ovaries were slightly yellow and thin. The undeveloped spermatheca was about 4mm high and 2mm wide, the lumen was narrow. The spermatheca and ovary of the juvenile crabs was cut longitudinally. The ovaries of the juvenile crabs showed a large amount of oogonia (Figure 6D). The spermatheca of two out of 20 Baltic Sea juvenile females (carapace width 34.4 and 34.7 mm) contained free spermatozoa and small accumulations of spermatozoa (Figure 6 schematic draw, figure 6A, B). The spermatozoa are surrounded by a fluid which is coloured light purple-greenish (Figure 6A, B). In the spermatheca of these juvenile females, the cuticle and a heavily folded glandular epithelium are visible (Figure 6C, E, F). In none of the dissected juvenile females, the special valve-like tissue could be detected.

*Summer adult female close to Baltic Sea*

The spermatheca of 2 out of 19 adult females collected in the Lake Westensee in July 2008 and 2009 contained free spermatozoa, which could be observed in the whole spermatheca (Figure 7A). Almost the whole spermatheca is filled with a green coloured fluid. The spermatheca was about 12mm high and 7mm wide and had an ovoid-like form. The ovaries of the adult females collected in July before molting were light yellow-orange and enlarged. In the dissected ovaries, mainly vitellogenic oocytes as well as several previtellogene oocytes are visible coloured green (Figure 7B). The nuclei contain the nucleoli, which are stained yellow and light orange respectively (Figure 7C).

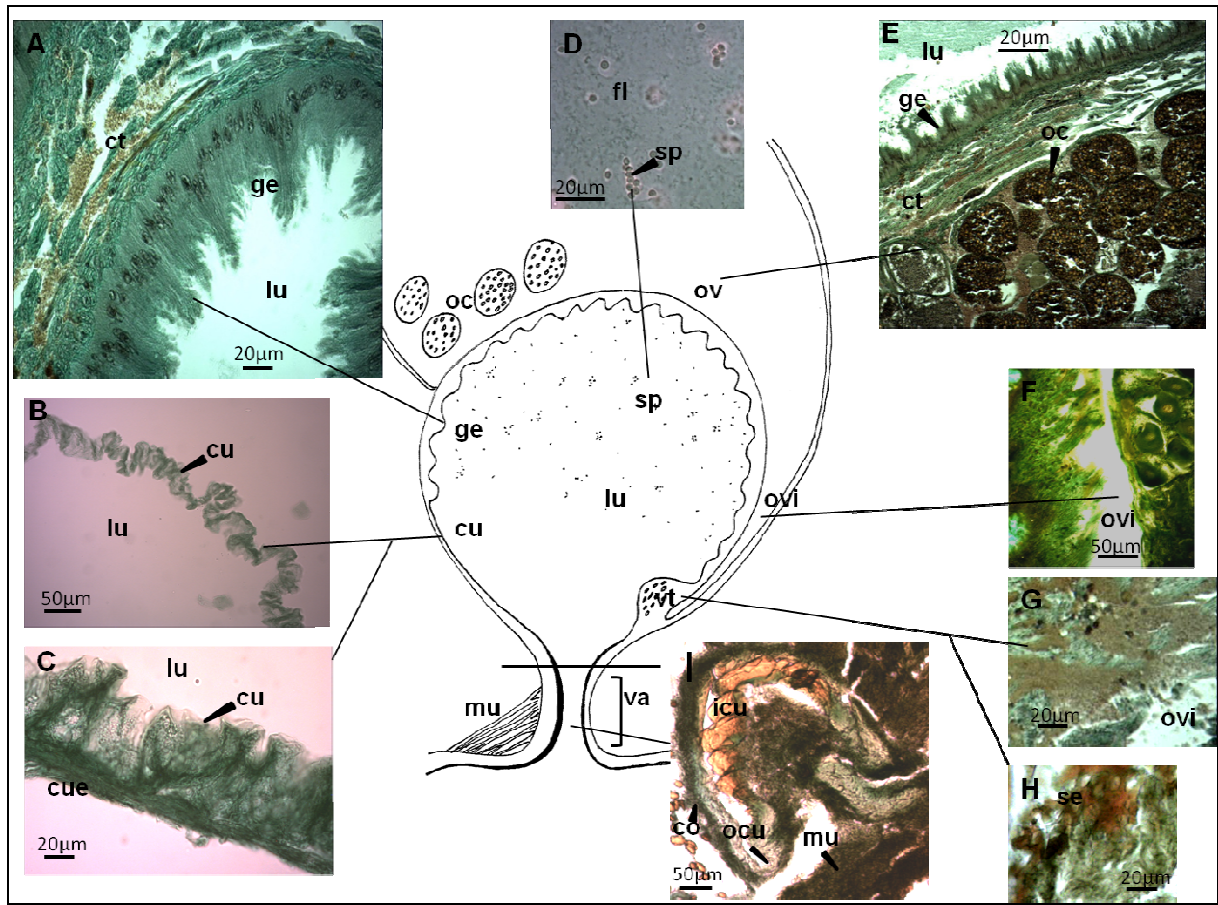
*Summer adult female close to North Sea*

In the summer adult females collected in the Soholmer Au, no spermatozoa were detected in the spermatheca. The spermatheca was about 10mm high and 6mm wide. The ovary had the same colour and size like the ovary in the summer adult females collected in the Lake Westensee.

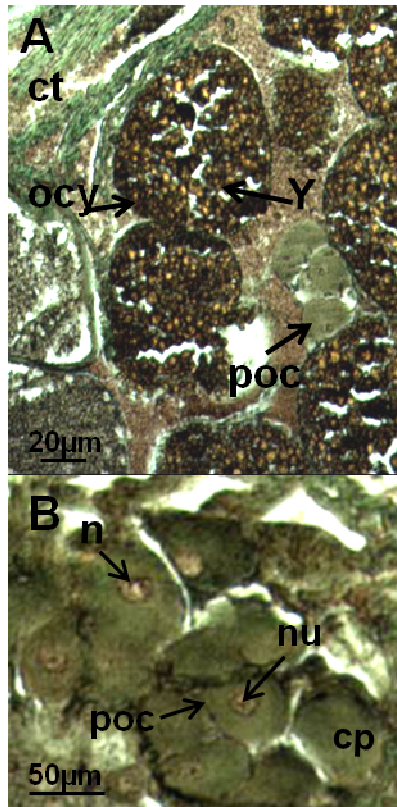
Table 2 shows an overview of all distinctive features observed in Baltic Sea.

**Table 2:** Special observations in analysed *Eriocheir sinensis* females from the Baltic Sea.

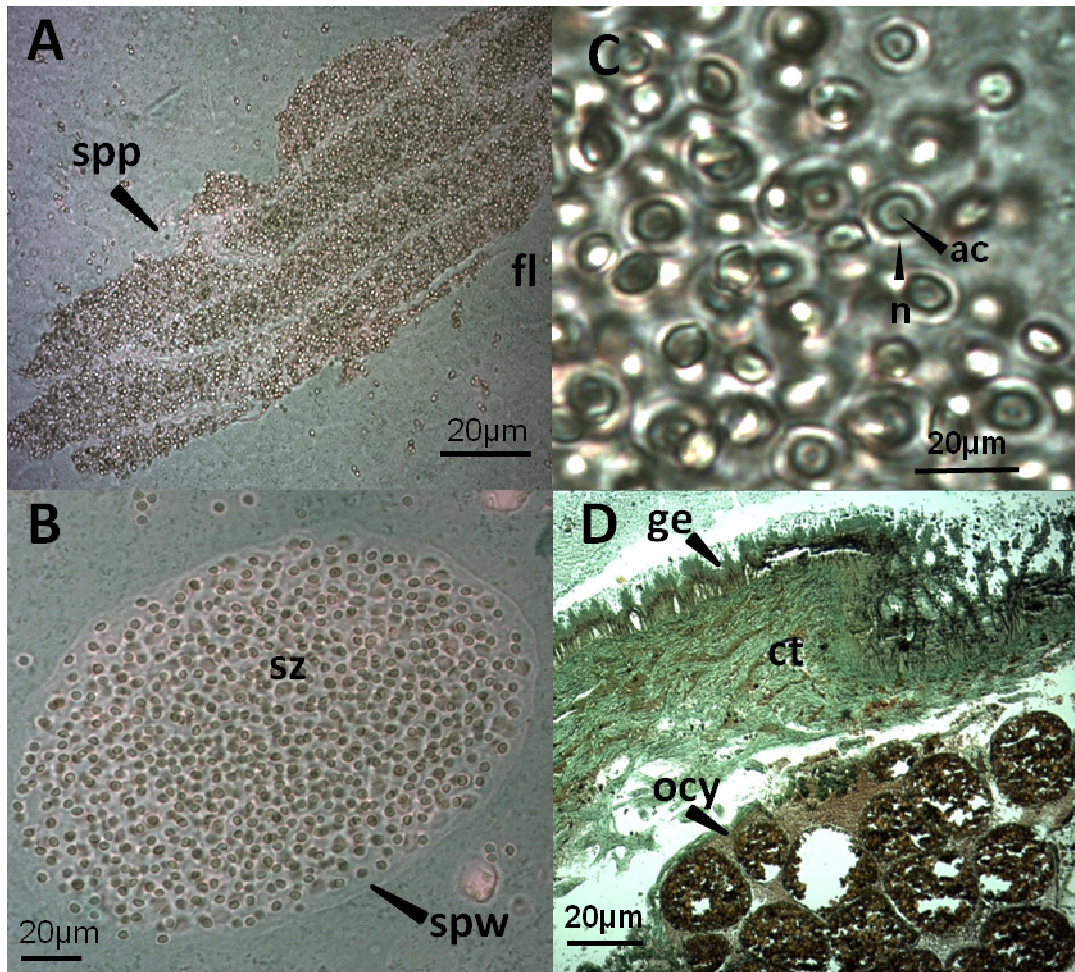
Location	Stage	N	Observation	Time
Lake Westensee	Adult females	2	Spermatozoa in spermatheca	July 2008, 2009
Eastern Kiel Canal, close to Kiel Fjord	Adult ovigerous females	14	Spermatophores in spermatheca	Sept.-Dec. 2008- 2010
Eastern Kiel Canal, close to Kiel Fjord	Juvenile crabs (carapace width 22.6 – 36.7)	35	Migrating with adult crabs	Sept.- Dec.2008- 2010
Eastern Kiel Canal, close to Kiel Fjord	Juvenile crabs (carapace width 34.4 and 34.7mm)	2	Spermatozoa in spermatheca	October 2010



**Figure 3:** Schematic drawing of the spermatheca of an ovigerous *Eriocheir sinensis* female, longitudinal view. Spermatheca about 7 mm wide and 17 mm high. **(A)** Magnification of glandular epithelium. **(B)** Heavily folded cuticle. **(C)** Magnification of the cuticle and single-layered cuticular epithelium. **(D)** Spermatozoa in the lumen of spermatheca. **(E)** Ovary with ripe oocytes. **(F)** Oviduct. **(G)** Valve-like tissue at the mouth of the oviduct. **(H)** Secretions at the valve-like tissue. **(I)** Cross section of vagina with inner and outer cuticle wall. co: columnar epithelium; ct: connective tissue; cu: cuticle; cue: cuticular epithelium; fl: fluid; ge: glandular epithelium; icu: inner vagina wall with flexible cuticle; lu: lumen of spermatheca; mu: muscle; oc: oocyte, oou: outer vagina wall with nonflexible part of cuticle; ov: ovary, ovi: oviduct; se: secretion; sp: spermatozoa; va: vagina; vt: valve-like tissue.

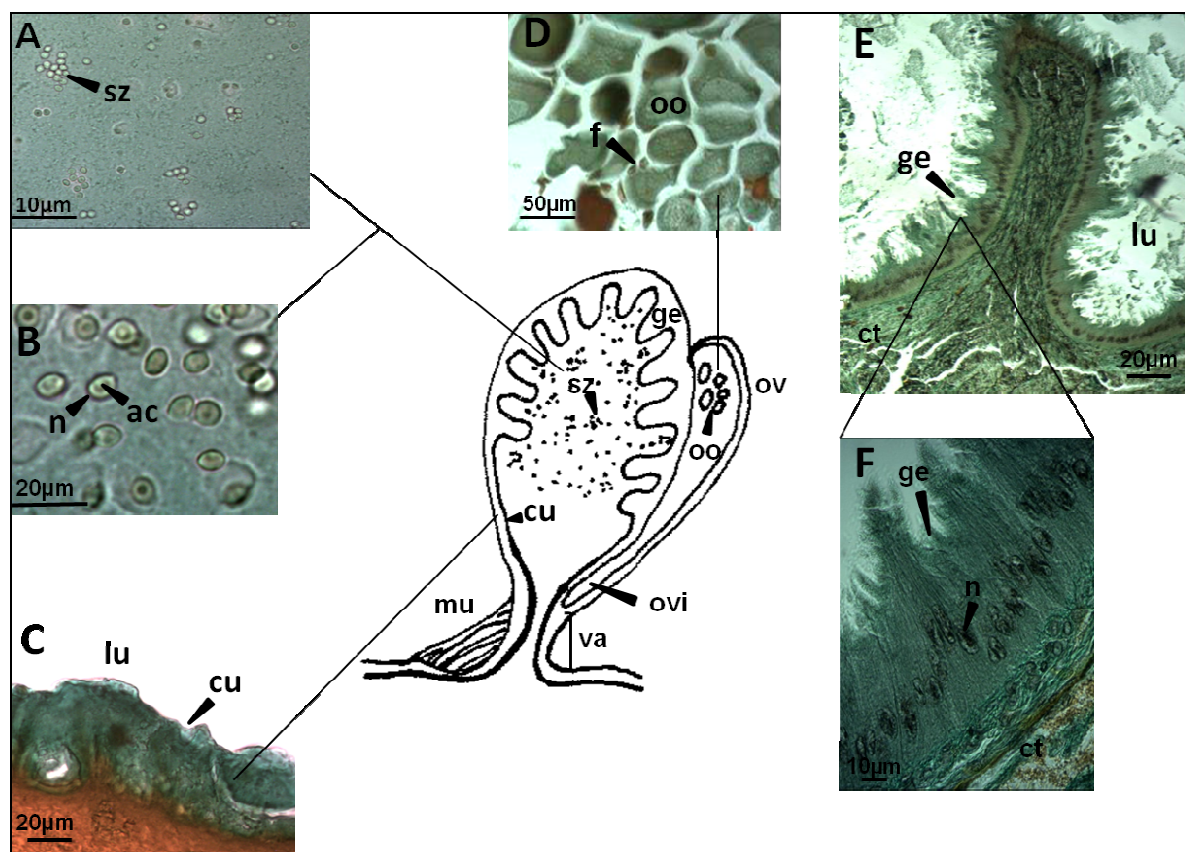


**Figure 4:** Different maturity stages of egg-cells in the ovary of an ovigerous *Eriocheir sinensis*. **(A)** Previtellogene oocytes and ripe eggs with yolk granules. **(B)** Previtellogene oocytes with a clearly visible nucleus and nucleolus. cp: cytoplasm; ct: connective tissue; n: nucleus; nu: nucleolus; ocy: oocyte with yolk granules; poc: previtellogene oocyte; y: yolk.

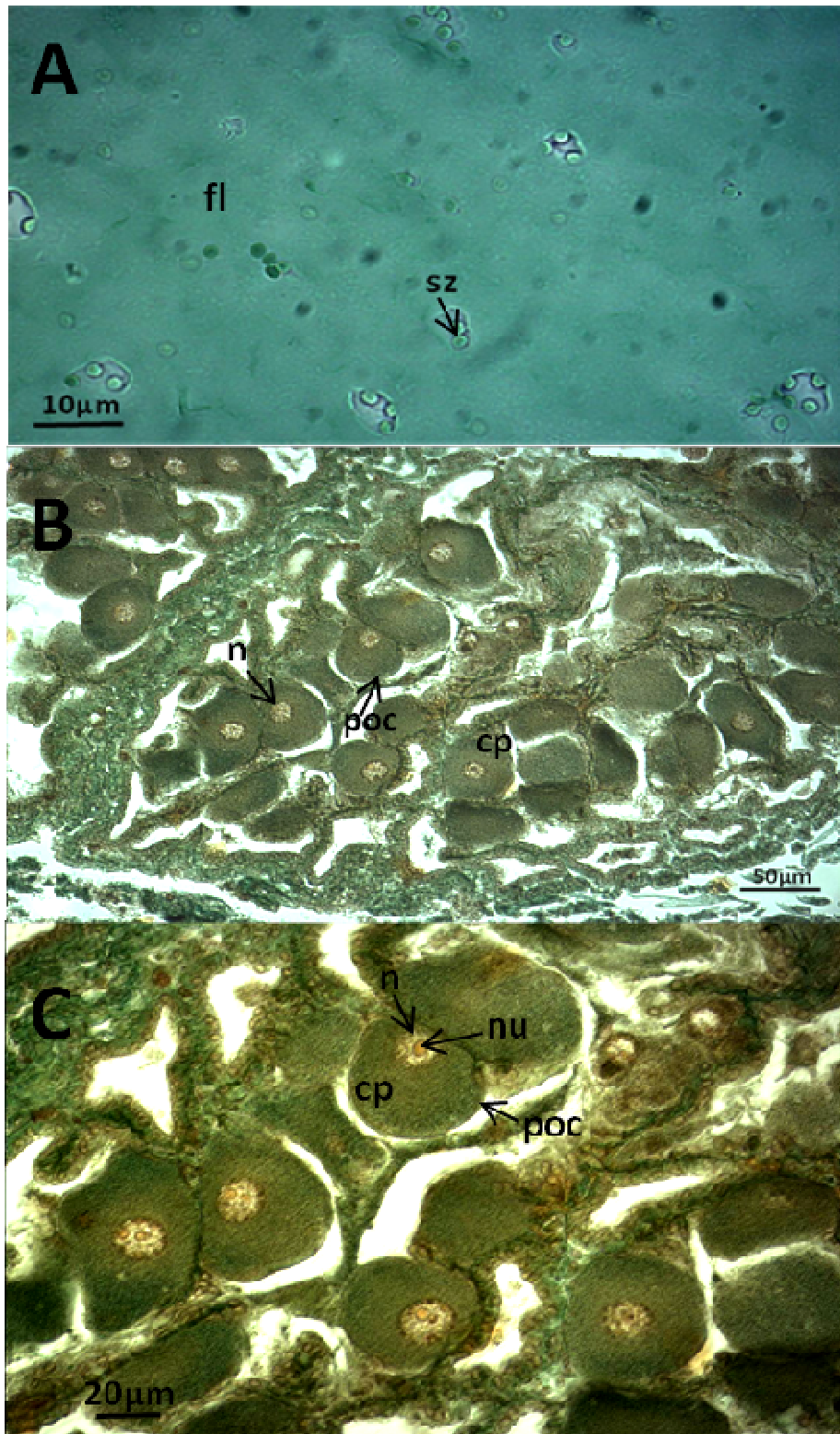


**Figure 5:** Histological cross sections of the spermatheca of an ovigerous female collected in Kiel Canal close to the Kiel Fjord in the mating season. **(A)** Dissolving spermatophore. **(B)** Spermatophore with clearly visible wall. **(C)** Enlargement of spermatozoa in spermatophore. **(D)** Ovary with mature oocytes. ac: acrosome; ct: connective tissue; fl: fluid; ge: glandular epithelium; n: nucleus; ocy: oocyte with yolk (ripe oocyte); spp: spermatophore; spw: spermatophore-wall; sz: spermatozoa.





**Figure 6:** Schematic drawing of the spermatheca of a juvenile *Eriocheir sinensis* female, collected in the Kiel Canal close to the Kiel Fjord in the mating season, longitudinal view. Carapace width was 34.4 mm and the pleon slightly round. Spermatheca was 3mm wide and 5 mm high. (A) Spermatozoa in the centre of spermatheca. (B) High magnification of spermatozoa in the centre of spermatheca. (C) Cuticle of the spermatheca wall. (D) Part of ovary with oogonia. (E) Heavily folded glandular epithelium of spermatheca wall. (F) High magnification of glandular epithelium. ac: acrosome; ct: connective tissue; cu: cuticle; f: follicle cells; ge: glandular epithelium; lu: lumen of the spermatheca; mu: muscles; n: nucleus; oo: oogonium; ov: ovary; ovi: oviduct; sz: spermatozoa; va: vagina.



**Figure 7:** Longitudinal section of ovary of adult *Eriocheir sinensis* female caught in July in the Lake Westensee (A) Spermatozoa in the spermatheca (longitudinal section) of an adult *Eriocheir sinensis* female, collected in the Lake Westensee in July. (B) Part of ovary with previtellogene oocytes (C) Oocytes in higher magnification. cp: cytoplasm; fl: fluid; n: nucleus; nu: nucleolus; poc: previtellogene oocyte; sz: spermatozoa.

## Discussion

### General findings in morphology of female reproductive tract

The spermatheca of *Eriocheir sinensis* corresponds to the basal type according to Diesel (1991): it is linked to the oviduct in the basic area where it is connected with the vagina (Figure 3 schematic draw). It was observed that the spermatheca wall of the basal type consists of a dorsal part lined with glandular epithelium and a ventral part lined with cuticle (McLay and Lopez-Greco 2011 and references therein). Generally it is assumed that the dorsal part has the function of sperm storage and in the ventral part the fertilization takes place like it was first shown for the brachyuran family Majidae by Diesel (1991). Recently it was also observed in the family Pinnotheridae (Becker et al. 2011) and the genus *Uca* (Lautenschläger et al. 2010). However, the role of the secretion produced from the glandular epithelium is not fully clarified yet. Functions like accommodation of spermatophores, dehiscence of spermatophores and producing sperm plugs for enclosing female ducts after copulation are suggested from several authors (Spalding 1942, Ryan 1967, Bauer 1986, Bawab and El-Sherief 1988, Diesel 1989). Furthermore the spermatheca glandular wall presumably produces nutrients for the storing of sperms (Subramoniam 1993), whereas Johnson (1980) proposed that the seminal fluid or own energy stores provides the required nutrients for the spermatozoa. Also in the specimens investigated in the present study a distribution in two different areas is clearly visible: the dorsal part with the glandular epithelium and the ventral part with the cuticle lying on a single-lined epithelium (Figure 3 schematic draw, 3 A-C). The spermatozoa and spermatophores were located in the dorsal part. According to the studies mentioned before, we assume that these dissimilar parts also have different functions like it was suggested for the brachyuran crab taxa *Uca* and Pinnotheridae (Lautenschläger et al. 2010, Becker et al. 2011). In contrast, Lee and Yamazaki (1990) described that the spermatheca wall consists of a single crumpled layer of columnar epithelium.

In the zone where the spermatheca opens into the vagina and the oviduct ends towards the vagina a special tissue was observed in all specimens. This special tissue has been only found in two other brachyuran crabs until now, in the genus *Uca*



(Lautenschläger et al. 2010) and the family Pinnotheridae (Becker et al. 2011). As mentioned in the introduction, Lee and Yamazaki (1990) found this so-called valve-like tissue between oviduct and spermatheca of *Eriocheir sinensis* for the first time. These authors observed that several days after egg-laying, the valve-like tissue was discharged into the cavity of the spermatheca and a new valve-like tissue was performed. This indicates that the female is able to produce several egg-batches in one mating season as it is generally known (Peters 1938b, Kobayashi and Matsuura 1999, Jin et al. 2002) and which is also supported by the different egg maturity stages in the ovary of the ovigerous females (Figure 4). In contrast to the study of Lee and Yamazaki (1990), the present study as well as the studies on *Uca* and Pinnotheridae showed secretions from the valve-like tissue into the lumen of the spermatheca (Figure 3H). Lautenschläger et al. (2010) suggest that this tissue also plays an important role for a controlled fertilization due to the location between the lumen of the spermatheca and the oviduct.

The vagina corresponds to the concave type (Figure 3I) according to Hartnoll (1968); Hoestland (1948) described this type for *Eriocheir* for the first time. The prominent muscle bundle helps to open and close the vagina actively during the copulation as well as egg-laying and hence these processes can be controlled (Hartnoll 1968, Lee and Yamazaki 1990). Several other crab taxa show this vagina type, for example *Cyclograpsus*, *Hyas*, *Majidae*, *Ocypodidae* and Pinnotheridae (Hartnoll 1968, Diesel 1991, Lautenschläger 2010, Becker et al. 2011). Besides this type also a simple type occurs, which is a round tube surrounded by longitudinal musculature (Hartnoll 1968) as described for *Carcinus maenas* and *Cancer magister* for example (Hartnoll 1968, Jensen et al. 1996).

### Special findings in Baltic Sea females

The observations and morphological analyses yield several special features especially for the Baltic Sea specimens.

One of the most interesting observations is the migration activity of definitively immature female crabs, indicated by a triangular pleon, versus a broadly round pleon in adult females (Figure 2). The histological analysis shows completely undeveloped

ovaries which only contain oogonia (see Figure 6D). However, despite these results spermatozoa were found in the spermatheca, indicating that these juvenile crabs have copulated. Since none of these migrating juvenile females had a valve-like tissue, we conclude that this tissue obviously do not play a role in copulation.

In the present literature, it is generally assumed that mitten crabs in Europe start migration at the age of 4-6 years when they become sexually mature (Anger 1991, Fladung 2000). These observed small specimens have mated even without a developed ovary as the spermatheca was filled with spermatozoa (Figure 6A, B). Such mating of juveniles has not been observed at the North Sea coast. According to laboratory experiments with *Eriocheir japonica*, Kobayashi (2003) stated that females "only copulate when the vitellogenesis is completed after the puberty moult, and females with immature ovaries cannot copulate". Our results contradict these conclusions at least for the Baltic Sea populations. For other crab taxa, there is published evidence of mating as for example between moulting from last juvenile stage to first adult stage of the brachyuran crab *Cancer magister* (Jensen et al. 1996 and references therein). The spermatozoa are stored across the moults until the females are sexually mature. Hankin et al. (1989) could show that *Cancer magister* females are able to produce viable eggs from sperm, which were retained for at least 2.5 years in the spermatheca. It is not known, after how many months or years *Eriocheir sinensis* can produce viable larvae from stored sperms in juvenile females. This has to be investigated in further laboratory experiments.

A further remarkable observation is the occurrence of spermatozoa in the spermatheca of adult females found in July before the migration and mating season in a freshwater lake nearby the Baltic Sea. Since mating season starts in late summer, the observed spermatozoa could not be fresh ones from the year these females were caught. This means that these crabs have returned to the freshwater habitats after the last mating season. Hence, mitten crabs at least in the Baltic Sea tributaries do not inevitably die after one mating season, as it was generally assumed so far (Peters 1938b, Cohen and Weinstein 2001 and references therein).

The observation of the spermatozoa in the spermatheca of the "summer- females" as well as the spermatozoa in the spermatheca of the juvenile crab indicates that spermatozoa can be stored for longer than one mating season. Besides the study of

Hankin et al. (1989) on *Cancer magister* mentioned before, several other studies showed that some crab species can fertilize egg-batches with stored sperms in successive mating seasons (Majoids Hinsch 1972, Diesel 1991; Ocypodoids Greenspan 1982, Paul 1984; Xanthoids Morgan et al. 1983).

A third interesting observation is a high number of spermatophores in the spermatheca of ovigerous females caught close to the Baltic Sea. It is still discussed, how long spermatophores exist in the spermatheca and when they are dissolving in free spermatozoa. It is assumed that in most cases spermatophores of *Brachyura* dissolve shortly after copulation (Klaus et al. 2009 and references therein). Benhalima and Moriyasu (1998) suggested two types of spermatophores: one type dissolves during copulation, the other type remains in the spermatheca and store the spermatozoa. According to Hou et al. (2010), the spermatophores of *Eriocheir sinensis* belong to the rapid-dissolving type. Accessory sex glands of the male reproductive system lead to the digestion of the spermatophores in the spermatheca soon after copulation. Thus, the spermatophores in the ovigerous Baltic Sea females are a clear hint that another copulation after egg-laying have occurred (Figure 5A,B). No spermatophores were found in the analysed North Sea females which could be a hint for a special feature in the mating behaviour of Baltic Sea females.

In summary we can conclude that at least in the region of the western Baltic Sea the reproductive behaviour of *Eriocheir sinensis* is more complex than presently known from other regions. The species shows a broad spectrum of reproduction strategies, which is unique for brachyurans and have not reported before. Multiple migrations in the life span of female Chinese mitten crab appear to be very probable and death after egg-laying is not a must. Furthermore, juvenile females migrate and copulate long before maturation and also egg-bearing females obviously copulate again. The glandular epithelium as a dominant part of the spermathecal wall of *Eriocheir sinensis* supports a possible long-term sperm storage: Furthermore, the special regenerating valve-like tissue also supports the possibility for several ovipositions also in the next mating season. This means that several assurances are part of the reproductive cycle to raise the probability of a successful reproduction as probable as possible.

The species seems to be in the process of adaptation to the environment they invaded about 90 years ago. Anger (1991) and also Montú (1996) could show that *Eriocheir sinensis* is able to produce a further zoea stage as well as a further megalopa stage under unfavourable conditions. Hence, the Chinese mitten crab seems to possess a certain flexibility to respond to certain environmental circumstances. With the aid of basic histological analyses an insight was gained, how flexible *Eriocheir sinensis* can respond to the unfavorable conditions of their invaded habitat. These results, in turn, could be necessary for the prediction, if *Eriocheir sinensis* would be able to establish in a new invaded habitat.

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## GENERAL CONCLUSIONS AND OUTLOOK

Introduced about 100 years ago most likely by ballast water of the merchant ships, the catadromous Chinese mitten crab is one of the most common and notorious alien species in Northern Germany. Occurring in high densities, the species causes economically high losses in the fishing industry, since it destroys the fish traps and feeds on the caught fish, which cannot be marketed anymore (Fladung 2000). Furthermore, *Eriocheir sinensis* causes substantial damages in the embankments due to its burrowing activity (Peters 1936, Rudnick et al. 2003). The species is also suspected to have negative impacts on native communities, due to feeding on the offspring of rare fish species and finally due to direct predation on decapods (Clark 1998, Veldhuizen and Stanish 1999, Fladung 2000, Rudnick 2000, Gilbey et al. 2008). However, information about many aspects concerning reproduction biology and genetic status has been sparse so far. This thesis shows that combination of field investigations, population genetics and morphological analyses is necessary to understand the mechanisms of successful establishment of an alien species. For *Eriocheir sinensis*, such combination of different methods has not been conducted before.

The results of these analyses lead to a distinct new picture of the biology of the Chinese mitten crab in Northern Germany. The species is able to reproduce in the Baltic Sea, which has been doubted until now. According to Anger (1991), the reproduction cycle of the Chinese mitten crab demands specific high salinities like occurring in the North Sea. Thus, the brackish Baltic Sea with lower salinities is an extreme habitat for a successful reproduction cycle of the mitten crab. No significant genetic differentiation between North Sea and Baltic Sea populations exist, whereas females reproducing in the Baltic Sea show a whole slew of special reproductive strategies, not found in North Sea females and not known before: (1) Juvenile females are regularly migrating during mating season and even are copulating; (2) adult females copulate more than one time in the mating season also after egg-laying and (3) spermatozoa can be stored until the next reproduction season or longer. As a consequence, Baltic Sea females do not inevitably die after one reproduction as it

has been assumed so far (Hymanson et al. 1999, Fladung 2000, Jin et al. 2002, Rudnick et al. 2003).

According to the results, *Eriocheir sinensis* exhibits an enormously broad spectrum of mating behaviour. Anger (1991) and later also Montú (1996) observed flexibility in the larval development in laboratory experiments: under unfavourable salinity and temperature, an additionally larval stage as well as an additional megalopa stage developed. Due to the enormous spectrum, *Eriocheir sinensis* is able to establish self-sustaining populations in different habitats. This extreme broad reproductive flexibility is unique for brachyurans and was not reported before. The relatively high genetic variability detected in Schleswig-Holstein presumably offers different possibilities to respond to different habitat conditions. Reports from its native range give a hint for a broad spectrum of life span and behaviour: two types of Chinese mitten crabs occur in China, the coastal and the large river type (Zhao 1999 cited in Cohen and Weinstein 2001). The latter spends 2 years in freshwater of large rivers before migrating into the estuaries. Since these crabs reach a large body size, they are harvested and marketed during migration time. The coastal type lives in the coastal streams, becomes mature with one year and has a much smaller body size. This type is called precocious crab (Jin et al. 2002). It is unknown, if genetic differences between both types exists (Zhao 1999 cited in Cohen and Weinstein 2001).

Ongoing introductions from genetically different Chinese populations could be a reason for the relatively high genetic variability and could explain the observed broad reproductive responses to the extremely different marine conditions of the North Sea and Baltic Sea. The multiple introductions from separated Chinese populations, which are leading to a new mixture of the gene pool in the invaded habitat, are the basis material for adaptive evolution. The relatively high mortality rate observed in the Baltic larval stages is presumably an indicator that the adaptation to the brackish Baltic Sea is still in process. Anger (1991) also assumed that *Eriocheir sinensis* is not already fully adapted to the conditions in the North Sea due to the high larval mortality at low temperatures between 4-8°C. These temperatures occur in spring in the marine coastal areas of Northern Germany. The reproduction situation especially in the western Baltic Sea can be the basis for a positive selection and speciation

process on the way to stable reproductive Baltic Sea populations of Chinese mitten crabs. Thus, the Chinese mitten crab is a very suitable model organism to study ecological and evolutionary processes in real time.

This thesis can serve as a basis for further studies on the status of *Eriocheir sinensis* as alien species in other regions, as well as for a comparison with other successful alien species. The thesis leads to several open questions: Is the broad spectrum of observed reproductive strategies also detectable in autochthonous populations as well as in other invasive populations of *Eriocheir sinensis*? Do the two types of *Eriocheir sinensis* found in China show genetic differences compared to each other and to European and American populations? Do they show special reproductive strategies like observed in this study? Do special reproductive strategies also occur in the male reproductive system and behaviour? Furthermore, the surprising feature that spermatozoa were found in the migrating Baltic Sea juveniles and in adult summer females is an interesting topic for further investigations. Laboratory experiments can solve the question, if the stored spermatozoa produce viable larvae.

In general, profound knowledge about the biology of invasive species like preferred habitat, reproduction strategies and life-cycle requirements is essential to develop a management plan as it is done currently for *Eriocheir sinensis* in the U.S. ([http://antaskforce.gov/spoc/mitten\\_crab.php](http://antaskforce.gov/spoc/mitten_crab.php)).

Population genetic methods are necessary to identify source populations and to determine genetic variability and genetic composition. These data are important for evaluation of the potential invasive success of the non-indigenous species. In this context, more genetic data from the Baltic Sea area should be obtained to get a comprehensive data set for detection of invasion pathways and identification of source populations as well as identification of the level of gene flow and genetic variability. In general, genetic data, knowledge about biology as well as analysis of ecological and economical impacts are essential for a risk analysis of the non-indigenous species and in turn for developing effective control efforts, preferably before the species get establish. Furthermore, this knowledge facilitates to determine potential endangered regions for successful dispersal and establishment of the Chinese mitten crab. In this context, also methods have to be developed to detect

new populations of *Eriocheir sinensis*. Therefore, educational outreach of the public is necessary, too.

The Chinese mitten crab is an established alien species in Schleswig-Holstein and must be accepted as part of the fauna in this region. Due to its broad flexibility and the intensive ship traffic (ballast water as main transport vector), *Eriocheir sinensis* has the potential to invade more regions of Northern Europe. Recent reports of mitten crabs at the Swedish coast confirm this assumption (Drotz et al. 2010). In Schleswig-Holstein, high densities will be common also in the future, probably with fluctuations as observed in the past in the Elbe-system, presumably due to fluctuations in temperature and water quality during the reproduction phase (Fladung 2000). Fishermen have already begun to sell the crabs to Chinese restaurants and marketers who resell them to China, where the species is a very popular delicacy. Furthermore, mitten crabs can be exploited for biogas production, chitosan production and animal feed production (Fladung 2000). For slight profit, very high regular numbers of crabs (7-8 tons per year) have to be marketed (Fladung 2000). However, only low numbers of crabs lead to an economically favourable situation, since then no serious damages on nets and fish occur.

Overall, the successful establishment of *Eriocheir sinensis* in Northern Germany and the potential for further invasions and introductions underline the need for measurements like ballast water treatment to prevent such spread and establishment of further alien species. Once established, it is very difficult or even impossible to dam potential ecological and economical damages and prevent further spread.



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## ANNEX

### Study 1

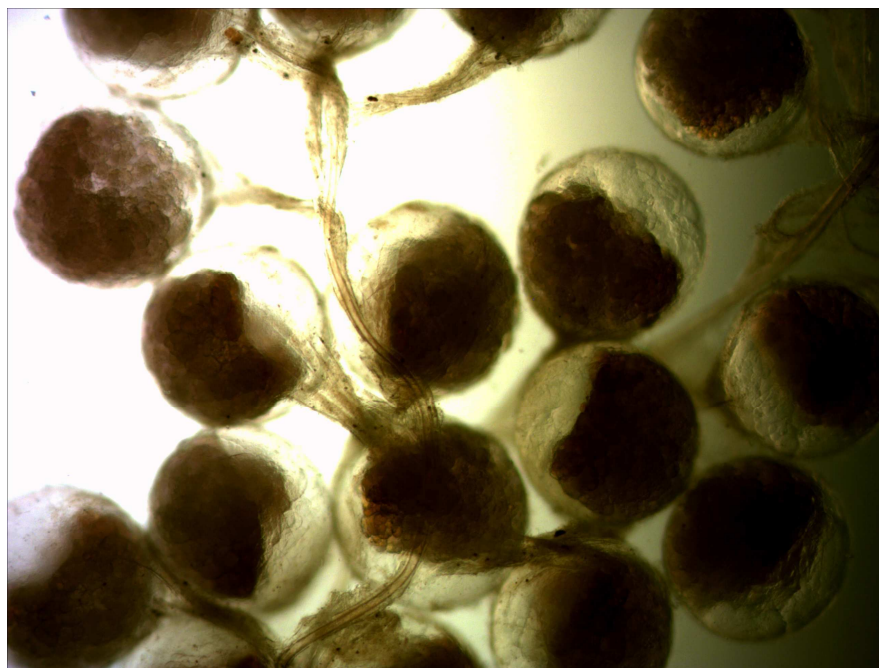


Figure 1: Eggs of ovigerous female caught in the Kiel Fjord April 2009.

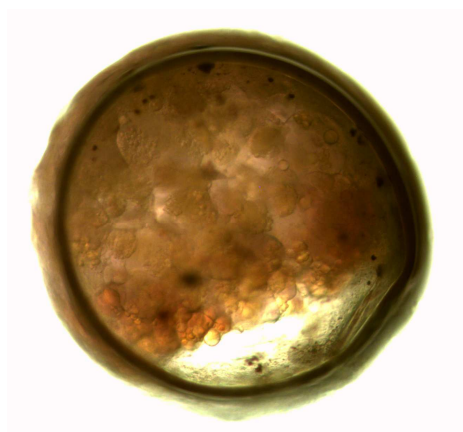


Figure 2: Enlarged egg of ovigerous female caught in the Kiel Fjord April 2009.





Figure 3: Hatching *Eriocheir sinensis* prezoa-larvae in the laboratory. Ovigerous female were caught in the Kiel Fjord April 2009 and kept in the laboratory.



Figure 4: *Eriocheir sinensis* zoea-larvae stage I reared in the laboratory under “Kiel Fjord” conditions. Eggs were taken from ovigerous female caught in the Kiel Fjord April 2009 and kept in the laboratory.



Figure 5: *Eriocheir sinensis* zoea-larvae stage II reared in the laboratory under “Kiel Fjord” conditions. Eggs were taken from ovigerous female caught in the Kiel Fjord April 2009 and kept in the laboratory.

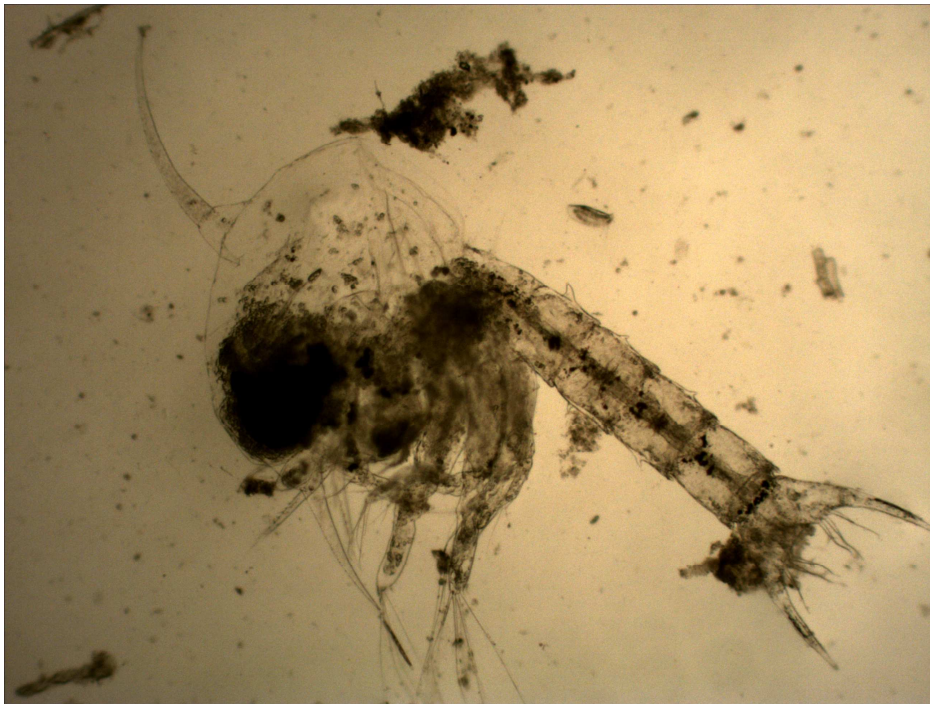
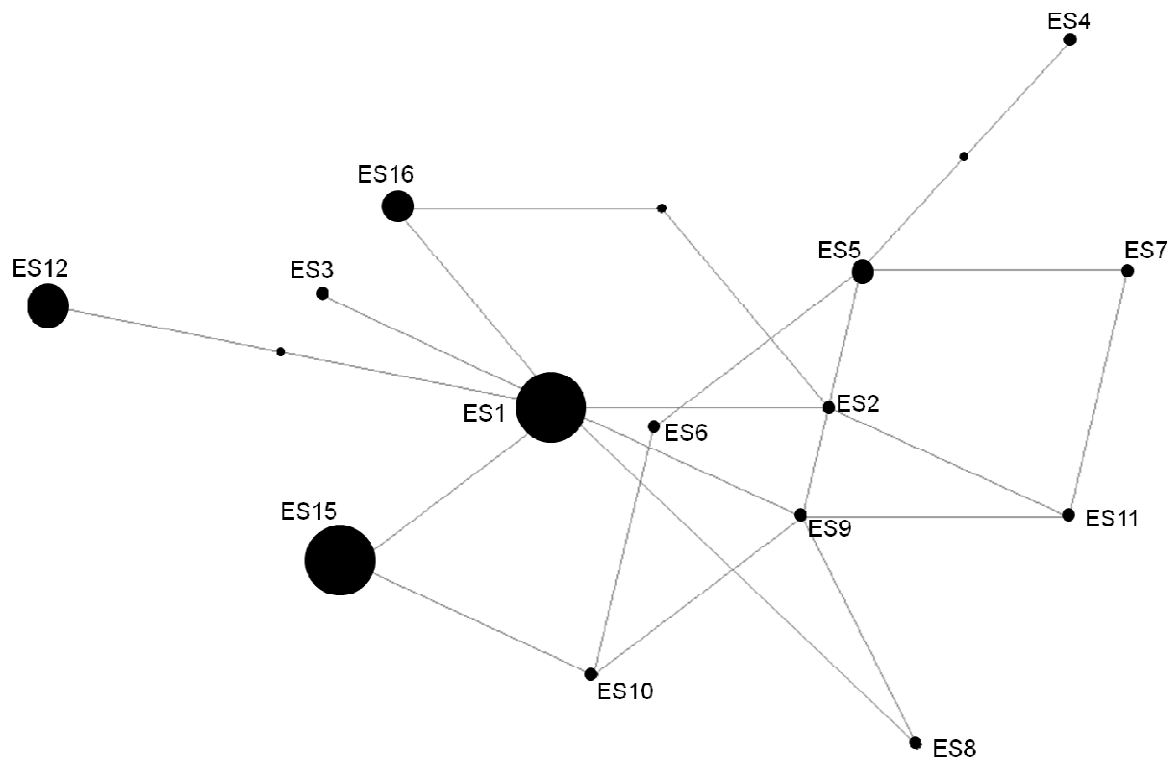
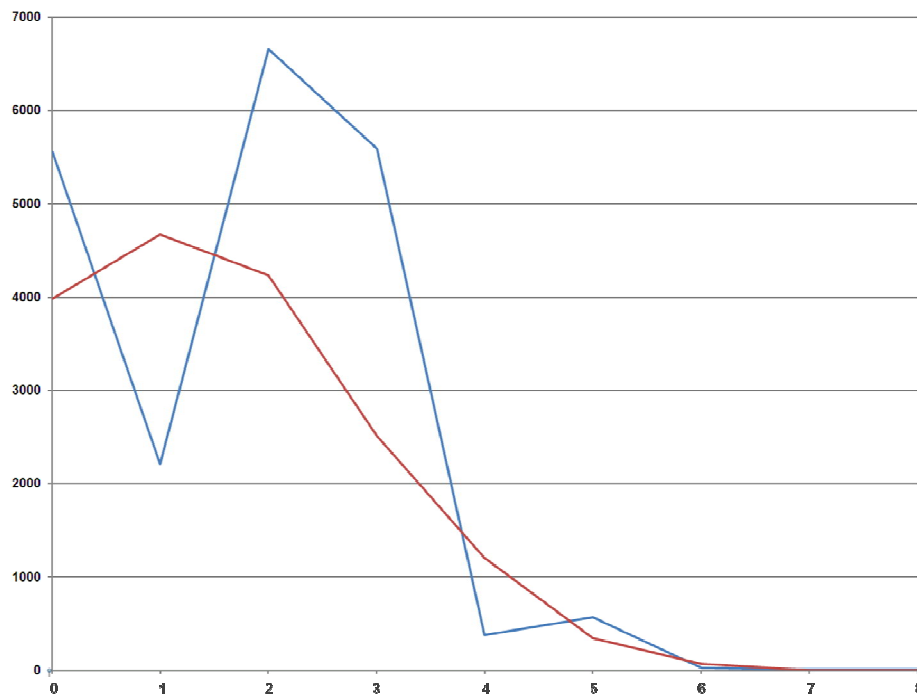


Figure 6: *Eriocheir sinensis* zoea-larvae stage III reared in the laboratory under “Kiel Fjord” conditions. Eggs were taken from ovigerous female caught in the Kiel Fjord April 2009 and kept in the laboratory.

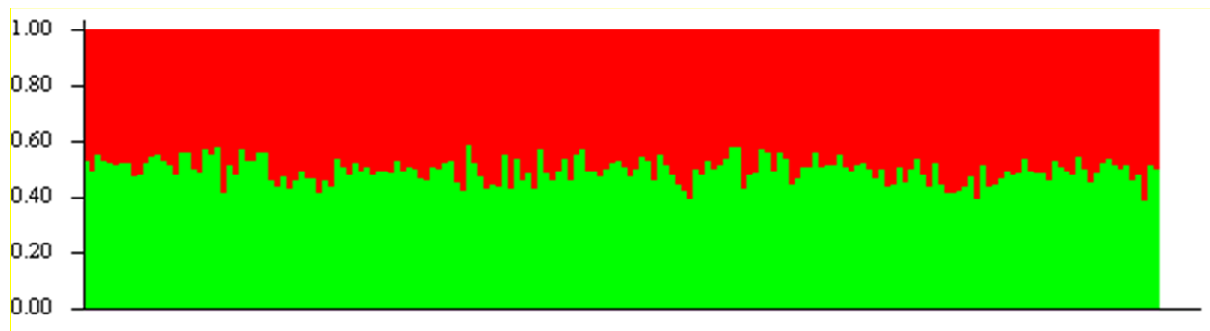
## Study 2



**Figure 1:** Median Joining-Network of 9 Haplotypes (ES8-H16) found in present study (GenBank Acc.nr: JQ956425 – 31) and 7 Haplotypes (ES1-ES7) found in the study of Hänfling et al. (2002), GenBank Acc.nr: AF 435114 to 435119. Haplotype ES1 and ES5 in the study of Hänfling - is identical with ES14 and ES13 respectively of the present study. The smallest black dots are hypothetical haplotypes. Size of black circles represents the frequency of the haplotypes found in the Northern German populations analyses in present study.



**Figure 2:** Mismatch distribution analysis. Distribution of pairwise sequence differences. Blue graph: observed distribution, red: simulated distribution



**Figure 3:** Bar plot of the Structure analysis: X-Axis: Individuals, Y-Axis: Probability of belonging to two assumed groups for every individual shown as subdivided colour bars.

# ANNEX

**Table 1:**  $H_0$  (observed heterozygosity),  $H_E$  (expected heterozygosity) and A (allelic richness) for every locus and population analysed in Northern Germany. AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Soholmer Au

Pop	Genetic diversity	Esin06	Esin18	Esin26	Esin36	Esin38	Esin42	Esin55	Esin67	Esin87	Mean
LF	Ho	0.8416	0.7833	0.8809	0.8782	0.72667	0.825	0.7166	0.8083	0.8785	0.81542
	He	0.8049	0.8643	0.8572	0.8178	0.90603	0.8865	0.8758	0.7659	0.875	0.8504
	A	9.974	9.119	10.187	10.878	11.127	9.27	10.696	9.05	10.284	10.065
E	Ho	0.7233	0.6875	0.658	0.724	0.751	0.73	0.7625	0.7122	0.6899	0.7153
	He	0.9032	0.8850	0.8790	0.8104	0.875	0.8629	0.875	0.7883	0.6673	0.8384
	A	10.618	9.035	9.458	9.847	9.723	9.785	9.395	9.98	8.871	9.6347
SO	Ho	0.79231	0.82	0.74167	0.86154	0.76122	0.8842	0.76923	0.85385	0.77692	0.80671
	He	0.90347	0.8351	0.79876	0.84012	0.82278	0.84842	0.90724	0.81448	0.63348	0.82265
	A	9.966	9.425	9.690	9.013	9.000	9.306	9.132	9.447	8.073	9.228
AU	Ho	0.6531	0.67667	0.671	0.6117	0.5646	0.6245	0.62788	0.6652	0.6451	0.63775
	He	0.89777	0.7867	0.76778	0.86002	0.78007	0.854	0.7977	0.7502	0.6895	0.7982
	A	8.474	8.492	7.331	7.623	7.167	7.788	7.643	7.478	7.498	7.7213
RE	Ho	0.868	0.74167	0.80436	0.73846	0.71538	0.82	0.88462	0.82	0.84615	0.80429
	He	0.8649	0.86436	0.8715	0.75641	0.925534	0.87755	0.88914	0.82612	0.85686	0.8591
	A	10.833	10.456	10.931	9.831	10.762	10.474	10.556	10.952	10.79	10.6206
LH	Ho	0.6787	0.626	0.62875	0.75556	0.769	0.658	0.7434	0.68421	0.7599	0.70039
	He	0.87692	0.86795	0.81882	0.70794	0.90769	0.83462	0.83333	0.82077	0.73333	0.8223
	A	9.000	8.2033	8.1804	8.000	8.945	8.047	8.4313	8.4891	8.12	8.3796
REL	Ho	0.80952	0.87619	0.88718	0.82381	0.826	0.70968	0.77931	0.74419	0.81163	0.8075
	He	0.84395	0.88985	0.84882	0.83964	0.91053	0.82126	0.92498	0.7699	0.7606	0.8455
	A	10.032	10.688	10.799	10.316	11.384	10.096	10.161	9.382	9.210	10.2298
SL	Ho	0.67221	0.6895	0.7883	0.6984	0.7642	0.7124	0.6462	0.6351	0.7025	0.6612
	He	0.708	0.7241	0.8004	0.7742	0.7991	0.7763	0.7013	0.7005	0.7916	0.7528
	A	8.091	8.0844	8.814	8.7762	8.000	8.0545	8.0341	7.0557	8.899	8.1806

List of shown figures

**General Introduction- 1:** The study area Schleswig-Holstein with the largest rivers, canals and fjords respectively, where the Chinese mitten crab is established. BC= Bongsieler Canal, EL= Elbe, ELC= Elbe-Lübeck-Canal, EID= Eider, KC= Kiel Canal, SL= Schlei, ST= Stör, TRA= Trave, TRE= Treene. Maps: Baltic Sea map by courtesy of Robert Sommer, Ecology center Kiel; Schleswig-Holstein map: [https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein\\_relief\\_location\\_map.jpg](https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein_relief_location_map.jpg).

**General Introduction- 2:** Life cycle of *Eriocheir sinensis*, taken from Anger 1991.

**General Introduction- 3:** The Baltic Sea. Map by courtesy of Robert Sommer, Ecology center Kiel.

**General Introduction- 4:** Cross section of reproductive system of a brachyuran female crab according to Hartnoll 1968.

**Study 1- 1:** The present study was conducted in Schleswig-Holstein (S-H), Northern Germany, at the Baltic Sea coast (Baltic Sea map by courtesy of R.Sommer, Ecology Center Kiel). In the map of Schleswig-Holstein and in the enlargement the places of *Eriocheir sinensis*-catches are shown. KB=Kiel Bight, KC=Kiel Canal, KF=Kiel Fjord, LF=Lake Flehmude, RE= River Eider.

**Study 2- 1:** Sample sites of *Eriocheir sinensis* and all other sites, mentioned in the text. Baltic Sea map by courtesy of Robert Sommer, Ecology Center Kiel. AU= Aukrug, E= Eckernförde, KC=Kiel Canal, KF=Kiel Fjord, LB = Luebeck Bight, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Soholmer Au. Maps: Baltic Sea map by courtesy of Robert Sommer, Ecology center Kiel; Schleswig-Holstein map: [https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein\\_relief\\_location\\_map.jpg](https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein_relief_location_map.jpg).

**Study 3- 1:** Schleswig-Holstein, Northern Germany with the sampling places. KC= Kiel Canal, KF= Kiel Fjord, LW= Lake Westensee, RE= River Eider, RT= River Treene. SO=Soholmer Au. Maps: Baltic Sea map by courtesy of Robert Sommer, Ecology center Kiel; Schleswig-Holstein map: [https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein\\_relief\\_location\\_map.jpg](https://fr.wikipedia.org/wiki/Fichier:Schleswig-Holstein_relief_location_map.jpg).

**Study 3- 2:** Adult and juvenile female of *Eriocheir sinensis*.



**Study 3- 3:** Schematic drawing of the spermatheca of an ovigerous *Eriocheir sinensis* female, longitudinal view. Spermatheca about 7 mm wide and 17 mm high. **(A)** Magnification of glandular epithelium. **(B)** Heavily folded cuticle. **(C)** Magnification of the cuticle and single-layered cuticular epithelium. **(D)** Spermatozoa in the lumen of spermatheca. **(E)** Ovary with ripe oocytes. **(F)** Oviduct. **(G)** Valve-like tissue at the mouth of the oviduct. **(H)** Secretions at the valve-like tissue. **(I)** Cross section of vagina with inner and outer cuticle wall. co: columnar epithelium; ct: connective tissue; cu: cuticle; cue: cuticular epithelium; fl: fluid; ge: glandular epithelium; icu: inner vagina wall with flexible cuticle; lu: lumen of spermatheca; mu: muscle; oc: oocyte, ocu: outer vagina wall with nonflexible part of cuticle; ov: ovary, ovi: oviduct; se: secretion; sp: spermatozoa; va: vagina; vt: valve-like tissue.

**Study 3- 4:** Different maturity stages of egg-cells in the ovary of an ovigerous *Eriocheir sinensis*. **(A)** Previtellogene oocytes and ripe eggs with yolk granules. **(B)** Previtellogene oocytes with a clearly visible nucleus and nucleolus. cp: cytoplasm; ct: connective tissue; n: nucleus; nu: nucleolus; ocy: oocyte with yolk granules; poc: previtellogene oocyte; y: yolk.

**Study 3- 5:** Histological cross sections of the spermatheca of an ovigerous female collected in Kiel Canal close to the Kiel Fjord in the mating season. **(A)** Dissolving spermatophore. **(B)** Spermatophore with clearly visible wall. **(C)** Enlargement of spermatozoa in spermatophore. **(D)** Ovary with mature oocytes. ac: acrosome; ct: connective tissue; fl: fluid; ge: glandular epithelium; n: nucleus; ocy: oocyte with yolk (ripe oocyte); spp: spermatophore; spw: spermatophore-wall; sz: spermatozoa.

**Study 3- 6:** Schematic drawing of the spermatheca of a juvenile *Eriocheir sinensis* female, collected in the Kiel Canal close to the Kiel Fjord in the mating season, longitudinal view. Carapace width was 34.4 mm and the pleon slightly round. Spermatheca was 3mm wide and 5 mm high. **(A)** Spermatozoa in the centre of spermatheca. **(B)** High magnification of spermatozoa in the centre of spermatheca. **(C)** Cuticle of the spermatheca wall. **(D)** Part of ovary with oogonia. **(E)** Heavily folded glandular epithelium of spermatheca wall. **(F)** High magnification of glandular epithelium. ac: acrosome; ct: connective tissue; cu: cuticle; f: follicle cells; ge: glandular epithelium; lu: lumen of the spermatheca; mu: muscles; n: nucleus; oo: oogonium; ov: ovary; ovi: oviduct; sz: spermatozoa; va: vagina.

**Study 3- 7:** Longitudinal section of ovary of adult *Eriocheir sinensis* female caught in July in the Lake Westensee **(A)** Spermatozoa in the spermatheca (longitudinal section) of an adult *Eriocheir-sinensis* female, collected in the Lake Westensee in July. **(B)** Part of ovary with previtellogene oocytes **(C)** Oocytes in higher magnification. cp: cytoplasm; fl: fluid; n: nucleus; nu: nucleolus; poc: previtellogene oocyte; sz: spermatozoa.

List of shown tables

**General introduction- 1:** Salinity ranges in PSU reported for *Eriocheir sinensis*. (References Jin 1998, Zhao 1999 is a personal communication and presentation respectively, cited in Cohen and Weinstein 2001).

**General introduction- 2:** Temperature ranges in °C reported for *Eriocheir sinensis*. (References Guo 1996, Jiang 1998, Jin 1998, Zhao 1999 are personal communications and presentations respectively, cited in Cohen and Weinstein 2001).

**Study 1- 1:** Relevant data for the specimens sampled. Sex and Museum accession number could not be given for every specimen.

**Study 1- 2:** Relevant data for the larvae reared in the laboratory. The larvae were reared from ovigerous females caught in the Kiel Fjord in spring 2009. The eggs of the females caught in early summer were all infected by fungi.

**Study 2- 1:** Sampling sites of *Eriocheir sinensis* in Northern Germany with coordinates as far as available.

**Study 2- 2:** Characteristics of the 9 microsatellite loci analysed in *Eriocheir sinensis* (Hänfling and Weetman 2003).

**Study 2- 3:** Distribution of the 9 found haplotypes across the 8 sampling sites. AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Sohlmer Au, n=sample size. All sequences for the haplotypes except ES13 and ES14 have been submitted to GenBank (Accession numbers: JQ956425 – 31)

**Study 2- 4:** Variability within the populations, given parameters based on mitochondrial DNA: haplotype diversity (HD), nucleotide diversity (ND in per cent), standard deviation in brackets. AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Sohlmer Au

**Study 2- 5:** Pairwise values of net nucleotide diversity of the analysed populations (in %) above the diagonal. Pair wise  $\Phi_{st}$  -Values (Distance-method Kimura 2P) of the analysed populations below the diagonal. Both parameters based on mitochondrial DNA-data. Normal= not significant ( $p>0.05$ ) before Bonferroni - Correction, **bold** and *italic*= significant after Bonferroni-Correction (Significance-Level  $p = 0.05$ , Bonferroni-corrected Significance-Level  $p = 0.0018$ ).

AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Sohlmer Au



**Study 2- 6:** Characterization of the 9 Microsatellite loci. bp: base pairs, A: number of different

**Study 2- 7:** Mean values of  $H_O$  (observed heterozygosity),  $H_E$  (expected heterozygosity), AR (allelic richness), mean number of alleles (A) and number of private alleles (PA) of the 9 analysed microsatellites for every population analysed in Northern Germany. AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, SO= Soholmer Au.

**Study 2- 8:** Pairwise  $F_{ST}$  values for *Eriocheir sinensis* sample sites. Significant  $F_{ST}$  - values after Bonferroni-Correction (Bonferroni-corrected Significance-Level  $p = 0,0018$ ) in bold and italic letters.

AU= Aukrug, E= Eckernförde, LF = Lake Flehmude, LH= Lake Hemmelsdorf, RE= River Eider, REL= River Elbe, SL= Schlei, Soholmer Au

**Study 3- 1:** Masson-Trichrome stain steps with duration of staining. (after Romeis 1989).

**Study 3- 2:** Special observations in analysed *Eriocheir sinensis* females from the Baltic Sea.

#### List of used chemicals

Agarose	ROTH, K297.3
Borsäure	SIGMA, 233-139-2
dNTP-Mix	MBI-FERMENTAS, R0242
EDTA-Disodium	GERBU, 1034
Ethanol (70 %)	WALTHER KG KIEL
Ethanol (96-100 %)	WALTHER KG KIEL
Ethidiumbromid-Lösung	SIGMA, E-8751
ET400ROX Größenstandard	GE Healthcare
Loading Dye (Gelladepuffer 6x)	Qiagen, 239901
Längenstandard (L100+)	Geneo, NM 100
MegaBACE Long Read Matrix	GE Healthcare

MegaBACE Running Buffer	GE Healthcare
MegaBACE Tween Buffer	GE Healthcare
Natriumacetat (NaAc)	MERCK, 1.06268.0250
Natriumhydroxid	MERCK, 106498
Primer: ES06, 18, 26, 36, 38, 42, 55, 67, 87; PMT1, PMT2	BioTeZ GmbH
RNase A	ROTH, 7156.1
Salzsäure, HCl 32 % (10 N)	ROTH, P074.4
<i>taq</i> -Polymerase, PCR-Puffer und MgCl <sub>2</sub>	PROMEGA, M3178
Tris	SERVA, 37190

#### List of used instruments

Automatical sequencer	APPLIED BIOSYSTEMS, 3730 XL DNA Analyser
Electrophoresis chamber	Pharmacia, GNA-100, GNA-200 EASY-CAST™ ELEKTROPHORESIS SYSTEMS, Model B2 BIOZYM, EasyPhor Medi
Freezer	LIEBHERR, automatic 29 PRIVILEG, 280
Heating plate with magnetic stirrer	KIKA® LABORTECHNIK, RH basic
Capillar sequencer	AMERSHAM BIOSCIENCES, MegaBACE™ 1000
Fridge	CLATRONIC, CTC
Microwave	PHILIPS WHIRLPOOL, Space Cube

Pipettes	GILSON PIPETMAN, diverse Volumina
Instant camera	POLAROID, DS-34 Direct Screen Instant Camera
Voltage controller	PHARMACIA, LKB Gene Power Suppli (GPS) 200/400
Thermocycler	BIOMETRA®, T-Gradient PERKIN ELMER, Gene Amp PCR System 2400
UV- Transilluminator	UVITECH, UV-Transilluminator SXT20M
UV-Linker	STRATAGENE , UV Stratalinker 2400
Embedding automat	W2110, PSI Ltd., Basel/Switzerland
Cutting machine	rotation mikrotome Biocut 1130 Reichert-Jung, Heidelberg
Thermal cubicle	Memmert GmbH, Schwabach
Light microscope	Leica DM RBE
Digital camera	Tucsen TCA 5 MP

**CURRICULUM VITAE****Personal information**

Name	Thurid Otto
Date of birth	12.05.1980
Place of Birth	Eckernförde
address	Harmsstrasse 54, 24114 Kiel
Nationality	german

**Scientific work  
experience**

12/2011-07/2012	Scientist at the Zoological Institute, Special Zoology/Population genetics of Christian-Albrechts-University (CAU) Kiel
Period from 2006-2011	Self-employed diploma-biologist: remittance work for diverse projects of Ecology Centre Kiel and BioConsult SH GmbH & Co. KG
10/2001-01/2006	Scientific Assistant in Ecology Centre Kiel and Zoological Institute CAU Kiel

**Education**

07/2008-07/2010	PhD-fellowship from the graduate support of the CAU Kiel
Since 07/2008	PhD-student in Zoological Institute/Zoological Museum CAU Kiel  <u>Title:</u> Reproduction biology and Population genetics of the Chinese mitten crab ( <i>Eriocheir sinensis</i> ) in Schleswig-Holstein

- 10/1999 - 06/2006      Student of Biology at CAU Kiel
- Title of Diploma-Thesis: The genetic status of the red deer (*Cervus elaphus*) in Northern Germany as assessed by mitochondrial D-loop-sequences.  
grade: 1.0
- 06/1999                  School leaving examinations (Abitur) at Jungmann -  
Gymnasium Eckernförde  
grade: 2.2

### Research cruises

- Period from 2007 to 2011      RV Walther Herwig III, RV Solea Johann Heinrich von  
Thünen-Institute Hamburg; North Sea, North Atlantic:  
plankton survey, fishery
- RV Tridens Netherlands; North Atlantic: fishery
- 01/2005, 09/2005,  
06/2006                  RV Heincke, RV Alkor, Leibniz-Institute of Marine  
Science (IFM-GEOMAR) CAU Kiel,  
North Sea, Baltic Sea:  
plankton survey, fishery

### conferences

- 02/2011                  Oral presentation: Population Biology of the Chinese  
mitten crab in Northern Germany.
- International Conference "BioSystematics", Berlin
- 12/2010                  Poster: Population genetics and reproduction biology of  
the Chinese mitten crab in Northern Germany.
- International Senckenberg Conference: Biology of  
Freshwater Decapods, Frankfurt
- 06/2010                  Oral presentation: First report of reproduction of the  
alien chinese mitten crab in the western Baltic Sea.
- International Crustacean Conference in Qingdao, China

- 04/2009      Oral presentation: Reproduction biology and Population genetics of the Chinese mitten crab (*Eriocheir sinensis*) in Schleswig-Holstein – first results  
  
German Crustacean Conference, Rostock
- 09/2006      1. prize for poster: The genetic status of the red deer (*Cervus elaphus*) in Northern Germany as assessed by mitochondrial D-loop-sequences and microsatellites.  
  
Conference of German Mammalian Society, Kiel
- 02/2006      Oral presentation: The genetic status of the red deer (*Cervus elaphus*) in Northern Germany as assessed by mitochondrial D-loop-sequences.  
  
Meeting of Graduate students of Evolution Biology, German Zoological Society, Potsdam

## Publications

- 2011      **Otto T** and Brandis D :  
  
First evidence of *Eriocheir sinensis* reproduction from Schleswig-Holstein, northern Germany, western Baltic Sea. *Aqua Invas* 6: 65–69.
- 2011      Niedziałkowska M, Jędrzejewska B, Honnen AC, **Otto T**, Vadim E, Sidorovich T, Perzanowski K, Skog A, Hartl GB, Borowik T, Bunevich AN, Lang J, Zachos FE:  
  
Molecular biogeography of red deer *Cervus elaphus* from eastern Europe: insights from mitochondrial DNA sequences. *Acta Theriol* 56:1–12.

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## CONTRIBUTIONS OF AUTHORS

### **Study 1: First evidence of *Eriocheir sinensis* reproduction from Schleswig - Holstein, Northern Germany, western Baltic Sea**

Investigation was designed by Thurid Otto. Surveys were organized and conducted also by Thurid Otto. Additionally, plankton samples from eastern Kiel Canal and Kiel Fjord from weekly surveys of IFM-Geomar were available, provided by Annegret Stühr. Laboratory experiments were conducted by Thurid Otto and manuscript were written by Thurid Otto with helpful comments of Dirk Brandis and Klaus Anger.

### **Study 2: Small-scale genetic structure and genetic variability of an established alien species, *Eriocheir sinensis*, in Northern Germany with a special focus on the Baltic Sea**

Specimens were collected by Thurid Otto. Investigation was designed by Thurid Otto, Günther B. Hartl and Dirk Brandis and laboratory work was conducted by Thurid Otto. Analysis of data and writing of manuscript were done by Thurid Otto with helpful comments of Frank Zachos and Günther B. Hartl.

### **Study 3: Reproduction biology of the alien Chinese mitten crab *Eriocheir sinensis* in Northern Germany with special focus on the Baltic Sea.**

Experimental set-off was designed by Thurid Otto, Dirk Brandis and Günther B. Hartl. Specimens for histology analyses were collected by Thurid Otto. Preparations of the female crabs were also conducted by Thurid Otto. Investigation and analyses of the sections and writing of manuscript were done by Thurid Otto with helpful comments of Dirk Brandis.

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## **DANKSAGUNG**

Mein großer Dank gilt allen Personen und Institutionen, welche die Durchführung dieser Dissertation ermöglicht haben.

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## **ERKLÄRUNG**

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig erarbeitet habe. Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft angefertigt worden. Die Dissertation ist nach Form und Inhalt meine eigene Arbeit und es wurden keine anderen als die angegebenen Hilfsmittel verwendet. Diese Arbeit wurde weder ganz noch zum Teil einer anderen Stelle im Rahmen eines Prüfungsverfahrens an einer Universität vorgelegt. Dies ist mein bisher erstes Promotionsverfahren und die Promotion soll im Fach Zoologie erfolgen.

Kiel, den

Thurid Otto

