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Genetic diversity of the non-native crab *Rhithropanopeus harrisii* (Brachyura: Panopeidae) in the Polish coastal waters – an example of patchy genetic diversity at a small geographic scale

by

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Abstract

The American panopeid crab species *Rhithropanopeus harrisii* (Gould, 1841) is listed as an non-native species in European waters. In Poland, it occurred in the 1950s at two sites at the Baltic Sea coast, the Dead Vistula River (DVR) and the Vistula Lagoon (VL). Almost 50 years later, two additional populations were identified in the Gulf of Gdańsk (GG) and its inner part, Puck Bay (PB). In the present study, we sequenced and analyzed part of the mitochondrial cytochrome oxidase subunit I gene of the four Polish populations of *R. harrisii* in order to assess their genetic diversity and connectivity. The analyzed sequences of a length of 989 base pairs revealed eight different haplotypes. The highest number of haplotypes (n=6) was observed in the population from GG, whereas the lowest (n=3) in VL. The most common haplotype was recorded in 37% of the analyzed individuals. Pairwise Φ_{ST} values were mostly non-significant, with the exception of the comparison between DVR and VL (Φ_{ST} = 0.267; P < 0.05) and between PB and VL (Φ_{ST} = 0.194; P < 0.05), indicating a restricted gene flow or different sources of colonization.

Key words: *Rhithropanopeus harrisii*, coastal waters, genetic diversity, restricted gene flow, invasion biology

Introduction

Human-mediated introductions of alien species are regarded as a global problem due to the ecological and economic consequences they can cause (Lowe et al. 2000, Rudnick et al. 2000, d'Udekem d'Acoz 2006). Our current knowledge about this matter is largely based on the presence and abundance data, whereas information on the origin and genetic diversity of non-native species, as well as the adaptive capacity and the functional role in new environments is rather sparse. Therefore, integrating genetics and ecology is crucial in the context of biological invasions. Insights from these fields, combined with the understanding of evolutionary processes, could determine whether an invasion may take place, succeed, or fail (Sakai et al. 2001, Lee 2002, Lawson Handley et al. 2011, Seebens et al. 2013, Markert et al. 2014). Molecular identification and population genetics render new and exciting tools to improve our understanding of the population dynamics during biological invasions and have already shed light on the management, prevention and restoration strategies (Holland 2000, Sakai et al. 2001, Le Roux & Wieczorek 2009).

The American Harris mud crab *Rhithropanopeus harrisii* has been described by Grozholz & Ruiz (1996) as one of the most widely distributed brachyuran invaders worldwide. Its native areas are saline to brackish waters of the northwest Atlantic from Canada to the Gulf of Mexico (Williams 1984). This species reached Europe by ship transport (Rodriguez and Suarez 2001, Projecto-Garcia et al. 2010) and was first described in the Netherlands (Maitland 1874). Thereafter, *R. harrisii* gradually spread into other European regions, including countries with Baltic coastlines, e.g. Germany (Nehring & Leuchs 1999), Denmark (Jensen & Knudsen 2005), Poland (Demel 1953, Kujawa 1957, Michalski 1957) and most recently Estonia (Kotta & Ojaveer 2012) and Finland (Fowler et al. 2013). In Polish coastal waters, *R. harrisii* occurred in 1951 and since then, a stable and dense population has been observed in the oligohaline waters of the Vistula Lagoon (VL) (Demel 1953, Rychter 1999) and Dead Vistula River (DVR) (Michalski 1957, Turoboyski 1973, Normant et al. 2004). However, in recent years, more frequent appearance as well as a sudden increase in the abundance of *R. harrisii* was noticed also in the Gulf of Gdańsk (GG) and in its inner part, Puck Bay (PB), where clusters of crabs were observed (Hegele-Drywa & Normant 2014). The sudden appearance of *R. harrisii* in these waters is somewhat surprising because, as mentioned before, for decades stable populations have occurred in the adjacent waters of DVR and VL (Turoboyski 1973; Janta 1996; Rychter 1997, 1999; Normant et al. 2004), and despite a direct connection with DVR, *R. harrisii* was absent for more than 50 years in the Gulf of Gdańsk (Hegele-Drywa & Normant 2014). According to Cronin (1982) and Cronin & Forward (1986), larvae of the Harris mud crab stay within estuaries, and do not drift far from the parental population. Such a behavior of larvae could be an explanation for local endemism. In general, however, estimates of dispersal distances are mostly indirect (Levin et al. 1993) and derived from inferences of oceanography (Lee et al. 1994), larval biology (Emlet et al. 1987), or the genetics of adult populations (Palumbi 2001). Moreover, most of the documented discontinuities in the gene flow along a coast occur due to natural barriers, water currents or genetic isolation by distance (Palumbi 2003, Cassone and Boulding 2006, Patarnello et al. 2007). It is worth mentioning that along with the patchy distribution of *R. harrisii* observed in European waters, the crab also demonstrates genetic heterogeneity among the investigated European populations (Projecto-Garcia et al. 2010). A similar situation was observed in the Texas freshwater reservoirs where Harris mud crab is distributed among five river systems and revealed genetic population structure among these populations (Boyle et al. 2010). This can be explained by multiple colonization events (founder effects) and pathways via ballast water, fouling communities on the hulls of vessels, or other human activities. Such a situation may also play a role in the Polish coastal populations of *R. harrisii* due to the existence of two large harbors in the Gulf of Gdańsk (GG). Moreover, the studied populations were collected from habitats characterized by diverse abiotic factors (e.g. temperature, salinity, type of bottom) as well as the availability and quality of potential food (Wiktor 1990; Kruk-Dowgiałło 1994; Żmudziński 1957; Pliński 1999; Osowiecki 1998, 2000; Kruk-Dowgiałło & Szaniawska 2008; Janas et al. 2004; Janas 2005; Łysiak-Pastuszak et al. 2006; Hegele-Drywa & Normant 2009, 2014).

Nevertheless, the occurrence of four not directly

connected populations on such small geographic scale, with two of them established more than 60 years ago, and two established relatively recently, contributed to undertaking this research. Furthermore, it should be emphasized that while the genetic diversity of the Harris mud crab was already examined (Boyle et al. 2010, Projecto-Gracia 2010), there is no genetic information available to date on this species in non-native regions on such a small scale. Therefore, it would be of great importance to evaluate the potential gene flow and dispersal capacity of this species, in order to monitor its future spread and to understand its genetic diversity. This shall be achieved through analyzing the population genetics of *R. harrisii* from four sites in the Polish coastal waters.

The objective of his study was to determine the genetic diversity of *R. harrisii* from four populations in the Polish coastal waters and to test the following hypotheses: 1) *R. harrisii* from recently established populations (PB, GG) in the Polish coastal waters is genetically similar to the older Polish populations (DVR, VL) and thus probably colonized locally, and 2) the small-scale geographic isolation of newly established populations as well as larval retention mechanisms in this species may result in differences in local genetic diversity.

Materials and methods

Specimens (n=80) were collected in 2010 and 2011 from four localities (n=20 from each locality) along the Polish coast: Puck Bay (PB), the Gulf of Gdańsk (GG), the Dead Vistula River (DVR), the Vistula Lagoon (VL) (Fig. 1) and preserved in 96% ethanol.

Genomic DNA was extracted from muscle tissue of walking legs using the Puregene kit (Gentra Systems). A fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified through the polymerase chain reaction (PCR; 40 cycles: 45 s at 95°C, denaturing; 1 min at 54-56°C, annealing; 1 min at 72°C, extension; final extension of 5 minutes at 72°C). The following primers were used COL 14R 5'-GCA TGA GCT GGT ATA GTA GG-3' and COH 12 5'-GGY ATA CCR

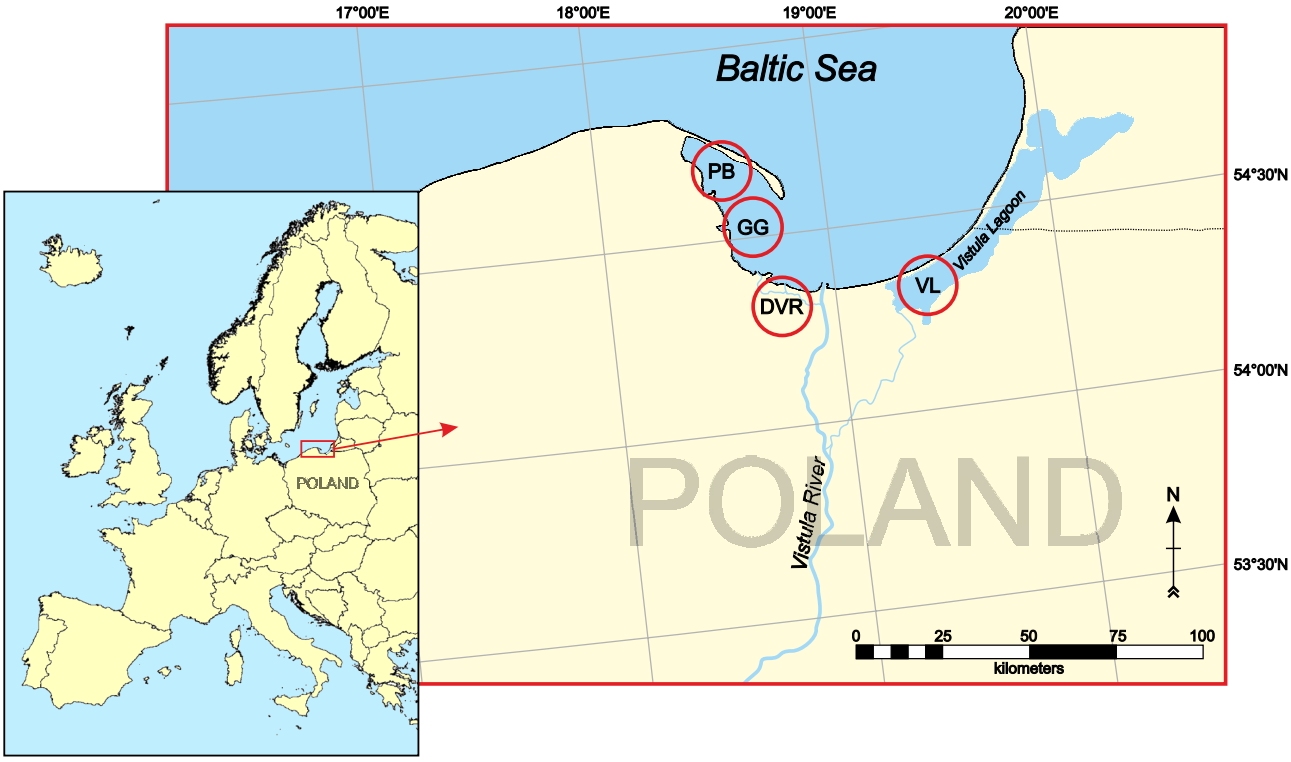

Figure 1

Rhithropanopeus harrisii collection sites in the Polish coastal waters: Puck Bay (PB), the Gulf of Gdańsk (GG), the Dead Vistula River (DVR), the Vistula Lagoon (VL)

TTT ART CCT AAR AA-3'. PCR products were outsourced for sequencing to LGC Genomics. The resulting sequences were edited and aligned with BioEdit (Hall 1999). The sequences were submitted to EMBL and are available under accession numbers: LN810557-LN810636.

Genetic diversity indices (nucleotide diversity π and haplotype diversity *h*) were assessed through DnaSP 4.0 (Rozas & Rozas 1999). The following additional tests were run with Arlequin 3.11 (Excoffier et al. 2005): AMOVA analysis (Φ-statistics) for the analysis of variance; neutrality tests Tajima's *D* and Fu's *Fs*, which test whether the sampled populations follow the neutrality model (i.e. genetic variability maintained by a balance between mutations and genetic drift; Avise 2004); mismatch analyses using the minimization of the sum of squared deviations (SSD) between the observed mismatch distribution and its expectation under the model of sudden expansion, discriminating distributions that result from expansion or from stationary populations (Harpending et al. 1993, Schneider & Excoffier 1999); and the raggedness index that quantifies the smoothness of the observed mismatch distribution, showing low values when the populations are stationary or suffered a bottleneck (Harpending et al. 1993, Harpending 1994). Moreover, a pairwise genetic distance (Φ_{ST}) was computed among populations from all locations (Excoffier et al. 2005). A statistical parsimony network of haplotypes was constructed using the program TCS 1.21 (Clement et al. 2000).

Results

A dataset of 80 sequences and an alignment length of 989 base pairs (bp) was obtained, revealing eight different haplotypes with 16 variable sites. The highest number of haplotypes within the populations (n=6) was observed in the GG population, whereas the lowest (n=3) was observed in the VL population. Haplotype A was the most common haplotype and was recorded in over 37% of the analyzed individuals, but only two haplotypes (B and C) were found in all populations, as haplotype A was missing from the VL population (Table 1).

Table 1

Haplotypes and the corresponding numbers of specimens carrying this haplotype of *Rhithropanopeus harrisii* from the Polish coastal waters: Puck Bay (PB), the Gulf of Gdańsk (GG), the Dead Vistula River (DVR), the Vistula Lagoon (VL)

Population	No. of individuals	Haplotypes	No. of individuals per haplotype
PB	20	A	10
		B	7
		C	2
		D	1
GG	20	A	7
		B	5
		C	5
		17GG	1
		18GG	1
		19GG	1
DVR	20	A	13
		B	2
		C	4
		D	1
VL	20	B	11
		C	8
		7VL	1

The haplotype network (Fig. 2) confirms the presence of three haplogroups (A, B, C). The highest haplotype diversity (0.784±0.053) was observed in GG and the lowest (0.553±0.111) in the DVR population, while the nucleotide diversity was the lowest within the populations from DVR and PB (Table 2).

We can conclude from the neutrality tests that all populations are close to a mutation-random drift

Table 2

Measures of genetic diversity of *Rhithropanopeus harrisii* from the Polish coastal waters: Puck Bay (PB), the Gulf of Gdańsk (GG), the Dead Vistula River (DVR), the Vistula Lagoon (VL)

Population	N	No. of haplotypes	No. of polymorphic sites	Haplotype diversity (h ± SE)	Nucleotide diversity (π ± SE)
PB	20	4	12	0.647 ± 0.072	0.00425 ± 0.001
GG	20	6	15	0.784 ± 0.053	0.00570 ± 0.001
DVR	20	4	12	0.553 ± 0.111	0.00406 ± 0.001
VL	20	3	11	0.563 ± 0.063	0.00537 ± 0.001

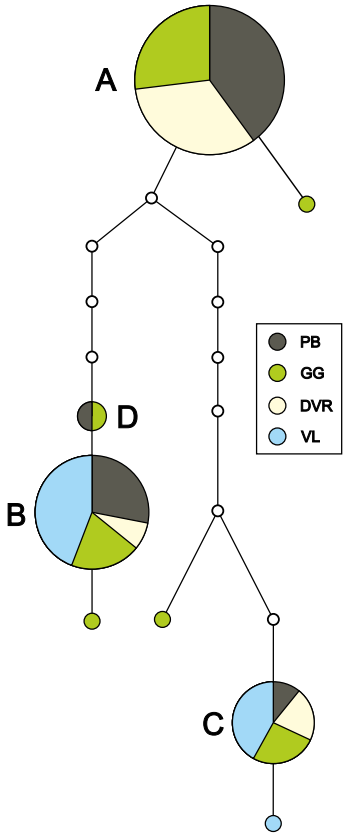

Figure 2

Minimum spanning network based on a 989 bp fragment of the COI gene of *Rhithropanopeus harrisii* from the Polish coastal waters: Puck Bay (PB), the Gulf of Gdańsk (GG), the Dead Vistula River (DVR), the Vistula Lagoon (VL), with four shared haplotypes constructed with TCS. White circles indicate missing haplotypes

equilibrium (neutral model), since the model was not rejected (Table 3). Despite being non-significant, high Tajima's *D* values (>2) obtained for VL may indicate association with genetic bottlenecks or founder effects. The SSD tests did not render similar results as all populations, except DVR, rejected the sudden expansion model. Although taking into account the raggedness index, the obtained high values might be due to stationary or bottleneck populations. Moreover, clear multimodal distribution of all four populations may allow us to exclude an experience of expansion event.

Although the AMOVA analysis suggests that the largest fraction of the variation resides within populations (81.14%), this result is not significant ($P = 0.25593$). However, a significant difference ($P< 0.0001$) is observed for the variation among regions (PB+GG and DVR+VL), even if the percentage of variation it represents is smaller (17.64%) (Table 4).

The highest pairwise genetic distances (Φ_{ST}) amounting to 0.267 ($P<0.05$) were noted between populations from DVR and VL, suggesting a significantly reduced gene flow. A similar situation was observed between populations from PB and VL (Table 5).

Discussion

Ecologists use molecular genetic techniques and analyses to address a wide variety of questions, including dispersal, genetic diversity of a species or source of an introduction (Selkoe & Toonen

Table 3

Measures of neutrality tests and mismatch distribution with their corresponding *P* values for the sampled populations of *Rhithropanopeus harrisii* from the Polish coastal waters: Puck Bay (PB), the Gulf of Gdańsk (GG), the Dead Vistula River (DVR), the Vistula Lagoon (VL)

Population	N	Tajima`s *D*	*P*	Fu`s *Fs*	*P*	SSD	*P*	Raggedness index	*P*
PB	20	0.873	0.815	4.686	0.980	0.176	<0.001	0.371	<0.001
GG	20	1.230	0.832	3.204	0.964	0.063	0.050	0.119	0.040
DVR	20	0.672	0.759	4.460	0.982	0.174	0.100	0.100	0.150
VL	20	2.528	0.993	8.126	0.999	0.537	<0.001	0.540	0.940

Table 4

Hierarchical analysis of variance of squared matrices of distances between all haplotypes of *Rhithropanopeus harrisii*

Source of variation	Variance components	% Variation	*P* value	Φ-statistic
Among regions	0.52083	17.64	<0.0001	$\Phi_{SC} = 0.01481$
Among populations within regions	0.03603	1.22	0.25247	$\Phi_{ST} = 0.18858$
Within populations	2.39605	81.14	0.25593	$\Phi_{CT} = 0.17638$

Table 5

Pairwise Φ_{ST} values among populations of *Rhithropanopeus harrisii* from the Polish coastal waters: Puck Bay (PB), the Gulf of Gdańsk (GG), the Dead Vistula River (DVR), the Vistula Lagoon (VL). Values with asterisks indicate significant differences ($P<0.05$).

Population	PB	GG	DVR	VL
PB	<0.00001			
GG	0.00785	<0.00001		
DVR	0.03509	0.01100	<0.00001	
VL	0.19358 *	0.08005	0.26680 *	<0.00001

2006). The aim of our study was to provide the corresponding data for the non-native brackish water crustacean species *Rhithropanopeus harrisii*. With the knowledge of physicochemical water parameters as well as food base (Osowiecki 1998, 2000; Paturej 2006; Hegele-Drywa & Normant 2009) of Harris mud crab from the Polish coastal waters, we tried to determine if the newly established populations were genetically similar, and thus derived from the older populations. Furthermore, the genetic relationships and gene flow among the four populations inhabiting this region were quantified and interpreted.

Eight haplotypes of the COI gene were found among 80 specimens of *R. harrisii* across the four sampled Polish populations. Such a reduced number of haplotypes for *R. harrisii* is not surprising in non-native regions. Similar results were obtained by Projecto-Gracia et al. (2010) for *R. harrisii* from the European waters based on a shorter fragment of the COI gene. On the other hand, Petersen (2006) found a haplotype diversity of 0.9568 in a native population of *R. harrisii* from the Neuse River (North Carolina, USA). Comparisons with data from this study suggest genetic impoverishment during the colonization process in Poland. However, haplotype numbers in other crab species from their native range have shown to vary from 30 to 45%, e.g. the lined shore crab *Pachygrapsus crassipes* and the estuarine crab *Hemigrapsus oregonessus* (Cassone & Boulding 2006, Petersen 2007).

Only two of the shared haplotypes for *R. harrisii* from the Polish coastal waters (B and C) were present in all populations, as the most common haplotype A was not recorded in the VL population. This pattern has at least three possible explanations, in addition to the random distribution of haplotypes from the overall gene pool: firstly, the VL population may have a genetically distinct founding source from that of the three other populations. Secondly, it may be a result of a much smaller founding population (genetic bottleneck), or thirdly, there were repeated introductions into the other populations but not into VL. The geographic location of VL, with a possible isolation effect by the Vistula Spit, may contribute to preventing the gene flow. The presence of unique haplotypes, even if at low frequencies, e.g. in GG, could either be explained by newly introduced haplotypes associated with the proximity of the commercial shipping harbors, or perhaps represent rare haplotypes that remain unsampled due to their low frequencies. However, even in cases of thorough sampling, it is possible that more haplotypes exist in unsampled specimens from the analyzed population (Muirhead et al. 2008). Despite non-significance, results obtained from both the Tajima`s *D* and Fu`s *Fs* tests support the postulate that Polish populations of *R. harrisii* are experiencing the bottleneck effect, possibly as a result of founder effects, which is usually observed in introduced populations (Fu 1997, Excoffier et al. 2005, Dlugosch & Parker 2008). Multimodal pairwise mismatch distribution and high values of the raggedness index are observed in Polish populations, which are typical for stationary or bottleneck populations (Harpending et al. 1993, Harpending 1994). Also according to Projecto-Gracia et al. (2010), bottleneck effects in other *R. harrisii* populations have occurred during colonization of non-native regions (e.g. European). Based on the above, we can assume that stationary populations without obvious features of expansion reveal different genetic diversity metrics. Also our results performed in AMOVA allow concluding that some populations from the Polish coastal waters are not connected by gene flow. We did not find the same pattern as Projecto-Gracia et al. (2010), as in our case variation among regions was significant and amounted to over 17%. On the other hand, taking into account the relatively small distances separating

the analyzed populations in our study as compared to relatively large distances between populations in the study of Projecto-Gracia et al. (2010), the results from these two studies are not directly comparable. Moreover, pairwise Φ_{ST} indicates a limited gene flow between populations in two cases only (VL-PB and VL-DVR), whereas the gene flow among other populations was not significantly restricted. In the case of the population from VL, which is the most distant sampling site in relation to other sites, the founding population was probably much smaller than in the case of PB or DVR, resulting in a more pronounced founder effect. Possibly the VL population did not experience as many new successful introductions as PB or DVR or was founded by fewer individuals. Similar observations were made by Boyle et al. (2010) for *R. harrisii* from different freshwater reservoirs in Texas.

The obtained results suggest that our first hypothesis was correct. *R. harrisii* from recently established populations (PB, GG) in the Polish coastal waters are genetically similar to the older Polish populations (DVR, VL) and thus probably colonized from the older population. Due to lack of distant haplotypes as well as the occurrence of the main haplotypes B and C in all the analyzed populations, we can assume that the colonization process took place locally. However, the largest values for haplotype as well as nucleotide diversities occurred in GG and the lowest in DVR. Such high values for haplotype and nucleotide diversities for GG and PB are surprising due to the recent establishment of these populations, but might be due to the open character of their habitat as well as the vicinity of commercial shipping harbors. The increase in haplotype diversity, as a measure of the uniqueness of a particular haplotype in a given population, could be explained by recurring introductions through ballast water or hull fouling communities. According to Walk & Modrzejewska (2011), every year most of the vessels arriving at the trade harbors in GG are from other parts of the Baltic Sea. The second largest group are ships arriving from the North Sea and the third group are vessels coming from the southern coast of Ireland, Great Britain and western coast of France. Projecto-Gracia et al. (2010) noted that in the European populations of *R. harrisii*, there is a tendency for older populations to have higher genetic diversity. If that is true, we would expect the populations of *R. harrisii* discovered more than 60 years ago in DVR and VL to be more diverse than the younger ones, but this was not supported by our data. Instead, there seems to be a bottleneck effect which has decreased the haplotype and nucleotide diversities in older populations due to a very small initial population size (Tajima 1989, Projecto-Gracia et. al 2010). What is most astonishing is the lack of range expansion over this period of time from DVR, in spite of the direct physical connection between these sites.

The obtained results also allow us to accept the second hypothesis: small-scale geographic isolation of the newly established populations as well as larval retention mechanisms of this species may result in differences of local genetic diversity. The extent to which different populations of the same species are linked by exchange of larvae, recruits, juveniles, or adults is referred to as connectivity. Patterns of connectivity in a network of protected or isolated areas are important to understand the supply of adults and larvae into and out of a reservoir (Palumbi 2003). All populations of the Harris mud crab analyzed in this study occur in waters surrounded by natural barriers. The direct access to PB and GG as well as DVR is prevented by the Hel Peninsula, which protects these areas from physical impact of the open water of the Baltic Sea, like storms and waves (Pruszak 2004). A similar situation is observed in VL which is separated from open waters of the Baltic Sea by the Vistula Spit, a sandy peninsula forested to secure dune stability (Różyński et al. 2013). Furthermore, environmental heterogeneity is expected to be an important factor determining the occurrence, range expansion and gene flow, with invasions accelerating as individuals encounter favorable conditions, and decelerating as they reach less favorable environments (Colautti & MacIsaac 2004, Urban et al. 2008, Galil et al. 2009). All localities at the Polish coast where *R. harrisii* specimens were found offer a different but wide variety of shelters, good food base, as well as proper physicochemical water parameters (Turoboyski 1973; Rychter 1999; Paturej 2006; Hegele-Drywa & Normant 2009, 2014). This can be confirmed by the appearance of new populations in water areas where they have not been observed before (Czerniejewski & Rybczyk 2008; Czerniejewski 2009, Kotta & Ojaveer 2012, Fowler et al. 2013, Hegele-Drywa & Normant

2014). The gene flow in marine systems is generally predicted to be correlated with larval dispersal potential (Scheltema 1975, Hedgecock 1986), which in turn may strongly influence the geographical range and genetic structuring of populations (Hedgecock 1986). As already mentioned, the Harris mud crab exhibits larval retention, which is manifested in vertical migrations of zoeae around the depth where no horizontal motion of water occurs. Migration follows the tides, as larvae ascend during the flood tide and descend during the ebb tide (Cronin 1982). This migration pattern is typical for species that are retained in estuaries and is also observed for copepods, mysids, and fish larvae (Forward et al. 2001). However, there are no tides in the Polish coastal waters in the Baltic Sea, meaning that only ambient currents might influence the dispersal potential, which then is determined primarily by the length of the larval pelagic stage (Scheltema 1971, Grantham et al. 2003). Additionally, according to Forward (2009), the time before the metamorphosis is reduced by exposure to estuarine water, adult odor, or high salinity, but is prolonged by exposure to offshore water or low salinity. Since larvae are retained in estuaries, but occur in many estuaries, this flexibility allows *R. harrisii* to delay metamorphosis until megalopae encounter a suitable area for their settlement. According to this information, *R. harrisii* is an eurytopic organism that has found favorable niches, and due to suitable conditions in the Polish coastal waters and the larval retention mechanism, reveal different genetic diversity in the studied populations.

According to our findings, *R. harrisii* apparently still expands its range in the European waters, but so far little is known about its invasiveness in non-native ecosystems. With this paper we present for the first time the small-scale geographic genetic structure of Harris mud crab populations in a non-native environment. The obtained results might confirm the invasive potential of this species as well as good adaptation to environments in the Polish coastal waters. Among other adaptations, this is manifested in inter-population genetic differentiation. Such genetic diversification might be important to the success of biological invasions (Mooney and Cleland 2001, Sakai et al. 2001) and possibly responsible for phenotypic variability which might crucial for the colonization success (Parker et al. 2003, Poulin et al. 2005, Lee et al. 2011). Moreover, it can be assumed that even if the species does not pose a threat to local biodiversity at present, this may change in the future. According to the Marine Strategy Framework Directive (Ojaveer et al. 2014), *R. harrisii* – as a relatively small organism not spreading radically, but with the increasing number of individuals in the vicinity of the parental population – should be still monitored to foresee its status in non-native regions. Therefore, it is necessary to associate ecological, morphological and genetic analyses together with environmental surveys, to prevent an unexpected introduction of Harris mud crab specimens in the waters where it was never present before.

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