MARINE BIOGEOGRAPHIC BOUNDARIES AND HUMAN INTRODUCTION
ALONG THE EUROPEAN COAST REVEALED BY PHYLOGEOGRAPHY
OF THE PRAWN *PALAEMON ELEGANS*

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14 **Abstract**

15 A phylogeographic analysis is carried out for the widely distributed European littoral prawn
16 *Palaemon elegans* in order to test for potential genetic differentiation and geographic structure.
17 Mitochondrial sequences were obtained from 283 specimens from the northeastern Atlantic, the
18 Baltic, Mediterranean, Black and Caspian Seas. Our study revealed a surprisingly complex
19 population structure. Three main haplogroups can be separated: one from the Atlantic (Type I) and
20 two from the Mediterranean (Types II and III). While the Mediterranean types occur in sympatry, a
21 clear phylogeographic break was observed along the Almería-Oran-Front separating Type I and
22 giving evidence for a genetic isolation of Atlantic and Mediterranean populations. Type III
23 represents the most distinct haplogroup with high levels of nucleotide divergence, indicating the
24 occurrence of a cryptic species with a Messinian origin. The colonization of the southeastern Baltic
25 Sea is most likely due to human introduction.

26
27 **Keywords:** Cox1, 16SrRNA, phylogeography, Messinian Crisis, cryptic species
28

29 INTRODUCTION

30 The common European littoral prawn *Palaemon elegans* Rathke, 1837 (Crustacea: Caridea:
31 Palaemonidae) is adapted to cope with extremely variable environmental conditions: it tolerates a
32 wide range of salinities, temperatures and oxygen (Berglund 1980; Berglund & Bengtson 1981).
33 The species can be found from hypersaline lagoons to tidal rock pools, shallow rocky coasts,
34 *Zostera*, *Posidonia* and *Cymodocea* sea grass meadows to partly brackish estuaries. It is also
35 common along man-made rock jetties and within harbours. The native distribution ranges from the
36 Atlantic Ocean (from Scotland and Norway to Mauritania including the Azores, Madeira and
37 Canary Islands) to the entire Mediterranean Sea and Black Sea (d'Udekem d'Acoz 1999).
38 Nowadays, the species is also found in the Aral and Caspian Seas, but this occurrence goes back to
39 unintentional introductions in the 1950s (Zenkevich 1963; Grabowski 2006). Since 2000, *P.*
40 *elegans* has also colonized the southern coast of the Baltic Sea, where it is already replacing the
41 native *Palaemon adspersus* Rathke, 1837 (Grabowski 2006). It is discussed, if its appearance there
42 is due to natural dispersal or to human introduction (Grabowski 2006). The broad ecological niche
43 and the recent range expansion of *P. elegans* make the prawn an important player within the
44 European marine littoral fauna.

45 The dispersal capacities of this species are probably high, since the complete larval development
46 takes place in the ocean with six to nine (e.g Fincham 1977) zoeal stages (depending on
47 temperature and other environmental factors), potentially resulting in high rates of gene flow and
48 panmictic population structure. Nevertheless, Berglund and Lagercrantz (1983) found significant
49 genetic heterogeneity by horizontal starch gel electrophoresis including six sampling sites along
50 the Atlantic coast from Sweden to France. Fortunato and Sbordoni (1998) also documented high
51 genetic variability with allozymes within the Mediterranean Sea. Previously, morphological
52 variations had been suggested by De Man (1915).

53 The narrow connection between the Atlantic Ocean and the Mediterranean Sea has been
 54 proposed to represent an important phylogeographic break in several marine species based on
 55 oceanographic models and phylogeographic studies (e.g. littoral fish species,; shrimps,
 56 Luttikhuizen 2008; turtles, Reece et al. 2005; cirripedes, Pannancciulli et al. 1997; bivalves,
 57 Quesada et al. 1995; krill, Zane et al. 2000, Triantafyllidis et al. 2005; sea urchins, Duran et al.
 58 2004; fish, Borsa et al. 1997, Galarza et al. 2009; cuttlefish, Pérez-Losada et al. 2002). A recent
 59 review of more than 70 papers by Patarnello et al. (2007) reveals a combined signature of
 60 vicariance, palaeoclimatic fluctuation and life-history traits on the Atlantic-Mediterranean
 61 phylogeographical patterns. The Mediterranean Sea was strongly affected by well documented sea
 62 level regressions and Pleistocene glaciations and was isolated several times from the Atlantic. In
 63 the late Miocene (Messinium), a sea level regression separated the Mediterranean Sea from the
 64 Atlantic, leading to full or partial desiccation of the Mediterranean Sea (Hsü et al. 1977). At the
 65 beginning of the Pliocene, Atlantic water flooded the Mediterranean Basin again, allowing Atlantic
 66 species to re-colonize the Mediterranean. In addition, sea level regressions limited the biotic
 67 exchange through the Strait of Gibraltar during the Quaternary glacial periods (Vermeij 1978), but
 68 for shorter time intervals. The opening and closing of the Strait of Gibraltar has made the
 69 Mediterranean a region of high endemism and a generator of diversity. This narrow oceanic street
 70 with uni-directional surface currents could still represent an extant barrier to gene flow between
 71 Atlantic and Mediterranean populations and be responsible for ongoing allopatric separation. It is
 72 possible that dispersal is prevented by oceanographic patterns or by behavioural mechanisms that
 73 act to prevent transport of larvae between populations (Palumbi 2003).

74 The Mediterranean crustacean fauna has been postulated to have originated by repeated or
 75 continuous multiple colonization events with adaptation to specialized habitats and adaptive
 76 radiation (Almaça 1985). This could have lead to marked genetic differences resulting in cryptic

77 speciation and a high proportion of endemism (28.6 % according to Hofrichter 2002 and Patarnello
78 et al. 2007) in the Mediterranean Sea. Knowlton (1993, 2000) indicated that the phenomenon of
79 cryptic species is widespread in the oceans. Belfiore et al. (2003) define them as morphological
80 indistinct lineages separated by species level genetic differences. Several studies have shown the
81 great utility of molecular markers in diagnosing endemism and cryptic speciation, even when
82 traditional markers fail or are ambiguous (Avisé 2004). Cryptic species of *Clavelina* (Ascidiae)
83 could be identified in the northwestern Mediterranean with mtDNA data (Tarjuelo et al. 2001).
84 *Carcinus aestuarii* Nardo, 1847 of the Mediterranean Sea is morphologically very similar to the
85 Atlantic *Carcinus maenas* (Linnaeus, 1758). Its species status was finally confirmed with genetic
86 analyses of the 16SrRNA gene (Geller et al. 1997) and the Cox1 gene (Roman & Palumbi 2004).
87 The purpose of this study is to determine the degree of genetic differentiation within *Palaemon*
88 *elegans* along the corresponding Atlanto-Mediterranean (mostly European) coastline, with focus on
89 a population-level comparison using DNA-sequences of two mitochondrial genes, 16S rRNA and
90 Cox1. Our comparison comprises animals from the Atlantic and Mediterranean coast, including
91 Norway, the North Sea, the Baltic Sea, the Black and Caspian Seas. Thereby, we also address the
92 questions, whether the Strait of Gibraltar has a measurable effect on gene flow between Atlantic
93 and Mediterranean populations and if restricted gene-flow is resulting in cryptic speciation.
94

95 MATERIALS AND METHODS

96

97 *Sampling*

98 Specimens of *Palaemon elegans* were collected from 32 different coastal localities, comprising the
 99 Baltic Sea (2004-05), Norwegian Sea (2007), North Sea (2004-05), Atlantic coast of Portugal
 100 (2006), Spain (2004-05), the Canary Islands (2005), the Mediterranean coast of Spain (2004-06),
 101 Croatia (2004-2005), Italy (2006), Greece (2003) Tunisia (2006), Turkey (2007), Ibiza (2003 and
 102 2007), the Black Sea (2005) and donated from the Caspian Sea (see Tables 1 and 2, Figure 1). The
 103 samples were collected with a hand net, most often from rock pools, along man-made rock jetties
 104 and within harbours. After collection, the samples were directly preserved in 95% ethanol.

105

106 *DNA isolation, PCR amplification and DNA sequencing*

107 A total of 283 specimens of *Palaemon elegans* were used for this study. For the genetic analyses,
 108 genomic DNA was extracted from the muscle tissue of the abdomen using the Puregene kit (Gentra
 109 Systems). We amplified mitochondrial DNA from the large subunit rRNA (16S) gene, and from
 110 the cytochrome oxidase subunit I (Cox1) gene by means of polymerase chain reactions (PCR) (4
 111 min 94°; 40 cycles with 45 sec 94° / 1 min 48-50° / 1 min 72° denaturing/annealing/extension
 112 temperatures respectively and 10 min final extension at 72°). The following primer combinations
 113 were used: 16L2 (5'-TGC CTG TTT ATC AAA AAC AT-3') (Schubart et al. 2002) and 16H3 (5'-
 114 CCG GTT TGA ACT CAA ATC ATG T -3') (Reuschel & Schubart 2006). In the case of Cox1,
 115 the species-specific primers COL6Pe (5'- AAG ATA TTG GAA CTC TAT AT-3') and COH6Pe
 116 (5'- GTG SCC AAA GAA YCA AAA TA-3') were newly designed and applied. PCR products
 117 were purified with Quick / Sure Clean (Bioline), ethanol-precipitated, resuspended in water and
 118 sequenced with the ABI BigDye terminator mix (Big Dye Terminator® v. 1.1 or 3.1 Cycle

119 Sequencing Kit; Applied Biosystems) in ABI Prism automated sequencers (ABI Prism™ 310;
 120 Applied Biosystems). Sequences were compared in DNA alignments of 520 basepairs (bp) 16S and
 121 621 bp Cox1 mtDNA.

122

123 ***Data analysis***

124 The sequences were analyzed and proofread with the program ABI Sequencing Analysis® 3.4
 125 (Applied Biosystems) and manually aligned with BioEdit (Hall et al. 1999), excluding the primer
 126 regions. No ambiguities were encountered during alignment. For genetic comparisons of
 127 populations, parsimonious networks for both genes were built with TCS (estimating gene
 128 genealogies version 1.21; Templeton et al., 1992). Loops in the networks were solved after the
 129 three criteria suggested by Crandall and Templeton (1993): (1) geographic criterion, (2) frequency
 130 criterion and (3) topological criterion. Genetic heterogeneity within populations was estimated as
 131 haplotype diversity (h) (Nei & Tajima 1981), nucleotide diversity (π) (Nei 1987) and the mean
 132 number of pairwise differences (k) computed with DnaSP 4.00 (Rozas et al. 2003). The Φ_{ST} values
 133 of the more variable Cox1 dataset were calculated with Arlequin 3.1 (Excoffier et al. 2005) in
 134 order to estimate genetic divergences among populations. These comparisons were performed
 135 within the different haplogroups (see below). AMOVA analyses based on genetic distances were
 136 done with the 16S and the Cox1 dataset.

137

138 ***Mismatch analysis***

139 To determine the historical demography of the species and its genetic subunits we analysed the
 140 mismatch distributions with the model of Rogers and Harpending (1992) for the Cox1 dataset. The
 141 mismatch distributions are used to assess fit of haplotype data to the sudden demographic
 142 expansion model (Rogers & Harpending 1992). We tested the null hypothesis of neutrality which

143 may be rejected when a population has experienced population expansion (Tajima 1989).
144 Therefore, Tajima's D -test (Tajima 1989) and Fu's (1997) F_s test and their significance levels were
145 estimated with DnaSP 4.00 based on 1000 simulated re-sampling replicates. Mismatch distribution
146 analyses, under the assumption of selective neutrality, were also used to evaluate possible historical
147 events of population growth and decline (Rogers & Harpending 1992). Past demographic
148 parameters, including τ (Li 1977), θ_0 and θ_1 and their probabilities (Rogers & Harpending 1992)
149 were estimated with Arlequin and DnaSP.

150

151 ***Molecular clock***

152 A relative-rate-test was carried out with MEGA 4.0 (Tamura et al. 2007) to test the constancy of
153 evolutionary rates. We applied the relative rate tests between the common haplotypes of Type I and
154 Type III and the common haplotypes of Type II and Type III using *Palaemon serratus* (Pennant,
155 1777) from Ibiza as an outgroup. The mitochondrial clock calibrated for Cox1 of snapping shrimp
156 (Alpheidae) of 1.4 % sequence divergence between pairs of lineages per Myr was used (Knowlton
157 & Weigt 1998) to date the timing of population isolation.

158

159 **RESULTS**

160 A subset of 53 individuals were used for the analysis of the 16S rRNA over a length of 520 bp,
 161 resulting in 17 haplotypes (hts) and 29 variable sites, with 17 parsimony informative sites. The
 162 genetic heterogeneity revealed a relative high haplotype ($h = 0.811 \pm 0.04$) and nucleotide
 163 diversities ($\pi = 0.01438 \pm 0.00056$) and $k = 7.48$ as the overall mean number of pairwise
 164 differences.

165 Due to the higher variability of the mitochondrial Cox1 gene the sample size was extended to 283
 166 individuals and a fragment of 621 bp was used. From Crete we only had one specimen which was
 167 donated by a colleague. Overall, 136 different hts were detected resulting in high haplotype ($h =$
 168 0.950 ± 0.0009) and nucleotide ($\pi = 0.0472 \pm 0.00060$) diversities. The mean number of
 169 pairwise differences was $k = 29.28$. A total of 131 variable sites (21.1 %) were detected with 85
 170 parsimony informative sites. Most substitutions involved transitions, with a transition/transversion
 171 ratio amounting to 3.3. Nine mutations resulted in amino acid substitutions and only two of the
 172 nine are fixed within haplogroups (see Figure 3).

173

174 ***Haplotype networks***

175 Within *Palaemon elegans*, the comparison of multiple sequences of 16S mtDNA and Cox1
 176 revealed the existence of three different haplogroups (referred to as Types I - III) for both of the
 177 genes (Figs. 2-3). Within these haplogroups there is always one most common haplotype, here
 178 accordingly referred to as HT I - III

179 For the 16S gene (Figure 2), 23 out of 53 sequences belong to the Atlantic Type I. Out of these,
 180 19 share a common haplotype (ht), which is suggested to represent the ancestral haplotype (HT I).
 181 Four additional haplotypes (hts) are separated by one transition from HTI. Type II is separated with
 182 at least six ($p=1.2\%$) and Type III with at least thirteen ($p=2.5\%$) fixed mutation steps from all

183 Type I haplotypes. Within Type II, the common ht (HT II), is recorded from five specimens (four
 184 other hts differ by one mutation and one by two mutations with respect to HT II). Type III is
 185 separated by at least nine differences ($p=1.7\%$) from hts of Type II and its common ht (HT III) was
 186 recovered from thirteen specimens (additional four hts are separated by one mutation and one ht by
 187 two mutations). The haplotype diversity, nucleotide diversity and the mean number of pairwise
 188 differences for each collection site are listed in Table 1.

189 In case of the Cox1 gene of *P. elegans* (Figure 3), the minimum spanning network shows a
 190 higher genetic differentiation in comparison to 16S and results in a multiple star-like minimum
 191 spanning tree. Type III hts could not be parsimony-connected according to the 95% significance
 192 criterion to the Type I-II network, and the corresponding sequences are separated by over 50
 193 mutation steps from those of the other two types. Type I was found in 80 specimens from Norway
 194 (Bergen), Germany (Eckernförde, Helgoland, Wilhelmshaven, Kiel), Portugal and Spain (Canary
 195 Islands, Cádiz, Granada, Málaga). The haplotype-diversity within this type was high ($h = 0.956 \pm$
 196 0.014 ; $N(ht) = 49$) and the nucleotide diversity relatively high ($\pi = 0.0013 \pm 0.00035$) (see also
 197 Table 2). The central ht of Type I (HT I) includes 14 individuals from different geographic regions;
 198 21 rare haplotypes are directly connected to HT I. Eleven specimens from northern Europe diverge
 199 as the so-called haplo-subgroup *b* from the main stem at the ht *a* (see Figure 2). Specimens
 200 belonging to ht *a* and its seven minimally diverged hts (haplo-subgroup *a*) are mostly from
 201 southern Europe (Portugal, Canary Islands, Granada), but also Norway. Most specimens of the
 202 Canary Islands were found in another haplo-subgroup around ht *c*, with four connected hts.
 203 Haplotype *a* is separated by four mutation steps and ht *c* by five mutation steps from the HT I on
 204 the shortest connecting line between HT I and HT II. HT I is separated from HT II by 12 mutation
 205 steps ($p=1.9\%$). Four fixed mutations separate all hts of haplogroups from Type I and II, whereas
 206 the most distant haplotypes within Type I differ by thirteen mutation steps.

207 The second haplogroup, Type II, includes 89 specimens exclusively from the Mediterranean
 208 Sea: Spanish coast (Ampolla, Salou, Villanova, Ebro, Girona, Ibiza), Italy (Fusaro, Calabria),
 209 Greece, Croatia, Tunisia and Turkey. We calculated similar values for the haplotype-diversity ($h =$
 210 0.930 ± 0.020 ; $N(h) = 52$) and nucleotide diversity ($\pi = 0.0056 \pm 0.0004$) as in Type I (see also
 211 Table 2). The postulated ancestral ht of Type II (HT II) is shared by 21 specimens and 27 hts are
 212 directly connected to HT II by only a few mutation steps in a star-like fashion. A haplo-subgroup
 213 around ht *d* is separated by three mutation steps and lies on the line connecting HT I and HT II.
 214 Twenty rare haplotypes diverge more or less directly from ht *d*. Within Type II, the most distant
 215 connections sum up to fourteen mutation steps.

216 Type III includes most of our specimens ($N=117$), but only 36 hts diverged here from the
 217 central ht resulting in a lower haplotype-diversity ($h = 0.771 \pm 0.041$) and nucleotide diversity (π
 218 $= 0.0029 \pm 0.00032$) (see also Table 2). The geographic distribution of Type III includes the
 219 Polish and German Baltic Sea and otherwise ranges from Mediterranean Spain (Garrucha, Mar
 220 Menor, Torreveja, Cabo de la Nao, Valencia, Ebro, Ampolla, Ibiza) to Italy (Fusaro, Livorno),
 221 Tunisia, the Black Sea and Caspian Sea. It was also found in one specimen from Croatia, Crete and
 222 Girona each. Type III has one common ht (HT III), which was found in 56 specimens. HT III is
 223 separated from HT I by 54 mutation steps ($p=8.7\%$) and from HT II by 53 mutation steps (8.5%).
 224 Out of these mutation steps, 26 are exclusive for Type III and therefore characteristic for this type
 225 (Table 3 and 4). Thirty-six hts or haplo-subgroups are separated by only a few mutation steps from
 226 HT III in a star-like fashion. Geographical overlap between Types II and III is so far recorded from
 227 the populations of Girona, Ampolla, Ebro, Ibiza, Fusaro, Croatia and Tunisia (see also Figure 4).
 228 Haplotype and nucleotide diversities and the mean number of pairwise differences for each
 229 collection site was always estimated only for the type which dominates the site as listed in bold in
 230 Table 2 in order to not confuse gene flow within types with possible cryptic species.

231 ***Population statistic parameters***

232 Analysis of molecular variance of the Cox1 dataset reveals a molecular variance between the three
 233 types of 93.68%, within populations of 5.98% and among populations within types of 0.33%. The
 234 AMOVA between all subpopulations reveals a significant mean value of overall Φ_{ST} of 0.94
 235 ($p>0.0001$). Pairwise Φ_{ST} values between populations of Types II and III are listed in Table 5. The
 236 comparison was done between all collection sites for which at least seven specimens were
 237 available. The Φ_{ST} values were analysed combining Type I and II (Table 5) and, due to the high
 238 genetic differentiation, separately within Type III (not shown). In the case of the few populations
 239 documenting geographical overlap between Type II and III (Ampolla, Girona, Ibiza, Fusaro,
 240 Croatia and Tunisia) the rare hts from the other type had to be excluded from the respective
 241 analysis to avoid overly pronounced differentiation due to possible influence of cryptic species.
 242 The number of included specimens is shown in Table 2. The analysis revealed significant
 243 differences between populations of Types I and II (Table 5). Within Type I and II, almost all
 244 possible Φ_{ST} comparisons including the Canary Islands, Norway (except to Kiel), and Helgoland
 245 (except to Cádiz) were significant. Within the Mediterranean Type II, the Φ_{ST} values are low and
 246 almost all of them non-significant. The same pattern was revealed for all Φ_{ST} values within Type
 247 III, being very low (>0.09) and only few of them being significant without a clear geographic
 248 pattern (not shown). To confirm restricted gene-flow between geographic regions in the
 249 distribution range of Type I we performed an additional AMOVA analysis. A comparison between
 250 the North Atlantic (lumping Norway, Helgoland, Wilhelmshaven, Kiel) and the Portuguese-
 251 Spanish Atlantic (lumping Portugal, Cádiz, Canary Islands) reveals a highly significant mean value
 252 of overall Φ_{ST} of 0.19031 ($p>0.0001$).

253

254

255 ***Mismatch analysis***

256 Three separate assemblages of mismatch distributions were constructed: (A) Type I; (B) Type II
 257 and (C) Type III based on the Cox1 results (Figure 5). The mismatch distributions for all types
 258 were not significantly different from the sudden expansion model of Rogers and Harpending
 259 (1992). The smooth unimodal mismatch distributions of the separated types and the statistics of the
 260 other neutrality tests, Tajima's D and Fu's F_s were significant and negative (Table 6), thus
 261 suggesting sudden population expansion. Negative values of Tajima's D suggest deviations from
 262 mutation-drift equilibrium, possibly caused by populations bottlenecks (Tajima 1989). The
 263 diversity indices and the magnitude of the values of Fu's F_s and of demographic estimates were
 264 similar in Type I and Type II. These results suggest that both types have undergone a sudden
 265 expansion around the same time. The steep display of the mismatch curve of Type III, the small τ
 266 and relatively small θ_0 are consistent with a recent sudden expansion from a small initial
 267 population (Rogers & Harpending 1992; Avise 2000; Patarnello et al. 2007), according to the idea
 268 that the size of the initial population is proportional to the steepness the leading face of the curve of
 269 the mismatch distribution (Rogers & Harpending, 1992). The diversity indices and the values of
 270 demographic estimates of Type III are different from these of Type I and II suggesting a distinct
 271 demographic history. The τ estimates appear to support that Type I and Type II have an older
 272 history than Type III. The pattern of the multiple star-like networks may suggest otherwise
 273 (Bremer et al. 2005), but Type III has the shortest maximum distances (10) between hts, again
 274 arguing for a more recent evolution than Types II and III.

275

276

277

278 **DISCUSSION**

279 For most marine species, a high level of gene flow is suggested, due to the preponderance of
 280 pelagic larval stages and the absence of obvious distribution barriers for them. However, the high
 281 level of extant marine biodiversity suggests that genetic differentiation and speciation must be
 282 common in marine systems (Mathews 2006, Galarza et al. 2009). This was confirmed for the
 283 European prawn *Palaemon elegans* in the present study.

285 ***Phylogeographic structure***

286 In our study, the 16S rRNA and the more variable Cox1 mitochondrial genes revealed a
 287 surprisingly complex population structure in the marine species *Palaemon elegans* and clearly
 288 distinct genetic lineages. Three haplogroups can be defined: Type I (Atlantic and Alboran Sea),
 289 Type II (entirely Mediterranean) and Type III (Mediterranean plus Baltic, Caspian and Black
 290 Seas). The separation of the common ht of Type III and of the common hts of Type I - II amounts
 291 to 2.5% in the 16S rRNA and 8.1% in the Cox1 gene. When considering fixed differences only,
 292 Type III can be distinguished by 1.5% of the 16S gene and 4.2% of the Cox1 gene of both other
 293 types of *P. elegans* (see Tables 3 and 4). Since no consistent morphological differences were found
 294 between the different genetic types so far, we suggest that *P. elegans* represents a species complex,
 295 with the existence of one cryptic species. Many marine cryptic crustaceans have been detected
 296 (Knowlton 1993), e.g. in the snapping shrimp genus *Alpheus* using the 16S and Cox1
 297 mitochondrial genes (Mathews 2006), in the seabob shrimp species *Xiphopenaeus kroyeri* and
 298 *Xiphopenaeus riveti* with Cox1 (Gusmão et al. 2006), and among the mysid shrimp *Mesopodopsis*
 299 *slabberi* with 16S and Cox1 mitochondrial genes (Remerie et al. 2006).

300 When considering the existence of a cryptic species within *P. elegans*, the taxonomic status of
 301 the species needs to be revisited. Since *P. elegans* was first described by Rathke, 1837 from

specimens of the Black Sea and in that water body we only encountered Type III sequences, the name *P. elegans* corresponds to our Type III populations and would thus be restricted to the Black Sea, Caspian Sea, southeastern Baltic Sea and all of the Type III specimens in the Mediterranean Sea. De Man (1915) recognized three forms within *P. elegans*, to which he referred to as *Leander squilla*. According to him, *L. squilla* var. *intermedia* can be distinguished from the typical form by a shorter ramus of the outer flagellum and by the shape of the second leg, the carpus appearing a little shorter than the chelae. This form was described from the Dutch province of Zeeland, the English Channel, the Irish Coast, Scotch waters, France and Portugal (De Man, 1915). Therefore, the name *Palaemon intermedius* (De Man, 1915) would become available for typical Atlantic (and the Mediterranean populations of Type II),. So far the three types could not distinguished by external morphological characters (confirmed as personal communication by González-Ortegón) and it remains to be tested whether hybridization is taking place.

Causes of dispersal limits

Although, there exist some indications that marine animals may have survived in the Black Sea during the last brackish-water (<7‰) period (e.g. the shrimp *Crangon crangon* according to Luttikhuizen et al. 2008) and Rathke (1837) described *P. elegans* based on specimens from the Black Sea, Type III animals probably do not have their origin there. The high differentiation between Type III and the other two types suggest an earlier separation. The Black Sea was primarily a freshwater basin and was flooded with Mediterranean saline water about only 6,800 to 9,630 years ago and as a result, the Mediterranean fauna was re-introduced into the Black Sea (Dimitrov & Dimitrov 2004). The geology of the Mediterranean Basin gives evidence for at least one major isolation event from Atlantic waters followed by massive decrease of the water level (Messinian Crisis in the Pliocene) (Por & Dimentman 1989). Assuming that marine animals

326 survived in the Mediterranean Basin during that time, despite existing evidence of hypersaline
 327 conditions, allopatric speciation from the Atlantic forms would have been the logical consequence
 328 (e.g. Schubart et al. 2001). The relative rate test was not significant between Type II and the other
 329 two types ($p = 0.75$ for Type I and III; $p = 0.76$ for Type II and III), suggesting similar rates of
 330 molecular change. Therefore, an estimate of divergence could be carried out, based on the
 331 calibration of a molecular clock of other carideans, the snapping shrimp of the genus *Alpheus*
 332 separated by the closure of the Isthmus of Panama (Knowlton & Weigt 1998). For calculation, we
 333 used pairwise distances of the sequences with the lowest and highest divergence to obtain the mean
 334 value and standard deviation of divergence per million years. Our results indicate that the
 335 divergence between the ancestors of Types I and II and Type III occurred 6.85 ± 0.85 Myr ago.
 336 This confirms the possibility that Type III ancestors were isolated from the “Atlantic” populations
 337 during the Messinian Crisis, since the beginning of the massive desiccation is dated around 5.96
 338 Myr ago (see Krijgsman et al. 1999) and isolation must have occurred before. For the European
 339 shore crab genus *Carcinus*, genetic differences of 2.5% in the 16S and 11% in the Cox1 between
 340 the Atlantic *C. maenas* and Mediterranean *C. aestuarii* were detected (Geller et al. 1997; Roman &
 341 Palumbi 2004). These distances are comparable to *P. elegans* with genetic differences of 2.5% in
 342 the 16S and 8.7% in the Cox1 genes. Demeusy (1958) proposed that the isolation during the
 343 Messinian Crisis between the Mediterranean and the Atlantic could have provided the geographic
 344 barrier permitting allopatric speciation within *Carcinus*. The same has been suggested for the split
 345 of the Atlantic *Brachynotus atlanticus* and the Mediterranean *B. foresti* (see Schubart et al. 2001).
 346 There is also strong support that the origin of the snail *Salenthydrobia ferrerii* correlates with the
 347 crisis (Wilke 2003). With the flooding of the Mediterranean Basin with Atlantic water following
 348 the Messinian Crisis, Atlantic forms were re-introduced into the Mediterranean Sea, apparently
 349 without reproducing with the local Type III ancestors, and progressively separated from Atlantic

350 Type I, possibly due to isolation by distance, later gene flow barriers like Pliocene/Pleistocene sea
 351 level regressions and ongoing limited genetic exchange due to oceanic currents (e.g. Almería Oran
 352 Front), giving rise to the new Mediterranean Type II populations. The demographic analyses of
 353 Type I and II support a similar sudden expansion around the same time. Within the polychaete
 354 *Lysidice ninetta* (Audouin & H. Milne-Edwards, 1833), the presence of intraspecific cryptic
 355 lineages was recorded by Iannotta et al. (2007). For this species, a re-colonization scenario into the
 356 Mediterranean Basin from the Atlantic following the Messinian Crisis has also been put forward
 357 (Iannotta et al. 2007).

358 While Type II is found only in the Mediterranean Sea, Type III has a broader distribution. It
 359 also occurs in the Baltic, Black and Caspian Seas and seems to dominate the Mediterranean Sea
 360 (see also Figure 4). Occasionally the two types were found in sympatry (e.g. northeastern Spain,
 361 southwestern Italy, Croatia, and Tunisia).

362

363 ***Invasive species***

364 Grabowski (2006) reported the rapid colonization of the Polish Baltic coast by *Palaemon elegans*,
 365 discussing its occurrence in the context of representing an introduced invasive species rather than an
 366 effect of natural range expansion. Much of the biological diversity of the Baltic Sea is of foreign
 367 origin, intentionally or unintentionally introduced by humans, for example with ballast water
 368 (Leppäkoski & Olenin 2000). Since the 1950ies, 98 introduced species have been recorded into the
 369 Baltic Sea and Kattegat, e.g. the mysid shrimp *Hemimysis anomala* (see Leppäkoski & Olenin
 370 2000), the Chinese mitten crab *Eriocheir sinensis* (see Ojaveer et al. 2007), the zebra mussel
 371 *Dreissena polymorpha* (see Orlova and Panov 2004) and the talitrid amphipod *Orchestia cavimana*
 372 (see Herku et al. 2006). Since our study revealed that all southern Baltic Sea specimens belong to
 373 the genetic Type III, which is otherwise confined to the Mediterranean Sea and the Black Sea, it is

indeed likely that *P. elegans* was introduced to the Baltic Sea by humans and therefore represents an invasive species. In support of that is also the finding that the Baltic Sea population shows the lowest haplotype diversity (0.2) of all studied populations (Table 2). A possible founder effect resulting in a genetic bottleneck can have lead to chance reduction in genetic variation so that the haplotype diversity was reduced compared to the ancestral population (see also Hedrick 2005). Limited mtDNA haplotype diversity was also detected in the Baltic populations of the cladoceran *Cercopagis pengoi* (see Cristescu et al. 2001). The authors suggest that the population was founded by a small number of colonizers from the Black Sea and has undergone a bottleneck (Cristescu et al. 2001). The cause of this translocation remains unknown. A similar case of a bottleneck can also be assumed for the population of *P. elegans* in the Caspian Sea. Again we have a relatively low haplotype diversity (0.47) compared to the population in the Black Sea (0.89). Several geographically isolated lineages from different species that currently inhabit fragmented habitats in the Ponto-Caspian region, show a limited genetic divergence, which might be attributed to such genetic bottlenecks (Grigorovich et al. 2004).

388

389 ***Physical barriers to gene flow***

Our haplotype networks of *Palaemon elegans* show a geographic distinction between Atlantic (Type I) and Mediterranean populations, but with an extension of the Atlantic population into the Mediterranean Basin (Alborán Sea). For a long time, the relatively shallow Strait of Gibraltar, representing the geographic boundary between the western Mediterranean and the Atlantic Ocean, was assumed to act as a potential barrier of gene flow. However, more recently it has been suggested that the Almería-Oran front between Cabo de Gata (province of Almería in southeastern Spain) and Oran in Algeria represents the more important hydrographic boundary between Atlantic and Mediterranean surface waters. Circular jetties predominate between this front and the Strait of

398 Gibraltar (Tintoré et al. 1988). It has been confirmed to act on population structure of different
 399 marine species, e.g. the cuttlefish *Sepia officinalis* (see Pérez-Losada et al. 2002, 2007), the pelagic
 400 euphausiid crustacean *Meganyctiphanes norvegica* (see Zane et al. 2000) and the scallops *Pecten*
 401 *jacobeus* and *P. maximus* (see Ríos et al. 2002). Here we postulate that it also represents the
 402 barrier to gene flow between Atlantic and Mediterranean populations of *Palaemon elegans*.

403 Another European physical marine geographic boundary confirmed by our data is the English
 404 Channel separating Great Britain from northern France and connecting the North Sea to the
 405 Atlantic. The hydrodynamic features of the English Channel may also shape gene flow and
 406 separate water masses on both sides (Salomon & Breton 1993; Jolly et al. 2005). For the Atlantic
 407 populations of *Carcinus maenas*, a slight but significant break between western Europe and
 408 northern Europe along the English Channel was found (Roman & Palumbi 2004). A genetic break
 409 was also observed for the common goby *Pomatoschistus microps* around the British Isles, with
 410 distinct haplotypes dominating at either side of the English Channel (Gysels et al. 2004).
 411 Population genetic structuring was recognized in the goose barnacle *Pollicipes pollicipes* due to
 412 coastal currents in the northeastern Atlantic (Quinteiro et al. 2007). In this study, we revealed a
 413 significant differentiation between the populations of the North Sea (east of the English Channel)
 414 and the open northeastern Atlantic, which must be assigned to the effect of the English Channel.
 415 The Φ_{ST} -values showed evidence of a strong correlation between genetic differentiation and
 416 geographical distance (from Norway to Cádiz). An isolation-by-distance pattern within the Atlantic
 417 is thus plausible.

418

419 **Conclusions**

420 Results of this study give clear evidence for the existence of three haplotype groups within
 421 *Palaemon elegans*. Due to the high genetic differentiation of one of the three haplogroups (2.5% in

16S and 8.7% in Cox1 among most common haplotypes), we propose the occurrence of a cryptic species within this complex (Type III), probably resulting from an isolation event during the Messinian Crisis according to molecular dating with the Cox1 gene. The genetic diversity of the different types is furthermore influenced by physical barriers like the Almería-Oran Front and the English Channel. Future results based on nuclear DNA and breeding experiments will determine, if members of the three types hybridise and can be used to confirm the presence of a cryptic species. Further sampling is needed for populations originating from the Atlantic coast, like the Irish Sea and North Sea to investigate more closely the phylogeographic separation within *P. elegans* in this region. This is of particular importance, because pairwise Φ_{ST} values among Atlantic populations show a higher genetic differentiation than those within types in the Mediterranean Sea and thus give evidence for a higher potential of local endemism.

ACKNOWLEDGEMENTS

For financial support we like to thank the European Union funding (FEDER), the Spanish “Ministerio de Educación y Ciencia, Plan Nacional I+D” (project CGL2004-01083), the DAAD (D/03/40344) and the Spanish “Ministerio de Ciencia y Tecnología – Acciones Integradas (HA2003-078). Our special thanks go to Carsten Müller, José Paula, Ines C. Silva, Urszula Janas, Sammy de Grave, Lapo Ragonieri, Enrique González-Ortegón, Ruth Jesse, Sara Fratini, Sonya Uzunova, Henrik & Sophia Schubart, Florian Gmeiner, Andrea Schott, Veronika Ebe and students of the Universtiy of Regensburg for helping to collect and sending specimens of *P. elegans*. Thanks are further due to Graciela Sotelo for helpful discussions of statistical tests. Two anonymous reviewers helped to improve the manuscript with their comments.

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447

448

449 **Figure Legends**

450
451 **Figure 1.** Geographic locations of collection sites of *P. elegans*. The different types are shown in
452 symbols; for the abbreviations of collection sites see also Table 1 and 2 (AOF = Almería-Oran-
453 Front).

454
455 **Figure 2.** Minimum parsimonious spanning network of the 16S rRNA gene constructed with TCS.
456 The network is based on the haplotypes of 53 individual prawns. Each circle represents a single
457 haplotype and its diameter is proportional to the frequency of the haplotype, with the smallest
458 circle representing a single individual. Each line represents one substitution; short transverse lines
459 indicate missing haplotypes. The shading corresponds to the different types.

460
461 **Figure 3.** Minimum parsimonious spanning network of the Cox1 gene constructed with TCS. The
462 network is based on the haplotypes of 283 individual prawns. Each circle represents a single
463 haplotype and its diameter is proportional to the frequency of the haplotype, with the smallest circle
464 representing a single individual. Each line represents one substitution; short transverse lines indicate
465 missing haplotypes. The shading corresponds to the different types. Haplotypes with amino acid
466 changes are marked with a star. See Table 1 and 2 for the abbreviations of the localities.

467
468 **Figure 4.** Histogram showing the distribution of the three types and illustrating the geographical
469 overlaps. The shading corresponds to the three types.

470
471 **Figure 5.** Mismatch distributions and τ values obtained from Cox1 gene sequence data for Type I
472 (A); Type II (B) and Type III (C) The dotted line represents the observed pairwise differences. The
473 solid curve is the expected distribution under the sudden expansion model.

Table 1. Localities; locality name of the sampled populations and the number of specimens used for genetic comparisons (N) of the 16S gene; the haplotype diversity (h) and nucleotide diversity (π) within the examined population and the three types of *Palaemon elegans*. The abbreviations refer to localities of the sampled population.

Collection site of <i>P. elegans</i>	Abbr.	N	Type	h	π
Germany: Kiel: Nord-Ostseekanal	Kiel	2	I	-	-
Germany: Helgoland	Hel	1	I	-	-
Germany: Wilhelmshaven (Niedersachsenbrücke)	Wil	3	I	-	-
Spain: Canary Islands: Gran Canaria (La Isleta)	Can	7	I	0.28	0.00055
Spain: Cádiz: Rota	Cád	6	I	0.6	0.00128
Spain: Málaga: Marbella: Cabo Pino	Mal	2	I	-	-
Spain: Granada: Almuñecar	Gra	2	I	-	-
Spain: Almería: Garrucha	Gar	4	III	0.5	0.00096
Spain: Murcia: Mar Menor	MM	1	III	-	-
Spain: Tarragona: Ebro Delta (2006)	Eb06	6	III	0.33	0.00128
Spain: Girona: L'Escala	Gir	2	II	-	-
Spain: Balearic Islands: Ibiza (Cala Llenya)	Ibi	5	III	1	0.01385
Greece: Kalami Beach	Gre	3	II	-	-
Croatia: Pula	Cro	4	II	0.83	0.00224
Black Sea: Bulgaria: Sozopol	BS	5	III	0.6	0.00115

480 **Table 2.** Localities; locality name of the sampled populations and the number of specimens used for genetic
 481 comparisons (N) of the *Cox1* gene; the haplotype diversity (h) and nucleotide diversity (π) within the examined
 482 population and the three types of *Palaemon elegans* (type which dominates the population in bold; the number of
 483 specimens which belong to the type in brackets). The abbreviations refer to localities of the sampled population.

Collection site of <i>P. elegans</i>	Abbr.	N	Type	h	π
Poland: Baltic Sea: Gulf of Dansk	Bal	10	III	0.2	0.00129
Norway: Bergen: Fantoft	Nor	8	I	0.89	0.00397
Germany: Kiel: Nord-Ostseekanal	Kiel	13	I (9) & III (4)	0.9	0.00483
Germany: Helgoland	Hel	11	I	1	0.0065
Germany: Wilhelmshaven (Niedersachsenbrücke)	Wil	4	I	1	0.00483
Portugal: Lisbon: Cabo Raso	Por	10	I	0.93	0.00569
Spain: Canary Islands: Gran Canaria (La Isleta)	Can	10	I	0.87	0.0039
Spain: Cádiz: Rota	Cad	10	I	0.78	0.0029
Spain: Málaga: Marbella: Cabo Pino	Mal	4	I	1	0.00403
Spain: Granada: Almuñecar	Gra	10	I	0.87	0.0033
Spain: Almería: Garrucha	Gar	10	III	0.91	0.0034
Spain: Murcia: Mar Menor	MM	2	III	0	0
Spain: Alicante: Torrevieja	Tor	10	III	0.84	0.00272
Spain: Alicante: Cabo Nao	Nao	5	III	0.7	0.00225
Spain: Valencia: Puig	Val	10	III	0.78	0.00186
Spain: Tarragona: Ebro Delta (2005)	Ebro	9	II	0.91	0.0049
Spain: Tarragona: Ebro Delta (2006)	Eb06	10	III	0.53	0.00129
Spain: Tarragona: Ampolla	Amp	10	II (8) & III (2)	0.84	0.00408
Spain: Tarragona: Salou	Sal	10	II	0.87	0.00433
Spain: Tarragona: Vilanova di Geltrú	Vil	10	II	0.93	0.00530
Spain: Girona: L'Escala	Gir	10	II (9) & III (1)	0.96	0.02344
Spain: Balearic Islands: Ibiza (Cala Llenya)	Ibi	10	II (3) & III (7)	0.96	0.04376
Italy: Sardegna: Palau	Sar	5	III	1	0.00386
Italy: Livorno	Liv	9	III	0.92	0.00403

Italy: Fusaro: Lago di Fusaro near Pozzuoli	Fus	10	II (7) & III (3)	0.98	0.04409
Italy: Calabria: Torre Melissa	Cal	7	II	0.95	0.00598
Greece: Kalami Beach	Gre	4	II	1	0.00832
Greece: Crete	Cre	1	III	-	-
Croatia: Pula	Cro	12	II (11) & III (1)	0.96	0.02344
Turkey: Phaselis	Tur	10	II	0.98	0.00686
Tunisia: Bizerte	Tun	9	II (2) & III (7)	0.86	0.02312
Black Sea: Bulgaria: Sozopol	BS	10	III	0.89	0.00573
Caspian Sea	Cas	10	III	0.47	0.00269

485 **Table 3.** Diagnostic sites of the 520 bp fragment of the 16S rRNA gene always separating Types I and II from Type
 486 III. Dots in the lower sequence indicate nucleotides that are identical to those in the upper sequence. Sequence position
 487 after universal primer 16Sar (Palumbi et al. 1991).

488

489

Type	148	152	227	229	303	323	344	461
I	G	A	G	C	G	T	T	T
II	.	.	.	.	.	.	.	.
III	A	G	A	T	A	C	C	G

490

491 **Table 4.** Diagnostic sites of the 621 bp fragment of the Cox1 gene always separating Types I and II from Type III.
 492 Dots in the lower sequence indicate nucleotides that are identical to those in the upper sequence. Sequence position
 493 after universal primer CO1472 (Folmer et al. 1994); S = C or G; R = A or G.

494

Type	68	110	125	140	152	161	221	233	245	281	296	299	341	344
I	G	C	A	G	C	A	C	C	C	A	A	A	C	G
II	.	.	.	.	.	.	.	.	.	.	.	.	.	.
III	A	T	C	T	T	G	T	A	T	C	G	G	T	A

495

496

Type	345	348	353	374	479	506	527	557	569	581	599	620
I	T	A	T	C	A	A	C	C	T	A	C	C
II	.	.	.	.	.	.	.	.	.	.	.	T
III	G	G	A	T	T	S	T	R	C	T	T	A

497 Table 5. Pairwise Φ_{ST} values between all populations of Types I and II. Significant values are presented in bold (significance level 0.05 was calculated
498 from 10000 permutations).

	Canary I	Norway	Kiel	Helgoland	Portugal	Cádiz	Granada	Ebro	Ampolla	Salou	Vilanova	Girona	Fusaro	Calabria	499 Croatia
Norway (8)	0.17209														500
Kiel (9)	0.27355	0.03415													501
Helgoland (11)	0.27268	0.26671	0.19290												502
Portugal (10)	0.32169	0.23885	0.10244	0.07197											
Cádiz (10)	0.47745	0.41803	0.23295	0.07046	0.00498										
Granada (10)	0.33987	0.25447	0.10156	0.09130	-0.04089	0.05229									
Ebro (9)	0.70204	0.72885	0.72288	0.66915	0.71533	0.78745	0.76480								
Ampolla (8)	0.71651	0.74359	0.73557	0.67276	0.72285	0.80423	0.77947	0.01669							
Salou (10)	0.74134	0.76526	0.75659	0.70848	0.74870	0.81389	0.79502	-0.01300	0.08247						
Vilanova (10)	0.70316	0.72738	0.72197	0.67116	0.71479	0.77951	0.76048	-0.02737	0.08579	-0.04507					
Girona (9)	0.75743	0.78253	0.77021	0.72001	0.76071	0.82891	0.81056	0.02130	0.15401	-0.04220	-0.04715				
Fusaro (7)	0.67896	0.70300	0.70007	0.63968	0.68959	0.77175	0.74598	-0.02288	-0.04135	0.03474	0.03525	0.10746			
Calabria (7)	0.68154	0.69962	0.69618	0.64125	0.68608	0.76846	0.74220	-0.02708	-0.07055	0.02922	0.03031	0.08436	-0.05686		
Croatia (9)	0.63850	0.66347	0.66741	0.61565	0.66337	0.73380	0.70874	-0.04633	-0.02965	0.01227	-0.00724	0.05263	-0.07758	-0.05414	
Turkey (12)	0.68543	0.70469	0.70432	0.67172	0.70779	0.76066	0.74253	0.06072	0.14896	-0.00813	-0.00615	-0.00526	0.11147	0.09474	0.07803

503 **Table 6.** Summary of statistics of the Cox1 gene; N, number of sequences; M, number of haplotypes; h, haplotypic
 504 diversity; n, nucleotide diversity; S, number of segregation (polymorphic) sites; k, mean number of pairwise differences
 505 between individuals; SD, standard deviation; the demographic parameters θ_0 , τ , θ_1 values of Tajima's D and Fu with
 506 probability values (P).
 507

Sample	<i>N</i>	<i>M</i>	<i>h</i> (SD)	<i>n</i> (SD)	<i>S</i>	<i>k</i> (SD)	θ_0 , τ , θ_1			<i>D</i> (<i>P</i>)		<i>Fu's</i> (<i>P</i>)
Type I	76	49	0.96 (0.014)	0.0013 (0.0004)	86	8.21 (3.72)	0.082	3.545	33.477	-1.76 (0.01)		-24.77 (0.001)
Type II	90	52	0.93 (0.020)	0.0056 (0.004)	59	3.47 (1.8)	1.019	2.992	15.566	-2.28 (0.001)		-26.17 (0.001)
Type III	117	37	0.77 (0.041)	0.0029 (0.0018)	43	1.83 (1.06)	0.896	1.235	6.401	-2.39 (0.01)		-27.72 (0.001)

508

509

510 Figure 1

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512 Figure 2

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Figure 3

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515 Figure 3

516

517 Figure 4

(A)

(B)

(C)

518
519 Figure 5

Figure

Figure 1

Figure

Figure 2

Figure

Figure 3

Figure

Figure 4

(A)

(B)

(C)

Figure 5