Cryptic by invasion: Morphological similarity of two invasive parasitic copepods infecting marine bivalves in their introduced range

M. Anouk Goedknegt, David W. Thieltges, Jaap van der Meer, K. Mathias Wegner and Pieternella C. Luttikhuizen

Submitted manuscript
Abstract

Cryptic species are distinct species originally lumped under the same scientific name and only discovered in retrospect after molecular examination. Here we describe a case of the reverse that can result from biological invasions: two species originally described separately that lack reliable diagnostic characters and are in fact a case of crypticism. These parasitic copepod species infecting bivalve hosts now co-occur along Northeast Atlantic coasts, after being introduced from the Mediterranean Sea (Mytilicola intestinalis) and Japan (Mytilicola orientalis). As the copepods currently have overlapping host ranges and geographical distributions, our knowledge on their distribution, impact and interactions depends crucially on reliable species identification. In this study, we evaluated the reliability of morphological identification with molecular methods and verified whether these species represent a case of crypticism in the invasive range. Based on seven morphological variables that were measured on 182 individual copepods originating from blue mussel hosts (Mytilus edulis), principal component analysis showed two relatively distinct but overlapping morphological species groups for females, and no clear separation in males. Consequently, a discriminant function analysis revealed that females can be discriminated reasonably well based on morphological characteristics (error rate of 7%) but males cannot (error rate 25%). Though morphology of the two invasive parasite species was significantly differentiated, and the shape of the dorsal appendages was identified as the most important trait for species identification, no diagnostic character was detected that could flawlessly discriminate between both species. Hence, M. intestinalis and M. orientalis may be considered as ‘cryptic-in-retrospect’ in their invasive range. This case can serve as a cautionary example for other taxa with shifting distributional ranges; when site of origin can no longer discriminate species, a closer examination of morphological and genetic differentiation is advisable.
Introduction

Cryptic species abound in most animal taxa and are an important component of earth’s metazoan biodiversity (Knowlton, 1993; Bickford et al., 2007; Pfenninger and Swenk, 2007). Typically, cryptic species are distinct biological species that were known under the same scientific name until molecular methods detected the presence of several distinct, often highly diverged species (Pfenninger and Swenk, 2007). It is not uncommon that differentiating morphological characteristics are then discovered in retrospect, allowing additional reliable morphological discrimination between the species - which are then called pseudo-cryptic species (e.g., Sáez et al., 2003; Luttikhuizen and Dekker, 2010). Here we investigate the opposite scenario that can result from species invasions: a case of possible ‘crypticism-in-retrospect’, where two invasive species co-occurring in an area were originally described in their native ranges as distinct species, while in fact a trustworthy diagnostic feature may not exist.

The parasitic copepod *Mytilicola intestinalis* was first described in the mussel *Mytilus galloprovincialis* in the Adriatic Sea (Steuer, 1902) and presumably spread with infected mussels as fouling on ships’ hulls to the Atlantic coast of Europe where it started to infect native bivalve species (Caspers, 1939; Hockley, 1951; Theisen, 1966; Korringa, 1968). The congeneric *Mytilicola orientalis* was first described in Pacific oysters (*Crassostrea gigas*) from Japan (Mori, 1935) and was co-introduced to British Columbia in 1938 with shipments of Pacific oysters (Carlton, 1979). Via subsequent oyster transports to France in the 1970s (His, 1970), the parasite spread northward with its host to the Dutch Delta (Stock, 1993) and the Dutch, German and Danish Wadden Sea (Elsner et al., 2011; Pogoda et al., 2012). The two species diverged a long time ago and accumulated a minimum interspecific genetic difference of 16.08% while showing very low intraspecific diversity for COI (cytochrome-c-oxidase I, a mitochondrial gene) (Elsner et al., 2011). Divergence time could be in the order of seven to eleven million years ago based on a molecular clock commonly used for crustaceans (Knowlton and Weigt, 1998; Luttikhuizen et al., 2008). Hence, their species status is not under discussion.

Currently, the two copepod species do not only co-occur along north-eastern Atlantic coasts but have overlapping host ranges as well, infecting blue mussels (Korringa, 1950; Stock, 1993; Elsner et al., 2011; Pogoda et al., 2012; Goedknegt et al., 2017), common cockles (*Cerastoderma edule*) (Lauckner, 1983; Thieltges et al., 2013a; Goedknegt et al., 2017) and Pacific oysters (Dare, 1982; Grizel, 1985; Aguirre-Macedo and Kennedy, 1999; Goedknegt et al., 2017). Although the original descriptions provide clear starting points for distinguishing between the species, it has been noted that morphological identification may be problematic now that both species co-occur and this may have resulted in species identification errors in the past (Elsner et al., 2011).

Here, we focus on the German and Dutch Wadden Sea and the Dutch Delta, where the geographical ranges of the two parasite species overlap. The aim of this study was to assess the reliability of morphological identification of *M. intestinalis* and *M. orientalis*. We approached this in three ways: First, we took quantitative morphological measurements of a large pool of *Mytilicola* spp. from blue mussel (*Mytilus edulis*) and Pacific oyster (*Crassostrea gigas*) hosts originating from different locations. Second, we verified the species identity of every copepod molecularly by applying a diagnostic RFLP assay (Restriction Fragment Length Polymorphism) (Goedknegt et al., 2017). And third, we applied multivariate morphometric analyses to investigate the accuracy of morphological identification and to identify trustworthy features in species discrimination. In addition, as methods of preservation are known to modify or destroy morphological characteristics (Knowlton, 1993; Nygren, 2014), we investigated the effect of
several storage methods on the morphology of both species. Our results thus estimate the reliability of morphological identification and storage protocols for future studies on the distribution, host range and impact of the two invasive copepods, and verify whether these species represent a case of ‘crypticism-in-retrospect’ resulting from species invasions.

**Materials and Methods**

**Sample selection**

To ascertain that identification is reliable for all *Mytilicola* individuals found in the introduced region, we used a large pool of *Mytilicola* spp. that originated from different locations and different host species. Copepods were collected in two regions that were important invasion pathways of both copepods; the Dutch Delta (6 locations; May 2012) and the Wadden Sea (9 locations; different months in 2010-2012; Fig 4.1; Supplementary Table S4.1). All *Mytilicola* individuals were freshly removed from their host and stored in 96% ethanol. The copepods originated from the two main hosts, Pacific oysters (*Crassostrea gigas*; mean shell length ± SE: 120.3 mm ± 2.8 mm) and blue mussels (*Mytilus edulis*; mean shell length ± SE: 47.3 mm ± 0.6 mm). We took almost twice as many *Mytilicola* samples from mussels than from oysters, because mussels are host to both *Mytilicola* species and oysters only to one (*M. orientalis*) (Goedknegt et al., 2017). Individual copepods were then randomly drawn from each region/host species combination until, when possible, all host-sex categories (i.e. males and females of *M. intestinalis* and males and females of *M. orientalis*, see Materials and Methods section *Morphological measurements and identification*) were filled with at least 20 individuals for the Dutch Delta and 25 individuals for the Wadden Sea, where we sampled more locations (Fig. 4.1, Supplementary Table S4.1). However, as expected, *M. intestinalis* was not found in Pacific oysters and therefore this host/parasite category remained empty.

**Morphological measurements and identification**

Morphological species identification of individual copepods (performed by a single observer) was based on the shape of the dorsal appendages (folded inwards and stunted in *M. intestinalis* and outwards and pointed for *M. orientalis*) (Mori, 1935; Elsner et al., 2011) and on the shape of the caudal ramus (thick and widely divergent in *M. intestinalis* and narrow and non-divergent in *M. orientalis*; Fig. 4.2) (Elsner et al., 2011). Regarding sex determination, individuals were considered to be females when the cephalosome is trapezoid shaped (Ho and Kim, 1992) and the second maxilliped was missing (too small to be shown in Fig. 4.2) (Steuer, 1902). Additionally, only in females the posterior end of the abdomen (where the egg sacs are attached to) was trapezoid in shape, a feature that is easy to observe and we refer to as ‘pre-tail’ in this study (Fig. 4.2) (unpublished observations M. A. Goedknegt).

After the pre-sorting into the four species-sex categories the same observer conducted quantitative morphological measurements. With a camera (AxioCam Icc3) attached to a stereo microscope (Zeiss V8 discovery), pictures were taken of each individual copepod and body size measurements were conducted with the software package AxioVision. Head length (maximum vertical diameter), body width (males: thinnest part of the animal, females: between the 4th and 5th segment) and body length were measured. The divergence of the caudal rami (Elsner et al., 2011) was measured as the tail angle. This was the angle from which each tail part diverted from...
the 0° line that divides the copepod vertically into two symmetrical parts. In addition, the number of segments carrying appendages was counted and the shape of the appendages was noted (stunted or pointy). For females, also length of the pre-tail (distance from posterior end of abdomen - where the egg sacks are attached - to the end of the tail) was measured. See Fig. 4.2 for more details on body measurements of both sexes of both parasite species.

Fig 4.1 Sampling locations of blue mussel (*Mytilus edulis*) and Pacific oyster (*Crassostrea gigas*) hosts. A) The sampled regions the Dutch Delta and the Wadden Sea (shaded area), with the islands Sylt (north) and Texel (south). B) Sampling locations around the island of Sylt. C) Sampling locations around the island of Texel. D) Sampling locations in the Dutch Delta. For exact coordinates see Supplementary Table S4.1.

Molecular identification

For the molecular identification of the species identity of each copepod individual we developed two taxon specific primers that were previously used for species identification (Goedknegt et al., 2017). Taxon specific primers were designed on the basis of previously published *Mytilicola intestinalis* and *M. orientalis* cytochrome-c-oxidase I sequences (Genbank accession numbers HM775191-M775197) (Elsner et al., 2011). The new primers (MOICOIf 5'-CTTAATTACAGGGGTMTGATCGG-3' and MOICOIr 5'-TCGATCTGTTAAAAGCATAGTAATYG-3') amplify a 534 bp fragment yielding a PCR product of 83 bp (base pairs) long.

A diagnostic RFLP (restriction fragment length polymorphism) assay was developed that could distinguish between *M. intestinalis* and *M. orientalis*. The restriction enzyme NciI recognizes and cuts the sequence CC/SGG (where S = C or G and / indicates the cut site). The PCR product of *M. intestinalis* is cut once by NciI, and that of *M. orientalis* is cut twice. In *M. intestinalis* this results in two restriction fragments of 366 and 217 bp long. In *M. orientalis* the resulting pattern consists of three fragments 286, 222 and 75 bp long. This difference between the species was visualized
on 2% agarose gels which were run for 120 min. at 60 V, followed by staining with ethidium bromide.

**Fig. 4.2** Schematic representation (not to scale) of both sexes of both introduced *Mytilicola* species. On the left both sexes of *M. intestinalis* and on the right both sexes of *M. orientalis* with indications of the body size measurements that were taken. Note the stunted appendages (folded in) of *M. intestinalis* and pointy appendages in *M. orientalis*. Drawings with courtesy of Felipe Ribas.

**Effects of storage method on morphology**

To investigate the effects of storage method on copepod morphology, we sampled males and females of both *Mytilicola* species from mussel hosts (mean ± SD: 50.0 ± 6.3 mm) which were collected at two locations at the island of Texel: Vlakte van Kerken and Mokbaai (location 5 and 9, respectively in Fig. 4.1C). These samples were randomly assigned to two groups: 1) samples were taken fresh from the host, measured, stored in 96% ethanol and measured again after two weeks (n = 33); and 2) samples were taken fresh from the host, measured, frozen (-20 °C) for six weeks, defrosted, measured, stored in 96% ethanol and measured again after two weeks (n = 29; for descriptions of morphological measurements see Materials and Methods section *Morphological measurements and identification*).
Statistical analysis
All statistical analyses were performed using the statistical software package R (R Development Core Team, 2016). Prior to the analyses, homogeneity and normality assumptions were checked by using boxplots, histograms and qqplots (Zuur et al., 2010).

Effects of origin
In order to rule out any potential biases of the origin of the parasite on morphological body measurements, we used linear models to investigate the relationships between parasite body size and 1) host species (only for *M. orientalis* which infects both host species), 2) host size (for each of three parasite-host combinations) and 3) region (for each of the two host species). Sex of the parasite was used as an additional factor in all models. For the region models, we used a linear model to test and account for the relationship between parasite size and host size for both host species. Subsequently, we used the residuals of this model as response variable in the region models (i.e., correcting for host size), together with the molecular identity of the parasite species (only for the mussel model). We used parasite body length for these investigations, because this variable correlated with other size variables (head length, width and pre-tail, the latter only for females; see Supplementary Fig. S4.1)

Multivariate morphometric analyses
As both parasite species only co-occur in blue mussels in the investigated region, only parasites originating from this shared host were used in the multivariate analyses. The morphology of each individual parasite was characterized via seven morphometric parameters: body length, head length, width, tail angle, number of segments and shape of appendages (see Materials and Methods section *Morphological measurements and identification*), in which the latter was a dummy-coded nominal variable. The variable ‘pre-tail length’ is only a female feature, resulting in seven different morphological variables for females and six for males. For this reason and the fact that sex is only morphologically assessed, the multivariate morphometric analyses were executed for each sex separately and were performed in two steps. First, a principal component analysis (PCA) was conducted to illustrate the relative contribution of each of the morphological variables for each parasite species. Prior to this analysis all the morphometric variables were checked for outliers, which were removed when the value was larger than the mean plus three times the standard deviation, and subsequently all morphometric variables were log transformed. Second, a discriminant function analysis (DFA) was performed to investigate the accuracy of morphological species identification. With this analysis, the hypothesis was tested that the *a priori* defined groups of parasites (molecular species identity) would differ significantly in their combination of morphometric variables. In the DFA, untransformed variables (excl. outliers) were standardized by using the decostand function in the vegan package in R (Oksanen et al., 2016). The DFA was executed by the lda function of the MASS package (Venables and Ripley, 2002) in which the Wilks lambda statistic was used to test for an overall group effect. To determine the most important variables in the discrimination between groups, a stepwise discriminant function analysis was employed. Based on the total sum of Mahalanobis distances, this stepwise approach removed variables to produce a discriminant function with only important variables. In addition to the analysis of the full morphological data set, we did several straightforward calculations on the proportions of correct identifications when using only the two morphological features that are the easiest to assess (tail angle and shape of the appendages).
**Effects of storage method**

Differences between body size measurements within Group 1 (fresh - ethanol samples) were tested with (paired) Welch t-test's when variables were normally distributed and with non-parametric Wilcoxon rank tests when this assumption was violated. Differences between measurements within Group 2 (fresh - frozen - ethanol samples) were tested with a repeated measures ANOVA. To test for differences between groups for both fresh and ethanol treatments, the Welch t-test's or non-parametric Wilcoxon rank tests were used.

**Results**

**Molecular results**

Of the 307 morphologically identified *Mytilicola* samples (Table 4.1), most individuals displayed one of the two expected banding patterns in the PCR product and could accordingly be assigned to one of the two *Mytilicola* species (for examples of banding patterns see Supplementary Fig. S4.2). Nevertheless, in 15 cases, banding patterns deviated from expectations in three different ways. First, the PCR fragment had remained uncut after the restriction reaction (one individual). Second, eight individuals displayed an ambiguous pattern consisting of bands from both species (e.g., lane 15 in Supplementary Fig. S4.2). Third and finally, incompletely digested patterns occurred in six individuals (e.g., lanes 1-8 and lane 16 in Supplementary Fig. S4.2). To be conservative all these individuals were omitted from subsequent analysis. This resulted in a pool of 292 molecular identified individual copepods that originated from three different regions and two different host species, falling in the following categories: *Mytilicola intestinalis* female (n = 40), *M. intestinalis* male (n = 47), *Mytilicola orientalis* female (n = 109) and *M. orientalis* male (n = 96).

**Table 4.1** Sample sizes of introduced *Mytilicola* species in different sub-categories. Subcategories involve host species (*Crassostrea gigas* and *Mytilus edulis*), parasite species (*Mytilicola orientalis* and *Mytilicola intestinalis*), sexes (males and females) and the two regions (Dutch Delta and Wadden Sea). Assignments were based on genetic (copepod species) and morphological (sex) identification.

<table>
<thead>
<tr>
<th>Host species</th>
<th>Copepod species</th>
<th>Sex</th>
<th>Dutch Delta</th>
<th>Wadden Sea</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Crassostrea gigas</em></td>
<td><em>M. orientalis</em></td>
<td>Males</td>
<td>22</td>
<td>31</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Females</td>
<td>24</td>
<td>32</td>
<td>56</td>
</tr>
<tr>
<td><em>Mytilus edulis</em></td>
<td><em>M. orientalis</em></td>
<td>Males</td>
<td>20</td>
<td>27</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Females</td>
<td>21</td>
<td>35</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td><em>M. intestinalis</em></td>
<td>Males</td>
<td>20</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Females</td>
<td>19</td>
<td>26</td>
<td>45</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>126</strong></td>
<td><strong>181</strong></td>
<td><strong>307</strong></td>
</tr>
</tbody>
</table>

**Effects of origin**

**Host species and size**

For *Mytilicola orientalis*, which uses both blue mussels (*Mytilus edulis*) and Pacific oysters (*Crassostrea gigas*) as host, host species identity was an important determinant for parasite body length as the copepods were significantly larger in oysters (linear model; $F_{1,193} = 6.721$, $p < 0.05$; Fig. 4.3). Additionally, in both host species, *M. orientalis* females were larger than males ($F_{1,193} = 599.470$, $p < 0.001$).
Furthermore, host size positively affected parasite body length in mussels (linear regression; *M. orientalis* $R^2 = 0.87$, $p < 0.001$; *M. intestinalis* $R^2 = 0.84$, $p < 0.001$), but not in oysters ($p = 0.202$; Fig. 4.4). For all parasite species and host combinations in this model, there was a significant difference between the parasite sexes regarding the relationship between host size and parasite body length (*M. orientalis* in mussels $F_{1,99} = 633.554$, $p < 0.001$; *M. intestinalis* in mussels $F_{1,87} = 329.325$, $p < 0.001$; *M. orientalis* in oysters $F_{1,97} = 200.376$, $p < 0.001$). No significant interactions between sex and host size were found for neither of the parasite-host combinations (*M. orientalis* in mussels $p = 0.365$; *M. intestinalis* in mussels $p = 0.830$; *M. orientalis* in oysters $p = 0.418$).

Region

Body length of both parasite species (corrected for host size) in blue mussels did not differ between regions ($p = 0.350$; Fig. 4.5). Furthermore, within the mussel host, *M. orientalis* was overall larger than *M. intestinalis* ($F_{1,179} = 15.568$, $p < 0.001$), but a significant interaction term with sex showed that *M. orientalis* males were smaller than *M. intestinalis* males ($F_{1,179} = 7.110$, $p < 0.01$). Furthermore, for both parasite species females were larger than males ($F_{1,179} = 1048.029$, $p < 0.001$).

![Boxplots of *Mytilicola orientalis* body length (µm) in both host species. In grey copepods originating from oyster (*Crassostrea gigas*) hosts, in white copepods originating from mussel (*Mytilus edulis*) hosts from the Dutch Delta and Wadden Sea, for both females (left) and males (right).](image)
Fig. 4.4 Relationship between host shell length (mm) and parasite body length (µm) per sex for each *Mytilicola*-host species combination. A) *Mytilicola orientalis* in blue mussels (*Mytilus edulis*). B) *M. intestinalis* in blue mussels. C) *M. orientalis* in Pacific oysters (*Crassostrea gigas*). Fitted lines are significant regressions.
In Pacific oysters, *M. orientalis* parasites were larger in the Wadden Sea than in the Dutch Delta ($F_{1,97} = 9.656, p < 0.01$) and females larger than males ($F_{1,97} = 240.112, p < 0.001$). In addition, a significant interaction term indicated that the relationship between body length and region was dependent on the sex of the parasite ($F_{1,189} = 9.297, p < 0.01$).

**Fig. 4.5** Parasite body length (corrected for host size) for both introduced *Mytilicola* species. In grey *Mytilicola intestinalis* and in white *Mytilicola orientalis* at each region for females (left) and males (right).

**Reliability of morphological identification**

*Principle component analysis*

Morphological differences between parasite species were illustrated with a Principal Component Analysis (PCA), with limited overlap between species for females ($n = 92$, Fig. 4.6A), but considerable overlap in males, as many individuals presented intermediate morphologies ($n = 90$, Fig. 4.6B). This sex-related difference in species partitioning had consequences for the assessment of the reliability of morphological species identification with the Discriminant Function Analysis (DFA) for both sexes.
DFA for females
In total, 92 females entered the discriminant analysis with the following prior probabilities per group: *M. intestinalis* 0.41 (n = 38) and *M. orientalis* 0.59 (n = 54). One discriminant function was extracted and this was highly significant (Wilks $\lambda = 0.227$, $F_{1,141} = 40.925$, $p < 0.001$), indicating a clear discrimination between groups. The first discriminant function with standardized discrimination coefficients for females is given by:

$$F_i = 1.33 \times \text{appendages}_i + 0.62 \times \text{pre-tail}_i + 0.21 \times \text{segments}_i - 0.55 \times \text{tail angle}_i - 0.07 \times \text{length}_i - 0.02 \times \text{width}_i$$

Discriminant distributions show minimal overlap between two groups and therefore the discrimination was relatively accurate (Fig. 4.7A). The classification of the groups was highly successful for females; 95% of *M. intestinalis* females and 91% of *M. orientalis* females were correctly assigned to one of the two *Mytilicola* groups.

The morphological features used for identification strongly differed in their importance for a correct assignment. The variable ‘shape of appendages’ had by far the highest coefficient in the discriminant function. Similarly, the backward selection procedure showed that the shape of appendages, length of the pre-tail and the tail-angle were the last variables to be dropped, indicating their importance in the discrimination between the two parasite species.

When only using the morphological features that were the easiest to assess (shape of appendages and tail-angle) a large proportion of individuals could still be correctly assigned to the genetically identified species. By using the shape of the appendages as morphological identification feature, 97% of the *M. intestinalis* females and 87% of the *M. orientalis* females were correctly identified. If the tail angle was used as the only identification criterion, for 81% of the *M. orientalis* females and 84% of the *M. intestinalis* females the species identification was correct. A tail-angle of 20° was hereby used as cut-off point based on the group averages (a tail-angle < 20° was handled as "narrow" (*M. intestinalis*) and ≥ 20° was treated as "wide" (*M. orientalis*)).

From all genetically identified females, 94% of *M. orientalis* and 97% *M. intestinalis* individuals were correctly identified by either using the shape of the appendages or the angle of the tail.

DFA for males
In total, 90 males entered the discriminant analysis with the following prior probabilities per group: *M. intestinalis* 0.51 (n = 46) and *M. orientalis* 0.49 (n = 44). For males, one discriminant function was extracted and was highly significant (Wilks $\lambda = 0.692$, $F_{1,88} = 6.152$, $p < 0.001$). The first discriminant function with standardized discrimination coefficients for males is given by:

$$M_i = 0.76 \times \text{appendages}_i + 0.49 \times \text{length}_i - 0.48 \times \text{segments}_i - 0.19 \times \text{head}_i - 0.06 \times \text{tail angle}_i$$

For males, discriminant distributions show more overlap between the two groups than for females (Fig. 4.7B). This also showed in the classification of males, which was less reliable than that of females; 78% of *M. intestinalis* males and 73% of *M. orientalis* males were correctly assigned to one of the two *Mytilicola* groups.

Like in females, the variable ‘shape of appendages’ had the highest coefficient in the discriminant function for males. Also, the backward selection procedure showed that the shape of appendages was the last variable to be dropped, indicating its importance in the discriminant function.
Fig. 4.6 Principal component analysis for each sex separately. A) Females (n = 92; 7 morphological variables). B) Males (n = 90, 6 variables). In grey, the individual parasites that were molecularly identified as *Mytilicola intestinalis*, in black *Mytilicola orientalis*.
When using only the shape of appendages as morphological identification feature, some males could still be correctly assigned to the genetically identified species. However, the success rate was not as high as that for females: 70% of the *M. orientalis* and 76% of the *M. intestinalis* males were correctly identified. The angle of the tail was less important in the discrimination between groups for males, but if this feature was used as the only identification criterion, 64% of the genetically identified *M. orientalis* and 63% of the *M. intestinalis* males were correctly identified. From all genetically identified males, 82% of *M. orientalis* and 78% of *M. intestinalis* individuals were correctly identified by either using the shape of the appendages or the angle of the tail.

![Discriminant distributions for each sex separately.](image)

**A**) Females (n = 92) of the parasitic copepods *Mytilicola* spp.. **B**) Males (n = 90). In grey *Mytilicola intestinalis* and in white *Mytilicola orientalis*. On the x-axis the discriminant score of the first discriminant function and on the y-axis the relative frequency of the observations. The light grey area indicates observations with erroneous morphological identification.

**Effects of storage method on morphology**

We compared two groups of *Mytilicola* spp. that differed in storage methodology. In both groups, *Mytilicola* individuals were removed fresh from their host, but in Group 1 the samples were stored directly in ethanol, while in Group 2 the samples were frozen prior to ethanol storage.

No significant differences were found in body size measurements of fresh samples between Group 1 and Group 2 at the start of the experiment (all p > 0.05). Within the first group (fresh-ethanol), samples were significantly smaller after being stored in ethanol (all p < 0.05), however the shape of the appendages and the number of segments remained the same (Table 4.2). Length measurements (body length, head length, width, pre-tail) were, on average, 17.4% shorter after storage in pure ethanol. Within the second group (fresh-frozen-ethanol) there was no significant difference in body length between fresh and frozen samples (all p > 0.05), but frozen samples were significantly smaller after storage in ethanol (p < 0.05, except for pre-tail p = 0.106), however these samples shrunk less (9.0%) than fresh samples (Table 4.2).
Table 4.2 Effects of storage method on *Mytilicola* spp. morphology. The percentage of shrinkage of morphological measurements after storing fresh samples in ethanol (Group 1) and storing frozen samples in ethanol (Group 2). Welch paired t-test results are given in the adjacent columns.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fresh-ethanol % shrinkage</th>
<th>t</th>
<th>df</th>
<th>p</th>
<th>Frozen-ethanol % shrinkage</th>
<th>t</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length</td>
<td>17.2</td>
<td>2.348</td>
<td>31</td>
<td>&lt; 0.05</td>
<td>7.4</td>
<td>3.059</td>
<td>28</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Head length</td>
<td>22.2</td>
<td>4.112</td>
<td>32</td>
<td>&lt; 0.05</td>
<td>8.3</td>
<td>2.739</td>
<td>28</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Width</td>
<td>14.1</td>
<td>2.624</td>
<td>31</td>
<td>&lt; 0.05</td>
<td>10.9</td>
<td>3.101</td>
<td>28</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Pre-tail length</td>
<td>16.0</td>
<td>2.437</td>
<td>14</td>
<td>&lt; 0.05</td>
<td>9.5</td>
<td>1.709</td>
<td>17</td>
<td>0.106</td>
</tr>
<tr>
<td>Average</td>
<td>17.4</td>
<td></td>
<td></td>
<td></td>
<td>9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Our analyses show that *M. orientalis* and *M. intestinalis* are significantly morphologically differentiated, but with considerable overlap, and that no accurate diagnostic character exists in the study area where both invasive species now co-occur. The error rate of assignment to species is not equal for the sexes and is more than three times higher in males (25%) than in females (7%). We therefore argue that this is a case of ‘crypticism-in-retrospect’ by invasion, in which the two species were originally described from different parts of the globe (the Mediterranean Sea and Japan) and from different hosts (blue mussels and Pacific oysters). Now that the species’ ranges and hosts overlap, a sharp distinction between their morphologies is not seen. Whether the morphologies of the populations that yielded the type specimens are more clearly differentiated would be interesting to find out, but this is unknown at present.

For both males and females, the shape of the appendages is the most important discriminant factor in the multi-variate analyses and using only the shape of the appendages for identification, the two species can still be correctly distinguished in many cases (females: 87-97% and males: 70-76%). The shape of the dorsal appendages (pointy or stunted) is generally easier to observe for females as they carry pointy appendages on almost every segment of the body, while for males only the 3\textsuperscript{rd} and 4\textsuperscript{th} segments are pointy (Ho and Kim, 1992; unpublished observations M. A. Goedknegt). Unfortunately, the most useful feature for distinguishing between both species is subject to personal observation bias as it is a qualitative feature (pointy vs. stunted appendages). Nevertheless, this feature has also been used by other authors (Mori, 1935; Elsner et al., 2011). Another straightforward morphological feature, the tail angle, has been mentioned as a useful distinguishing feature in previous studies (Elsner et al., 2011), but in our data this variable was only useful for discriminating between species for females. Other morphological measurements such as body length, width, the length of the head and the pre-tail (females only) did not discriminate between species in the discriminant analysis. Interestingly though, these features are sometimes related to the origin of the parasites. Copepod body length is significantly affected by location (only parasites originating from oysters), host size (only parasites originating from mussels) and host species (only for *M. orientalis*) and because of collinearity, other body size measurements are likely affected by the origin of the parasites as well. These results suggest that a series of more comprehensive morphological measurements will not increase the reliability of morphological identification. In addition to morphological measurements, the number of
segments is not an important identification feature as it only discriminates between sexes (and life stages; Gee and Davey, 1986a). Therefore, of all the morphological variables, the shape of appendages and, to a lesser extent, tail angle can be considered as the most reliable and feasible identification features.

Storage both in ethanol and by freezing is recommended if species identification is based on the shape of the appendages, because this feature is not affected by the storage method. However, the storage of fresh samples in ethanol is advisable for molecular identification. If morphological measurements are intended, shrinkage in body size (body length, head length, width, pre-tail length) must be taken into account compared to fresh samples. Our study indicates that this shrinkage can be reduced if samples are first frozen and then transferred to ethanol. Regarding species identification, the method of choice will depend on the level of accuracy required for the research question at hand. If 100% accuracy is needed, then identification with molecular methods will be required for both sexes. However, if lower accuracy is acceptable, identification can be based on the shape of the appendages leading to 87-97% and 70-76% accuracy in females and males, respectively. Nevertheless, as these data were collected by one experienced observer, the reliability of morphological identification is likely to vary among multiple and/or unexperienced observers.

Although our analyses showed that *M. orientalis* and *M. intestinalis* are morphologically differentiated, there is considerable overlap in morphological features between the two species and no 100% accurate diagnostic character seems to exist in the study area where both invasive species now co-occur. We therefore argue that the two parasitic copepods are a case of ‘cryptism-in-retrospect’ by invasion. Although both species have originally been described separately for the Mediterranean Sea and Japan, their recent invasion along the North Atlantic coast has led to an overlap in geographic and host range where a sharp distinction between their morphologies is not seen. The fact that morphology is found to be somewhat differentiated between locations and hosts may yield clues as to the possible cause(s) of crypticism in these species. Several hypotheses have been put forward that may explain the phenomenon of cryptic species in an evolutionary context, among others those are: convergent morphological evolution, morphological stasis by stabilizing selection, relaxed selection on morphology, selection for phenotypic plasticity, and high levels of genetic variability for morphology related to large effective population size (Potter et al., 1997; Bickford et al., 2007; Egea et al., 2016). Furthermore, particular environments or taxa may harbour more cryptic species than others, although this is still debated (Knowlton, 1993; Nygren, 2014; Bickford et al., 2007; Pfenniger and Swenk, 2007). The observed differences in morphology among hosts and locations reject stabilizing selection as a potential cause for the similar morphology between these molecularly diverged species. In contrast, the morphological differentiation is consistent with several other possible causes, including plasticity, relaxed selection and large effective population sizes. Whatever the exact mechanisms, the phenomenon of reversed crypticism in these two species has several important implications. First, researchers may draw erroneous conclusions about the distribution and host range of both *Mytilicola* species. For instance, a study in the Exe estuary in the UK reported the presence of *M. intestinalis* in Pacific oysters (Aguirre-Macedo and Kennedy, 1999), while this parasite-host relationship has never been reported later when the existence of a second related species was known and it may therefore actually not exist (Elsner et al., 2011; Goedknegt et al., 2017). This conclusion is also supported by the lack of successful artificial infections of Pacific oysters with *M. intestinalis* (Elsner et al., 2011; personal communications M. E. Feis). Hence, most likely the parasite species encountered was actually *M. orientalis* and the researchers were probably not aware of the recent introduction of a new *Mytilicola* species due to the
morphological similarities of the two parasitic copepods. Second, studies on the impacts of the two parasite species on (often ecologically and commercially important) bivalve hosts, gave contradictory results (e.g., Lauckner, 1983; Steele and Mulcahy, 2001) and this may also be the result of misidentifications.

**Conclusions**

Our results show that no reliable diagnostic character can flawlessly discriminate between two co-occurring invasive parasite species (*Mytilicola orientalis* and *M. intestinalis*), which have originally been described separately. This case of ‘crypticism-in-retrospect’ can serve as a cautionary example for other taxa with shifting distributional ranges, e.g. due to global change or human-mediated introduction (Lenoir and Swenning, 2014). When site of origin can no longer discriminate between species, a closer examination of morphological and genetic differentiation will be advisable.

**Acknowledgments**

The authors wish to thank everyone who provided samples, including Tabea Stier, Anne-Karin Schuster and Carolin Wendling. We are grateful to Anneke Bol for help with the molecular analysis and to Felipe Ribas for the schematic representations of the parasites. Project partners Dr. Kees (C.J.) Camphuysen and Dr. Christian Bushbaum are thanked for their general support. This work was funded by the Netherlands Organization for Scientific Research (NWO) and the German Bundesministerium für Bildung und Forschung (BMBF) (NWO-ZKO project 839.11.002).
Supplementary material

Table S4.1 Sampling locations, coordinates for each site and the month and year of host collection.

<table>
<thead>
<tr>
<th>Region</th>
<th>Location</th>
<th>Coordinates</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wadden Sea</td>
<td>1</td>
<td>55.0175 °N, 8.2605 °E</td>
<td>Sept 2010/May 2012</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>54.4748 °N, 8.1841 °E</td>
<td>May 2012</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>53.0951 °N, 4.5332 °E</td>
<td>August 2010</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>53.0858 °N, 4.5427 °E</td>
<td>May 2012</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>53.0646 °N, 4.5434 °E</td>
<td>February 2011</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>53.0041 °N, 4.5903 °E</td>
<td>March 2011</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>52.5580 °N, 4.5412 °E</td>
<td>March 2011/May 2012</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>52.5605 °N, 4.4888 °E</td>
<td>November 2010</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>53.0022 °N, 4.4541 °E</td>
<td>May 2012</td>
</tr>
<tr>
<td>Dutch Delta</td>
<td>10</td>
<td>51.4048 °N, 8.1179 °E</td>
<td>May 2012</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>51.4017 °N, 4.0617 °E</td>
<td>May 2012</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>51.3743 °N, 3.5507 °E</td>
<td>May 2012</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>51.4121 °N, 3.4723 °E</td>
<td>May 2012</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>51.3729 °N, 3.4213 °E</td>
<td>May 2012</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>51.3141 °N, 3.5824 °E</td>
<td>May 2012</td>
</tr>
</tbody>
</table>