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Morphotypes of *Cytheridella illosvayi* (Ostracoda) Detected by Soft and Hard Part Analyses

Claudia Wrožyna^{1,3}, Werner E. Piller¹, and Martin Gross²

¹)University of Graz, Institute of Earth Sciences, NAWI Graz, Heinrichstrasse 26, A-8010 Graz, Austria

²)Universalmuseum Joanneum, Department for Geology & Palaeontology, Weinzöttlstrasse 16, A-8045 Graz, Austria

Abstract

Quantitative approaches dealing with soft- and hard-part morphological variability of ostracods are very rare for most species. Here we present intraspecific limb and shell variability of the Neotropical freshwater ostracod species assumed as *Cytheridella illosvayi*. For the first time such an analysis considered adult females and males, as well as juveniles. Variability of the appendages included the antennule, second antenna as well as the first and second thoracopod and was analysed in terms of ratios of podomeres, setae and limbs. Limb variability of adult and juvenile individuals (down to A-3) is generally low. The highest variation is shown in podomere proportions of the antennae, while thoracopods and setae provide minor and/or non-significant influence on the variability. Based on discrimination analyses shell parameters (i.e., shell length, position of the transversal sulcus) emerge to be more important for differentiation of groups than limb ratios. Adult females exhibit a large size range in which two clearly separated morphotypes exist. Although differences in limbs are small their existence can be proven also on the basis of proportions between podomeres of the antennae. Males and juveniles, however, do not display a separation into corresponding subgroups or morphotypes. The presence of two morphologically similar females and only one type of males indicates the coexistence of female morphotypes which may represent either two (cryptic) species or a mixed reproduction population in which parthenogenetic and sexual reproduction coexists.

Keywords

Ostracods; Neotropics; *Cytheridella*; morphological variability; morphotypes

Introduction

Ostracods represent one of the most diverse groups, with an estimated >20 000 living species, of which only approx. 10 000 have been described (Rodriguez-Lazaro & Ruiz-Muñoz, 2012). At present, ostracods are popular model organisms for research on the evolution of reproductive modes (e.g., Martens, 1998; Schön & Martens, 2012) and used as

³)Corresponding author; claudia.wrozyna@uni-graz.at.

proxies for climate and ecosystem changes (e.g., De Deckker & Forester, 1988; Horne, 2007; Wrožyna et al., 2010). They are one of the few invertebrate groups with an extensive fossil record, but are also rather unique as a fossil group with such a diverse modern representation (Schön et al., 1998).

Systematic, ecologic and evolutionary studies of ostracods rely heavily on morphological information. Traditionally, morphological analyses of ostracods are focused either on shell characteristics or on the appendages, but with limited linkage between them. In ostracod taxonomy, dimensions of the carapace (usually length and height) are commonly considered (e.g., Meisch, 2000) but not of the limbs. In contrast to shell variability, soft parts of ostracods are disregarded and lack sound documentation. Commonly, variability of the appendages was studied either due to ontogenetic concerns (e.g., Smith & Kamiya, 2003, 2005) or focused on specific features such as the male copulatory organs (e.g., Martens, 2000). Moreover, most studies refer to qualitative data only from adults and predominantly of females.

Thus, investigations related to intraspecific variation of ostracod taxa are based on shells and do not include information on the appendages (Neil, 2000). First approaches focused on individual limbs such as the first (Maddocks, 2000) or second antenna (Danielopol, 1978). Although efforts aligned for comparative description of ostracod soft parts have increased, these data mostly deal with qualitative differences (Martens et al., 1998a).

In this study we present data on morphological variability of both soft and hard parts of the ostracod species *Cytheridella ilosvayi* Daday, 1905, common in Central and tropical South America. Generally, little is known about the distribution of ostracod species, their biology and ecology in the Neotropics. The genus *Cytheridella* (Limnocytheridae, Timiriasevinae) has a global distribution during the Cenozoic ranging from central Europe, India and Africa to North and South America (Purper, 1974; Colin et al., 1997). Modern species occur in Africa (Karanovic, 2012) and the Neotropics (Purper, 1974), but information on species biogeography and ecology is restricted to the two species *C. ilosvayi* and *C. boldii* Purper, 1974 from North, Central and South America. Modern (American) *Cytheridella* has a wide geographical range covering the subtropics and tropics from approximately 30°N to 30°S (Sarasota County, Alvarez Zarikian et al., 2005; Tampa Bay, Willard et al., 2007; Florida; Quintana Roo, Mexico, Gabriel et al., 2009; Yucatan peninsula including Lago Petén Itzá, Guatemala, Pérez et al., 2010a, b; Hispaniola, Perez-Gelabert, 2008; Brazil, Upper Paraná floodplain, Higuti et al., 2007; Mormul et al., 2010; Rio Grande do Sul, Purper, 1974). *Cytheridella* occurs in various aquatic ecosystems, but shows preferences for rivers or at least slightly flowing conditions (Pérez et al., 2010a).

Methods

Sampling localities and study organism

Two surface samples were taken in September 2009 from two lake habitats in western Brazil, State of Amazonas near the city of Eirunepé (fig. 1). The localities Barro Branco (6°50'18.3"S 69°45'37.0"W) and Lago Comprido (6°43'52.7"S 69°44'33.9"W) are abandoned channels of the Tarauacá River. Samples were taken with a hand net from the

margins of the water bodies. At Barro Branco samples were taken between roots of floating plants (*Eichhornia*) (BB01-09) and from the surface sediments of the lake shore (BB02-09). Sample BB02-09 consisted of whitish mud with abundant leaf litter. Lago Comprido samples comprise a surface sample (LC01-09) of whitish mud and abundant leaf litter and a surface sample (LC02-09) covering the uppermost 10 cm. This sample consisted of whitish mud. Barro Branco samples contained a high number of ostracods with >80% living (appendages visible) specimens. Less ostracod material was available in Lago Comprido samples. The samples were clearly dominated by *Cytheridella ilosvayi*. Additional taxa were *Darwinula stevensoni* (Brady & Robertson, 1870) and *Stenocypris* sp. The population of *C. ilosvayi* was dominated by females.

All figured specimens are stored at the Museu Paraense Emílio Goeldi, Belém.

Supplementary material is stored at the Universalmuseum Joanneum, Graz.

Sample preparation

All samples were washed in the field through 200 μ m sieves. The sieve residuals were stored in ethanol (96%) immediately after sieving. Ostracods were completely picked from the samples under a binocular (Zeiss Discovery.V8). Specimens with opened valves were selected randomly for soft part dissection. Soft parts were detached from both valves using entomological needles, dissected, and mounted with Hydro-Matrix® (Micro-Tech-Lab, Graz, Austria) onto cover slides. Photographs for analysis of soft and hard parts were taken using a Zeiss Axioplan 2 and a digital camera Leica DFC 320. Stacked pictures were compiled with the software Helicon Focus Pro (Helicon Soft, Kharkov, Ukraine). Species identification of *Cytheridella ilosvayi* was carried out in accordance with Löffler (1961), Purper (1974) and Karanovic (2012).

Abbreviations applied to limb morphology

A1, first antenna; A1EV-EIII, fifth to third podomere of the first antenna; A1EIVe2, second anterior seta on the fourth podomere of A1; A2, second antenna; A2EIV-EII, fourth to second podomere of A2; A2 EIIIGM, apical claw of second antenna; T1, first thoracopod; T1EIV-EII, fourth to second podomere of T1; T1EIIe, antero-distally e-seta on the second podomere of T1; T2, second thoracopod; T2EIV-EII, fourth to second podomere of T2; T2EIIe, antero-distally e-seta on the second podomere of T2 (based on Meisch, 2000; Karanovic, 2012). See also fig. 2.

Biometric analyses of limbs

The morphology of limbs of 38 specimens were investigated using a transmitted light microscope at 100 $\times$ magnification. A total of 16 linear distances (lengths of segments and setae mentioned in the abbreviation list above) were taken on each of the 38 specimens using the Image Access 3.0 software (Imagic Bildverarbeitungs AG). In order to analyse symmetry between left and right limbs, each measurement was performed on both sides of 20 specimens. The distance measurements were further converted into 24 ratios: A1EIV/EIVe2, A1EV/EIV, A1EV/EIII, A1EIV/EIII, A2EIII/EIIIGM, A2EIV/EIII, A2EIV/EII, A2EIII/EII, T1EIV/EII, T1EIV/EIII, T1EIV/EII, T1EIII/EII, T2EII/EIIe, T2EIV/EIII, T2EIV/EII, T2EIII/EII, A1/A2, T1/T2, A1EIVe2/A2EIIIIGM, T1EIIe/T2EIIe, A1/T1, A2/T2,

A1/T2, *A2/T1* (ratios in italics were selected for further statistical analyses). In order to include the proportion of the limbs among each other, we added the length of each considered podomere referred to as the length of the limb (e.g., *A2/T2* refers to the sum of *A2EIV*, *EIII* and *EII* vs. sum of *T2EIV*, *EIII* and *EII*). Drawings of the limbs were performed with CorelDraw® X4 (Corel), based on photographs of the limbs.

Male copulatory organs were also separated and checked for their morphological variability.

Analysis of shell variability

In order to analyse variability of the shells, we have measured length, height and the length between the anterior margin and the transversal sulcus (referred to hereafter as 'length sulcus'). SEM pictures were checked for ornamentation, width and hinge.

Statistical analyses

First, the differences of the limb ratios between left and right limbs from 20 specimens were tested with two-sample *t*-tests performed with the Microsoft Excel® 2007. Multivariate statistical analyses were performed with SPSS 19 (IBM, 2010). For all 24 limb ratios the mean, range, variance and standard deviation were calculated. Prior to statistical analyses the linear measurements of the shell parameters were log-transformed. Out of all ratios measured, thirteen were selected (see above) for discrimination analyses based on normal distribution, homogeneity of variance (Levene's test) and significance of differences of variable means (Tukey's range test in conjunction with an ANOVA). Discrimination analysis was performed for (a) limb ratios, (b) shell parameters (length, height, L/H, length sulcus) and (c) both limb ratios and shell parameters.

Results

Shell characteristics

The length-height plot shows ontogenetic growth stages (see fig. 3). Accordingly, our dataset comprises instars down to A-3. Through the presence of fully developed copulatory organs the first three size classes were identified as adult females and males, respectively. Adult females reveal a large size range forming two well-separated size clusters. The larger females have lengths ranging from 1110-1140 μm and heights ranging from 613-653 μm ; while length of the smaller females ranges between 921-992 μm , and a height between 518-578 μm . Further, mature characteristics such as well-developed inner lamella, pore-canal (fig. 4), and eggs (variable number of 9 to 12) were observed in both female morphotypes. Male shells are smaller than the females (806-881 μm length, 451-485 μm height). Beside size there are no other (qualitative) shell characteristics (e.g., ornamentation) that are different between the two groups of females (for details see figs. 4 and 5).

Variability of the limbs

Analyses of left and right limb ratios revealed that both are significantly symmetrical ($P < 0.05$). Thus, the analysis of one limb (left or right) of each pair is sufficient for morphological analysis.

The variability of all morphological traits is displayed in fig. 6. Results of thirteen selected limb ratios that were used for multivariate statistics are summarised in table I and displayed in fig. 7. Also, table II presents a correlation matrix in order to display correlations between the ratios.

The majority of the limb ratios reveal relatively small ranges of all instars, e.g., A2EIV/EIII. The largest variability occurs at the first antenna (A1) and the second antenna (A2). In particular, the ratios A1EIV/EIVe2, A1EIV/EIII, A2EIII/EII, as well as A1EIVe2/A2EIIIIGM reveal large variation. Less variation is displayed by the first and the second thoracopod (T1 and T2) and the terminal podomere of A2. Also, ratios between limbs such as A1/A2, T1/T2, A2/T2 and A1/T2 exhibit only minor variation. Generally, limb ratios are either increasing or decreasing from A-2/3 to adult males and females (fig. 7). In most cases, smaller females and males exhibit similar means of the ratios. Means of limb ratios of the larger females do not continue the previous trend in limb ratios (e.g., A2EIII/EIIIIGM, fig. 7). This pattern is most conspicuous in the individual limb ratios (A1EV/EIII to T2EIII/EII). Males show relatively high variability of ratios related to the podomeres EII and EIII of the second antenna and/or second thoracopod. Highest variability is shown for ratios of the second antenna, whereas, the ratios A2EIV/EII and A2EIII/EII are relatively variable. Also, the length of EIII of the second thoracopod shows relatively high variation which becomes noticeable for the 'between limbs' ratios. However, males with, e.g., a short EIII or EII at A2 do not mandatorily have also short EIII at T2.

Ratios considering limbs among each other and those of thoracopods show high correlations (table II). Ratios of the first and second antenna are less frequently correlated. These correlations are in most cases weak (e.g., A2 EIV/EIII to A2 EIII/EII: $r = 0.37$). There is only one negative correlation between A2EIII/EII and A2EIV/EIII ($r = -0.37$).

Discrimination analysis of the limb ratios exhibits a significant separation of the specimens belonging to the different groups (according to the length-height plot): Wilk's Lambda: 0.05, Chi square: 84.39, P : 0.03. Classification results show that 84.2% of the specimens were correctly classified in terms of statistics. In the scatter plot (fig. 8) the large females (Female 1) are assembled in the left lower quadrant associated with negative loadings on axis (function) 1 and 2. Opposite, the smaller females (Female 2) have positive loadings on axis 1 similar to the males, thus, overlapping each other. Juveniles build a separate cluster with higher loadings on axis 2. Loadings of all groups vary around 0 on axis 3 except males which show a trend to more positive values and have a more scattered distribution.

The first canonical function accounted for 53.1% of explained variability and discriminates mostly between the adult females Female 1, Female 2 and males. The second and third functions accounted for 29.9% and 13.3% of explained variability, respectively. Discrimination function 4 explains 3.7% of variability (table IV).

Standardised discrimination function coefficients of function 1 (table IV) do not show a distinct pattern. Except A2EIV/EIII and T1/T2 all variables have relatively high values. Variables with highest coefficients for function 1 are A2/T2 (-2.87), T2EIII/EII (2.09), and A1/A2 (-2.06). Also, A1EV/EIII, A1EV/EIII (1.99), A2EIII/EIIIIG2 (1.93) and A1EIV/EIII

(−1.89) have high values. The number of high function coefficients of function 2 is lower. The variable with the highest coefficient is T2EIII/EII (3.18). Except A2EIV/EII, variables of antennas have low impact on function 2. Instead, the proportions between limbs are similar to the pattern on function 1, and have very high function coefficients (i.e., A2/T2, A1/T1, and A1/T2). Function 3 is predominantly influenced by variables referring to limb proportions, i.e. A1/T2, A2/T2. Second antenna variables (A2EIV/EII, A2EIII/EII) affect function 3. Limb proportions are important for function 4 with the difference that the variables of A2 contribute more. Canonical loadings, representing correlations between the investigated limb traits and the discrimination functions of function 1 are highest for A1EIV/EIII (0.32), and A2EIV/EII (−0.09). For the second function highest correlations occur for A2EIV/EIII (0.26) and A2/T2 (−0.25). Highest correlations for function 3 represent the ratios A1/T1 (0.61), A1/T2 (0.47), T1/T2 (0.41) as well as T1EIII/EII (0.3) and T2EIII/EII (0.24). Thus, discrimination of the specimens based on limb traits cannot be described by a single parameter. However, the majority of the explained variability (53.1%) is associated with features of the first and the second antennas. In particular, the ratios A1EIV/EIII and A2 EIV/EII appear to provide a large contribution to the separation of the specimens into different groups.

A highly significant discrimination (Wilk's Lambda = 0.01, Chi square = 150.52, $P = 0.00$) between the groups occurs for the shell parameters. Classification results indicate that 100% of originally grouped specimens were correctly classified. The first canonical function accounts for 98.4% of the explained variability, the second and third for 1.1% and 0.4%, respectively (table II). This distinct separation effect is clearly visible in the scatter plot (fig. 8). The groups are aligned along axis 1, and sorted by instars. Thus, the large females have the most positive loadings and the juveniles A-2/A-3 the most negative loadings on axis 1. The variability range of the loadings of the groups on axis 2 is quite small. Standardised discrimination function coefficients are given in table IV. The highest coefficient on function 1 is length (1.24). The 'length sulcus' (1.02) and L/H (0.85) are highest coefficients on functions 2 and 3, respectively. Accordingly, canonical loading of the shell length was highest for function 1 (0.86), distance to the sulcus had the highest correlation with function 2 (0.87), and L/H ratio showed the highest correlation on function 3. Thus, the length of the shells is the main discriminating parameter. Minor influence is given by the position of the transversal sulcus. The height of the shells has no influence on the separation.

Corresponding to the previous discrimination analyses highly significant separation of the groups is also observable for both limb ratios and shells (Wilk's Lambda = 0.00, Chi square = 216.21, $P = 0.00$; table II). Classification results show that 100% of originally grouped specimens were correctly classified. The first canonical function accounts for 94.1% of explained variability, the second and third functions explain 3.8% and 1.6%, respectively (table II). The scatter plot (fig. 8) shows that the smaller females, males, A-1 and A-2/3 instars are aligned along axis 1 with increasing loadings. The large females are well separated from the other groups associated with highest positive loadings on axis 2. The groups are relatively widely distributed on axis 3. Standardised canonical discriminant function coefficients are summarized in table III. Highest values reach shell length (−1.76), L/H ratio (1.31), ratio between T1/T2 (1.22) as well as A1/T1 (−0.94). All variables of antennas, especially the second (e.g., A2EIV/EII: 6.4), have very high coefficients for

function 2. For the first time, shell height (3.58) provides some effect on discrimination. Highest canonical correlation is provided for shell length (−0.61) on function 1. Thus, according to negative correlation coefficients differentiation between the individuals decreases with decreasing size. The highest loading on function 2 is given to length sulcus (−0.36) aside A2EIV/EIII (−0.24) and A1EIV/EIII (0.21). More complex is the situation of the canonical loadings on function 3. The highest loadings on function 3 have L/H (0.31), A1/T1 (0.28) and A1/A2 (0.27).

Discussion

Intraspecific variations of limbs and shells

Intraspecific variability of ostracods mostly covers variations in shape, size and length ratio of claws, setae and segments. These phenotypic variations are thought to reflect either underlying genotypic variability or are environmentally induced (ecophenotypic) or are a combination of interactive factors between both genotype and the environment (Martens et al., 1998a). There are no (qualitative) differences between the detailed descriptions by Purper (1974) and our material, except spines on the claw of T2 of females. In the present material, all females except four specimens of the small female group did not exhibit these spines.

No differences in shell or limb morphology between individuals from the different localities appeared. The majority of limb traits of *Cytheridella ilosvayi* have relatively low variability ranges (see fig. 4, table I). However, a large amount of variability is displayed by the antennula and second antenna which is not observed in other ostracod species thus far (e.g., Iepure et al., 2007, 2008). Relative length of setae is considered as an important parameter causing intraspecific variability (e.g., Yin et al., 1999; Iepure et al., 2007, 2008). The performed measurements on *C. ilosvayi* included one seta at each limb. But only one ratio (A2EIII/EIIGM) has been selected for multivariate analyses according to statistical tests for significance and normal distribution. Thus, the assumed higher influence of setae on the intraspecific variability might be attributed to difficulties in performing accurate measurements. After Maddocks (2000), the proportions of the podomeres are more important for intraspecific variability than those of the setae. However, no statistical analysis has been applied to test this hypothesis thus far. Our discrimination analyses of limb traits from *C. ilosvayi* support this hypothesis since they revealed a much higher influence of podomere ratios than of setae. In particular, the ratios of the fused podomere EIV and EIII of the antennula (A1EIV/EIII), as well as the ratio between EIV and EII of the antenna (A2EIV/EII) predominantly affects the explained variability (table V). To a minor degree, the ratios between limb pairs also affect the variability, i.e., A2 and T2. Further, the results demonstrate that the two female groups can be well separated from each other by podomere ratios (fig. 8). Considering the limb ratios as morphological trajectories (fig. 7) it is noticeable that the larger females deviate in most cases from the previous trend (e.g., A2EIII/EIIGM, fig. 7). The larger females display either lower or higher ratios than the smaller females. Naturally, one could expect a continuation of the development. This is most obvious in the antennae (i.e., A1EIV/EIII to A2EIV/EIII), while there is a higher correspondence with the other groups for the ratios between limbs (e.g., A1/A2). Contrary to

the deviations between the two females, the limb ratios of males (i.e., A1EIV/EIII and A2EIV/EII) are closer to that of the smaller females. This implies that the males might be the counterparts of the smaller females. The relatively large variation of ratios of the second antenna of the males could hint to a separation into two types of males (fig. 7). However, individual analyses are too small.

There is a discrepancy between the discrimination function coefficients and the correlation coefficients regarding limb ratios. Thus, high function coefficients of the proportions between limbs (e.g., A2/T2, table IV) indicate a high contribution to the discrimination function. Opposite, the correlation coefficients of the structure matrix (table V) reveal a strongly reduced number of variables correlating with the discrimination functions. The high number of function coefficients in analysis 1 (limb ratios) is probably related to relatively high degree of interdependency between the variables. There are some highly correlated ratios, especially those which contribute one to another. For instance, T1EIII/EII is well correlated with T1/T2 ($r = 0.77$). The correlation matrix (table II) shows, however, that these ratios which have high correlation coefficients in the structure matrix do not show high interdependencies with other ratios. Since these coefficients are 'partial', they reflect an association after the influence of all other variables has been removed, and therefore it seems inappropriate to assess the importance of the limb ratio variables by the size of their coefficients (cf. Williams, 1983).

Considering both limb traits and shell features the length of the shells appears to be the most important variable for discriminating groups. Further, the position of the sulcus is responsible for a certain amount of variability and differentiation. According to low function coefficients and canonical correlations, the length of setae do not have an influence on the intraspecific variability of *C. ilosvayi*. Ratios related to thoracopods, especially ratios among limbs (e.g., A1/T1, A2/T2) have several high discrimination function coefficients. Their low absolute correlations with each discrimination function appear predominantly at function 3 (table V), indicating that thoracopods or their podomere ratios are stable and do not permit discrimination of specimens and instars, respectively.

As observed in other ostracod species, our data reveal no sexual dimorphism in the limbs (cf. Cohen & Morin, 1990) between males and smaller females. However, the majority of limb ratios of the larger females deviate from that of the males (table I).

So far, the low number of studies from different families and/or genera as well as the differences in chaetotaxy of the families prevents a definite assessment of the importance of setae and podomere ratios for the intraspecific variability of individual taxa. Comparisons with literature data are limited due to the use of different limbs, limb traits and/or setae and segments. Since measurements on the complete appendages including all setae would be extremely time consuming, a standard procedure would be needed that allows relatively fast processing. Limb traits that we used were selected according to available literature data and the manageability (easy to recognise and measure). Although terminal claws of the walking legs are found to display large variability (Yin et al., 1999; Iepure et al., 2008) we excluded them from the analyses because these are often slightly distorted and cannot be aligned evenly on the slide. The same applies for setae, which are often affected by ontogenetic

changes (presence, style, position; Broodbakker & Danielopol, 1982). Thus, comparison between individuals of the last development stages is hindered. Accordingly, we choose setae that are present in instars down to A-3. Although it is reported that taxa of the subfamily Timiriaseviinae show higher variability in the third walking leg (Martens, 2000), we excluded this from our data since the bended position in *C. ilosvayi* precluded accurate measurements of that limb (T3).

Cytheridella morphotypes: cryptic species or mixed reproduction?

Two types of females that are predominantly differentiated by size (length) with the presence of only one type of males suggest either cryptic taxa or sympatric occurrence of mixed reproduction modes (i.e., sexual and parthenogenetic females).

Purper (1974) reported dimensions of female carapaces of *C. ilosvayi* with a length range of 957 to 1137 μm . This covers the ranges of our data set (Female 1: 1110-1140 μm length; Female 2: 921-992 μm length). Although Purper (1974) examined >1500 specimens, two (size) separated females were not mentioned. Carapace dimensions from a Guatemalan population were much smaller (females: 770-980 μm length; males: 634-787 μm length; Pérez, 2010), but the occurrences of two female morphotypes is also not reported. Beside size there are also various limb characters which differentiate the two females (fig. 7). For instance, the apical claw of the second antenna is proportionally larger than in the smaller females. The question remains, however, why there is only one type of male. Although there are hints of separation of males (see fig. 7, e.g., A1/A2) it could be possible that the morphological differences of the males are even smaller than in females (limbs) and could not have been differentiated by this kind of morphological analyses. The morphologies of the hemipenis did not reveal any differences or allowed a separation into groups. Assuming the possibility that potential male morphotypes have a strong morphological similarity, one could assign the same to the juveniles which also did not allow any separation. Because *C. ilosvayi* does not exhibit sexual dimorphism in T3 we could not differentiate A-1 in males or females. The low number of available *C. ilosvayi* specimens, however, precludes extensive analyses to test statistical significance of possible differentiation of males and A-1.

Although the knowledge of the existence of cryptic species exists for nearly 300 years, the discovery of cryptic species has increased exponentially over the past two decades because of the availability of DNA sequence data. Molecular analyses revealed exceptional cryptic diversity in some non-marine ostracods, with more than 40 cryptic taxa for *Eucypris virens* (Jurine, 1820) (e.g., Bode et al., 2010). Knowledge about cryptic ostracod species is predominantly restricted to information about genetic divergence (Adolfsson et al., 2009; Bode et al., 2010). Information about presence, extent, and style of morphological differences between cryptic species (including adults as well as juveniles) is missing.

Ostracods are known for their exceptionally wide range of different reproductive modes. Reproduction can be either sexual (species are fully sexual) or asexual (which are known as all-female population, e.g., Darwinulidae). Some of these parthenogenetic species can also reproduce sexually (e.g., *Limnocythere inopinata*; Horne & Martens, 1999). Often these species have a geographically restricted distribution which is termed geographic parthenogenesis (Martens et al., 1998b). Additionally, some ostracod species can coexist as

sexually and asexually reproducing forms (e.g., Bellavere et al., 2002). The presence of two female morphotypes and only one type of males could point to a mixed reproduction population in which males and both sexual and asexual females occur sympatrically. Although it is known that most Cyprididae and a high number of candonid and cytheroid non-marine species have mixed reproduction (Martens et al., 1998b) this phenomenon is thought to be highly underestimated (Chaplin, 1993). Ostracod species groups exhibiting mixed reproduction include *Candonocypris novaezelandiae* (Baird, 1843) (cf. Chaplin, 1992, 1993), *Heterocypris incongruens* (Ramdohr, 1808) (cf. Bellavere et al., 2002) and *Eucypris virens* (e.g., Schön et al., 2000). In *H. incongruens*, coexisting female morphotypes are differentiated by a lobe-like expansion of the selvage on the posterior margin of the left valve (Bellavere et al., 2002). Sexual and parthenogenetic females of *E. virens* do not show consistent morphological differences of the limbs (Martins et al., 2009). *Candonocypris novaezelandiae* reveals two different morphs differentiated by size and colour (Chaplin, 1992, 1993). Thus, sexual/asexual reproduction might be expressed more often in shell characters. Neither parthenogenetic nor mixed reproduction populations of *C. ilosvayi* have been documented so far (Löffler, 1961; Purper, 1974; Pérez et al., 2010a, b). A reason for the lack of two female morphotypes could be the geographically limited distribution of these mixed reproduction populations (cf. Martens et al., 1998b). Many European species are represented throughout their geographical range by parthenogenetic populations while sexual populations are either unknown or restricted to a few, usually southern (circum-Mediterranean) localities, e.g., *Eucypris virens* and *Heterocypris incongruens* (cf. Horne & Martens, 1999). Based on repeated samplings Chaplin (1992) reported successive displacement of a sexual lineage by a closely related parthenogenetic form in a spatially heterogeneous and biotically diverse environment which could be related to human disturbance. This would underline the importance of ecological and climatological impacts on the reproduction mode and possibly on sympatric speciation. So far, most extant representatives of the subfamily Timiriaseviinae are known to be sexual except the parthenogenetic genus *Kovalevskiella* and sexual and/or asexual *Frambocythere* (Carbonel et al., 1986; Colin, 2011). The fossil (Miocene) species *Cytheridella danielopoli* Purper, 1979 found close to our sampling localities in the Brazilian Amazon are thought to be sexual (Gross et al., 2013). Thus, the pre-existence of parthenogenetic *Cytheridella* lineages is not documented.

Benthic non-marine ostracods likely recognise their mate through direct contact by their limbs. Copulation has been observed between males and parthenogenetic females but with limited success (Horne et al., 1998). Thus, the reason for the larger size of Female 1 could be attributed to mate behaviour, and thus copulation of the larger parthenogenetic female with males might be precluded. However, although males can build diverse structures in their copulatory organs, this does not necessarily correspond to female genitalia, although they may reproduce successfully (Broodbakker, 1982). Bellavere et al. (2002) interpreted the different body size of sexual and asexual females according to niche differentiation and assuming differences in the ecological adaptation of the morphotypes. Another reason could be the enlargement of the brood pouch of parthenogenetic females, which likely increases the number of eggs and/or juveniles and improving the survival of offspring. Nine to twelve

eggs were counted in both female types, and there can only be speculations about the meaning of the size differences between the two female groups until more data are collected.

Based on morphological coherence and analogous to *E. virens* (Martens et al., 1998b), we consider both females as morphotypes of *Cytheridella*. Final clarification of whether these morphotypes represent cryptic species or mixed reproduction mode has to be determined by molecular biological analyses.

Conclusion

The morphological approach of this study provides quantitative information on hard- and soft-part variability of the ostracod *Cytheridella*.

Analyses of limbs and shells revealed that variability of soft part morphology is generally low. Highest variability is found in the antennula and second antenna. Thoracopods and setae have only minor influence on variability. This pattern is also shown by juveniles A-3 up to A-1. Further, males as well as females are symmetrical (left and right) at least for the antennula, second antenna as well as both thoracopods T1 and T2. This study documents that proportions between podomeres can be easily obtained and provide important information about variability.

Discrimination analyses that maximise the separation between groups indicate that shell parameters appeared to be more important for differentiating groups. In particular, shell length and the position of the transversal sulcus account for the majority of explained variability of the specimens, even in combination with limb ratios. Important limb traits discriminating the specimens are (pen)-ultimate podomeres of the antennae. Although there is some indication that proportions between limbs affect the variability, high interdependency of limbs and traits may exceed the actual influence.

Moreover, morphological analyses revealed two types of females that are separated primarily by size (length) and to a minor degree by podomere ratios of the antennae. Thus, the larger females ($\geq 1000 \mu\text{m}$) were characterized by smaller A1EIV/EIII, A2EIII/EIIGM and larger A2EIV/EII ratios, respectively, than small females ($\leq 1000 \mu\text{m}$) and males. The presence of two morphologically similar females and only one type of male may indicate either cryptic species in which the males cannot be identified or a mixed reproduction population in which parthenogenetic and sexual reproduction coexists.

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Fig. 1.
Map of sampling localities Barro Branco and Lago Comprido (white circles), State of Amazonas, western, Brazil.

Fig. 2. Simplified appendages of *Cytheridella ilosvayi* Daday, 1905 used for morphological analyses: the antennule (A1), the second antenna (A2), the first and second thoracopod (T1, T2). Indicated are the measured lengths (dashed line) of podomeres and setae, respectively. Roman numerals refer to fifth to third and fourth to second podomeres, respectively, of the respective limb. For abbreviations see also abbreviation list in the text.

Fig. 3. Carapace size in terms of length and height of *Cytheridella ilosvayi* Daday, 1905 in adult females, males and juvenile instars down to A-3.

Fig. 4. Stacked pictures of each left and right shells of the two female morphotypes (♀₁, ♀₂), males (♂), and juveniles (A-1 to A-3) of *Cytheridella ilosvayi* Daday, 1905.

Fig. 5.
SEM pictures of left and right shells of females (♀₁, ♀₂), males (♂), and juveniles (A-1 to A-3) of *Cytheridella ilosvayi* Daday, 1905.

Fig. 6.

Variation ranges of the 24 measured limb ratios represented as boxplots for all investigated individuals (total), and separately for instars (A-1 and A-2/3), males and both female morphotypes (females 1 and 2). For codes of the morphological traits see abbreviation list in the text. Black dots represent outliers.

Fig. 7.

Selected limb ratios of adult females, males and juveniles of *Cytheridella ilosvayi* Daday, 1905, displaying morphological trajectories (square = females 1, cross = females 2, diamond = males, triangle = A-1, oval = A-2/3). This figure is published in colour in the online edition of this journal, which can be accessed via <http://booksandjournals.brillonline.com/content/journals/15685403>.

Fig. 8.

Scatter plots of the first against the second and third, respectively, discrimination functions for analyses of limb ratios, shell parameters and limb ratios combined with shell parameters (square = females 1, cross = females 2, diamond = males, triangle = A-1, oval = A-2/3). This figure is published in colour in the online edition of this journal, which can be accessed via <http://booksandjournals.brillonline.com/content/journals/15685403>.

Table 1

Selected limb ratios and shell parameters of *Cytheridella ilosvayi* Daday used for the discrimination analyses

	A1 EV/EIII	A1 EIV/EIII	A2 EIII/EIHGM	A2 EIV/EIII	A2 EIV/EII	A2 EIII/EII	T1 EIII/EII	T1 EIII/EI	T2 EIII/EII	A1/A2	T1/T2	A1/T1	A2/T2	A1/T2	Length (μm)	Height (μm)	Length sulcus (μm)	Length/height
Female 1	Mean	1.28	1.69	1.55	0.28	0.75	2.72	1.04	1.15	0.62	0.68	0.91	0.92	0.571	125.2	622.3	457.0	1.8
	SD	0.11	0.44	0.08	0.02	0.09	0.35	0.03	0.06	0.02	0.02	0.15	0.04	0.02	18.02	12.12	23.15	0.05
Female 2	Mean	1.17	1.96	1.87	0.24	0.70	2.94	1.08	1.09	0.63	0.67	0.82	0.94	0.58	950.8	542.7	370.8	1.8
	SD	0.17	0.29	0.15	0.02	0.07	0.19	0.13	0.08	0.01	0.05	0.09	0.03	0.02	18.65	13.68	21.99	0.04
Male	Mean	1.17	1.96	1.90	0.25	0.70	3.05	1.08	1.08	0.70	0.70	0.92	0.91	0.64	848.4	510.7	340.8	1.8
	SD	0.11	0.19	0.20	0.02	0.18	0.36	0.07	0.07	0.11	0.02	0.14	0.06	0.10	22.06	131.52	17.77	0.04
A-1	Mean	1.21	1.79	1.85	0.26	0.75	2.86	1.02	1.06	0.66	0.74	0.85	0.96	0.63	736.0	412.6	343.9	1.8
	SD	0.05	0.08	0.07	0.02	0.06	0.19	0.16	0.09	0.09	0.01	0.12	0.02	0.08	23.44	9.24	42.05	0.05
A-2/3	Mean	1.11	1.66	1.78	0.25	0.74	3.01	0.97	1.02	0.63	0.77	0.80	0.99	0.62	646.1	358.9	291.2	1.8
	SD	0.09	0.14	0.16	0.04	0.06	0.31	0.08	0.25	0.03	0.01	0.01	0.04	0.01	55.98	24.23	23.86	0.04

Table II

Pearson correlation matrix for selected limb ratios in *Cytheridella ilosvayi* Daday

	A1 EV/EIII	A1 EIV/EIII	A2 EIII/EIIIGM	A2 EIV/EIII	A2 EIV/EIII	A2 EIV/EIII	A2 EIII/EIII	T1 EIII/EIII	T2 EIII/EIII	A1/A2	T1/T2	A1/T1	A2/T2	A1/T2
A1 EV/EIII	Correlation	1	0.704**	-0.060	0.330*	0.205	-0.085	-0.235	0.155	0.116	-0.086	0.003	0.106	0.085
A1 EIV/EIII	Significance (two-way)	0.000	0.719	0.043	0.217	0.612	0.156	0.352	0.489	0.608	0.987	0.525	0.614	
	N	38	38	38	38	38	38	38	38	38	38	38	38	38
	Correlation	0.704**	1	0.135	-0.047	-0.078	0.006	-0.142	0.068	0.160	-0.204	-0.077	-0.014	0.001
A2 EIII/EIIIGM	Significance (two-way)	0.000		0.420	0.781	0.642	0.969	0.394	0.685	0.336	0.220	0.646	0.936	0.996
	N	38	38	38	38	38	38	38	38	38	38	38	38	38
	Correlation	-0.060	0.135	1	-0.064	-0.029	0.262	0.128	0.037	0.707**	-0.016	-0.029	-0.012	0.056
A2 EIV/EIII	Significance (two-way)	0.719	0.420		0.704	0.862	0.112	0.445	0.825	0.000	0.923	0.861	0.944	0.736
	N	38	38	38	38	38	38	38	38	38	38	38	38	38
	Correlation	0.330*	-0.047	-0.064	1	0.389*	-0.370*	-0.076	-0.139	0.010	0.019	0.016	0.010	-0.030
A2 EIV/EII	Significance (two-way)	0.043	0.781	0.704		0.016	0.022	0.648	0.406	0.952	0.911	0.922	0.951	0.857
	N	38	38	38	38	38	38	38	38	38	38	38	38	38
	Correlation	0.205	-0.078	-0.029	0.389*	1	0.493**	-0.201	-0.020	0.268	-0.126	-0.015	-0.054	0.081
A2 EIII/EII	Significance (two-way)	0.217	0.642	0.862	0.016		0.002	0.226	0.903	0.103	0.450	0.929	0.749	0.630
	N	38	38	38	38	38	38	38	38	38	38	38	38	38
	Correlation	-0.085	0.006	0.262	-0.370*	0.493**	1	-0.186	0.102	0.351*	-0.146	-0.007	-0.027	0.149
T1 EIII/EII	Significance (two-way)	0.612	0.969	0.112	0.022	0.002		0.264	0.541	0.031	0.381	0.965	0.874	0.372
	N	38	38	38	38	38	38	38	38	38	38	38	38	38
	Correlation	-0.235	-0.142	0.128	-0.076	-0.201	-0.186	1	0.379*	0.114	0.771**	0.731**	0.316	0.335*
T2 EIII/EII	Significance (two-way)	0.156	0.394	0.445	0.648	0.226	0.264		0.019	0.496	0.000	0.000	0.054	0.040
	N	38	38	38	38	38	38	38	38	38	38	38	38	38
	Correlation	0.155	0.068	0.037	-0.139	-0.020	0.102	0.379*	1	0.198	0.605**	0.642**	0.870**	0.886**
A1/A2	Significance (two-way)	0.352	0.685	0.825	0.406	0.903	0.541	0.019		0.234	0.000	0.000	0.000	0.000
	N	38	38	38	38	38	38	38	38	38	38	38	38	38
	Correlation	0.116	0.160	0.707**	0.010	0.268	0.351*	0.114	0.198	1	0.009	0.218	-0.047	0.279
	Significance (two-way)	0.489	0.336	0.000	0.952	0.103	0.031	0.496	0.234		0.957	0.189	0.778	0.090
	N	38	38	38	38	38	38	38	38	38	38	38	38	38

	A1 EV/EII	A1 EIV/EII	A2 EII/EIIGM	A2 EIV/EII	A2 EIV/EII	A2 EII/EII	T1 EII/EII	T2 EII/EII	A1/A2	T1/T2	A1/T1	A2/T2	A1/T2
T1/T2													
	Correlation	-0.086	-0.204	-0.016	0.019	-0.126	-0.146	0.771**	0.605**	0.009	1	0.867**	0.631**
	Significance (two-way)	0.608	0.220	0.923	0.911	0.450	0.381	0.000	0.000	0.957	0.000	0.000	0.000
N		38	38	38	38	38	38	38	38	38	38	38	38
A1/T1													
	Correlation	0.003	-0.077	-0.029	0.016	-0.015	-0.007	0.731**	0.642**	0.218	0.867**	1	0.625**
	Significance (two-way)	0.987	0.646	0.861	0.922	0.929	0.965	0.000	0.000	0.189	0.000	0.000	0.000
N		38	38	38	38	38	38	38	38	38	38	38	38
A2/T2													
	Correlation	0.106	-0.014	-0.012	0.010	-0.054	-0.027	0.316	0.870**	-0.047	0.679**	0.625**	1
	Significance (two-way)	0.525	0.936	0.944	0.951	0.749	0.874	0.054	0.000	0.778	0.000	0.000	0.000
N		38	38	38	38	38	38	38	38	38	38	38	38
A1/T2													
	Correlation	0.085	0.001	0.056	-0.030	0.081	0.149	0.335*	0.886**	0.279	0.631**	0.737**	1
	Significance (two-way)	0.614	0.996	0.736	0.857	0.630	0.372	0.040	0.000	0.090	0.000	0.000	0.000
N		38	38	38	38	38	38	38	38	38	38	38	38

Italicised values indicate correlation coefficients that represent highest absolute correlations between a variable and a discrimination function.

* Correlation is significant on the 0.05 level (two-way).

** Correlation is significant on the 0.01 level (two-way).

Table III

Eigenvalues and Wilk's Lambda of discrimination analyses for limb ratios, shells, and limb ratios and shells

Function	Eigenvalue	% of variance	Cumulative %	Canonical correlation	Test of functions	Wilk's Lambda	Chi-square	df	Significance
Limb ratios									
1	2.835	53.1	53.1	0.860	1 to 4	0.049	84.385	52	0.003
2	1.593	29.9	83.0	0.784	2 to 4	0.188	46.746	36	0.108
3	0.712	13.3	96.3	0.645	3 to 4	0.488	20.067	22	0.579
4	0.196	3.7	100.0	0.405	4	0.836	5.018	10	0.890
Shells									
1	51.504	98.4	98.4	0.990	1 to 4	0.010	150.515	16	0.000
2	0.586	1.1	99.6	0.608	2 to 4	0.512	21.787	9	0.010
3	0.213	0.4	100.0	0.419	3 to 4	0.811	6.802	4	0.147
4	0.016	0.0	100.0	0.126	4	0.984	0.518	1	0.472
Limb ratios and shells									
1	100.913	94.1	94.1	0.995	1 to 4	0.000	200.621	68	0.000
2	4.030	3.8	97.8	0.895	2 to 4	0.045	80.394	48	0.002
3	1.684	1.6	99.4	0.792	3 to 4	0.228	38.391	30	0.140
4	0.631	0.6	100.0	0.622	4	0.613	12.725	14	0.548

Table IV

Standardised canonical discrimination function coefficients for analyses of limb ratios, shells, and limb ratios with shells

	Function			
	1	2	3	4
Limb ratios				
A1EV/EIII	<i>-1.89</i>	0.02	0.34	-0.80
A1EIV/EIII	<i>1.99</i>	0.12	-0.14	0.38
A2EIII/EIIIIGM	<i>1.93</i>	0.42	0.34	<i>-1.65</i>
A2EIV/EIII	-0.16	<i>0.91</i>	0.61	0.59
A2EIV/EII	<i>0.89</i>	-0.25	-0.83	-0.87
A2EIII/EII	-0.68	-0.14	0.89	<i>1.08</i>
T1EIII/EII	<i>-0.82</i>	<i>-1.01</i>	0.02	-0.15
T2EIII/EII	<i>2.09</i>	<i>3.18</i>	-0.41	-0.35
A1/A2	<i>-2.06</i>	-0.61	-0.81	<i>2.76</i>
T1/T2	-0.36	-0.43	0.17	0.80
A1/T1	<i>1.05</i>	<i>1.73</i>	0.61	-0.59
A2/T2	<i>-2.87</i>	<i>-2.65</i>	<i>-1.98</i>	<i>3.08</i>
A1/T2	<i>1.18</i>	<i>-1.28</i>	<i>2.25</i>	<i>-3.00</i>
Shells				
Length	<i>1.24</i>	-0.24	-0.01	-0.25
Height	-0.13	-0.20	0.39	0.94
Length sulcus	-0.19	<i>1.02</i>	-0.23	0.28
Length/height	-0.60	0.53	<i>0.85</i>	-0.26
Limb ratios and shells				
A1EV/EIII	0.66	<i>-1.27</i>	-0.86	0.85
A1EIV/EIII	0.09	<i>1.12</i>	<i>1.08</i>	-0.30
A2EIII/EIIIIGM	-0.77	<i>2.15</i>	0.37	1.78
A2EIV/EIII	0.18	<i>-3.79</i>	<i>1.44</i>	-6.06
A2EIV/EII	-0.13	<i>6.40</i>	-0.49	9.98
A2EIII/EII	0.58	<i>-4.07</i>	0.48	-6.81
T1EIII/EII	0.11	-0.50	<i>-1.37</i>	0.35
T2EIII/EII	-0.46	<i>1.55</i>	<i>2.34</i>	1.25
A1/A2	0.06	<i>-2.04</i>	-0.65	-2.86
T1/T2	<i>1.22</i>	-0.45	0.66	-1.52
A1/T1	<i>-0.94</i>	0.46	<i>1.75</i>	1.04
A2/T2	0.26	<i>-2.63</i>	<i>-2.89</i>	-3.41
A1/T2	0.22	<i>1.89</i>	-0.50	2.90
Length	<i>-1.76</i>	-0.63	0.07	-1.41
Height	0.21	<i>3.58</i>	-0.19	5.93
Length sulcus	0.46	-0.30	-0.47	0.97

	Function			
	1	2	3	4
Length/height	<i>1.31</i>	−0.02	0.74	1.22

Ratios between limbs, e.g. A1/A2, include only the last three podomeres. Length sulcus is related to the distance between the margin and the transversal sulcus. Italicised values indicate correlation coefficients that represent highest absolute correlations between a variable and a discrimination function.

Table V

Structure matrices of three discrimination analyses (limb ratios, shells, limb ratios and shells) representing common correlations of a variable and a discrimination function within groups

	Function			
	1	2	3	4
Limb ratios				
A1EIV/EIII	<i>0.323</i>	0.197	−0.031	−0.061
A2EIV/EII	<i>−0.091</i>	0.035	0.011	0.065
A1EV/EIII	−0.063	0.287	0.001	−0.166
A2EIV/EIII	−0.229	<i>0.261</i>	0.048	−0.063
A2/T2	−0.010	<i>−0.248</i>	0.237	−0.021
A1/T1	−0.028	−0.031	<i>0.610</i>	0.033
A1/T2	0.040	−0.191	<i>0.473</i>	0.009
T1/T2	−0.098	−0.162	<i>0.408</i>	0.082
T1_EIII/EII	0.032	−0.059	<i>0.299</i>	−0.017
T2EIII/EII	0.067	−0.047	<i>0.244</i>	0.055
A1/A2	0.137	0.114	0.240	<i>0.473</i>
A2EIII/EIIIIGM	0.236	−0.081	0.045	<i>0.392</i>
A2EIII/EII	0.125	−0.127	0.242	<i>0.360</i>
Shells				
Length	<i>0.858</i>	0.265	0.439	−0.009
Length sulcus	0.230	<i>0.869</i>	−0.279	0.336
Length/height	−0.002	0.289	<i>0.904</i>	−0.316
Height	0.156	−0.106	0.445	0.876
Limb ratios and shells				
Length	<i>−0.610</i>	−0.297	0.207	0.088
Length sulcus	−0.160	<i>−0.360</i>	−0.067	0.345
A2EIV/EIII	−0.012	<i>−0.240</i>	0.065	0.094
A1EIV/EIII	−0.036	<i>0.205</i>	0.183	0.089
A2EIII/EIIIIGM	0.000	<i>0.197</i>	0.108	−0.194
A2EIV/EII	0.002	<i>−0.080</i>	−0.001	−0.029
A2/T2	0.036	<i>0.046</i>	−0.010	0.029
Length/height	0.003	−0.122	<i>0.307</i>	−0.094
A1/T1	0.034	−0.028	<i>0.279</i>	0.172
A1/A2	−0.004	0.070	<i>0.273</i>	−0.135
Height	−0.112	0.013	<i>0.183</i>	0.054
A1/T2	0.039	0.066	<i>0.154</i>	0.107
A2EIII/EII	0.019	0.118	<i>0.141</i>	−0.131
T1EIII/EII	0.019	0.033	<i>0.127</i>	0.094
T2EIII/EII	0.013	0.057	<i>0.126</i>	0.044
T1/T2	0.040	−0.048	<i>0.098</i>	0.045

	Function			
	1	2	3	4
A1EV/EIII	-0.025	-0.115	0.099	0.150

Italicised values indicate correlation coefficients that represent highest absolute correlations between a variable and a discrimination function.