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Cryptic diversity with wide salinity tolerance in the putative euryhaline *Testudinella clypeata* (Rotifera, Monogononta)

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Aquatic faunas in fresh, brackish, and salt waters are usually well defined and differ amongst these three habitats. Nonetheless, some animals are known to be euryhaline, namely present across wide salinity ranges. The wide tolerance of putative euryhaline species has, however been refuted in some cases by DNA taxonomy, which has uncovered cryptic diversity with narrow ecological niches. We aim to improve knowledge on the putative euryhalinism of microinvertebrates and test whether it might actually be a real phenomenon or if euryhaline species are mostly a consequence of our previous inability to identify cryptic species with narrow salinity ranges, as discovered in *Brachionus plicatilis*. Using morphological analyses and DNA taxonomy, we investigated the species reality and distribution of a putative euryhaline rotifer species, *Testudinella clypeata*, and evaluated whether cryptic species are ecologically and/or geographically segregated. Different DNA taxonomy approaches concurred in revealing the presence of seven cryptic species within the *T. clypeata* morphospecies, which, in contrast to what has been previously detected, are actually euryhaline. Moreover, differences in analysed morphological traits were not significantly different amongst cryptic species. This suggests that DNA taxonomy improves our estimates of the actual diversity of microscopic species, in contrast to the morphological approach.

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ADDITIONAL KEYWORDS: DNA taxonomy – ecological distribution – ecological tolerance – geographical distribution – generalized mixed Yule coalescent (GMYC) model – microscopic organisms – meiofauna – salt waters – traditional taxonomy.

INTRODUCTION

Salinity is an abiotic factor greatly influencing aquatic organisms (Schönborn, 2003; Ziemann & Schulz, 2011). As reported by Ekman (1953), aquatic environments may be classified according to differences in biological communities, with respect to salinity: fresh (0–0.5‰), brackish (0.5–20‰), and salt waters (> 20‰) host different organisms that are adapted to live in narrow salinity ranges. Nonetheless, many species are supposedly euryhaline (i.e. reported from wide salinity ranges). Euryhaline microinvertebrates are known for instance amongst Tardigrada, Gastrotricha, and Rotifera (e.g. Boaden, 1976; Nelson, 2002; Wallace *et al.*, 2006). The euryhaline status of halophilous (=living in

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saline waters) rotifer species can be challenged by the fact that morphological species with wide ecological tolerances have been discovered to be complexes of cryptic species, with each taxon potentially adapted to a narrower ecological range (e.g. Ortells, Gómez & Serra, 2003; Suatoni *et al*., 2006; Obertegger, Fontaneto & Flaim, 2012). The best known example is the species complex *Brachionus plicatilis* Müller, 1786 (Rotifera, Monogononta). This rotifer taxon inhabits salt and brackish waters and was previously thought to be euryhaline because it was reported from habitats with salinities ranging from 1 to 97‰ (Epp & Winston, 1977; Walker, 1981). However, DNA taxonomy revealed more than 20 cryptic species (Gómez, 2005; Suatoni *et al*., 2006; Fontaneto *et al*., 2007; Gribble & Welch, 2012), and differential adaptation to salinity has been demonstrated for taxa studied so far (Gómez, Carmona & Serra, 1997; Ortells *et al*., 2003; Campillo *et al*., 2011). Indeed, investigations performed under environmentally controlled conditions indicated that salinity differentially affects life history traits and reproduction rates in different species within the *B. plicatilis* complex (Campillo *et al*., 2011). Salinity is consequently considered one of the major determinants creating and maintaining local co-existence of multiple cryptic species in rotifers, as they may avoid competition through temporal and spatial displacement that correlates with variation in salinity (Gómez *et al*., 1997; Ortells *et al*., 2003).

Nonetheless, the occurrence of potentially euryhaline species is quite common amongst halophilous rotifers. This phylum comprises more than 2000 recognized species of microscopic aquatic animals (80–1000 μm in length); most species inhabit only freshwater but more than 400 species are known from brackish and salt waters (Fontaneto, De Smet & Ricci, 2006). Amongst these latter 400 species, at least 170 species are reported also from freshwater habitats. The other about 200 species have been found in brackish or salt waters only but most of them, such as the *B. plicatilis* complex, have been reported for habitats with wide salinity ranges and are still considered euryhaline species. So, several species identified on morphological characters by traditional taxonomy have been reported in habitats at wide salinity ranges. Are these rotifer species actually ecologically tolerant? Alternatively, do they display the same scenario as the *B. plicatilis* complex, with a large amount of cryptic species adapted to a limited salinity range? Only application of a molecular approach such as DNA taxonomy could address this question and determine whether euryhaline species exist within Rotifera.

The occurrence of cryptic species amongst rotifers is very common, indeed cryptic taxa were recently reported for another putative euryhaline monogonont rotifer, *Testudinella clypeata* (Müller, 1786) (Curini-Galletti *et al*., 2012). This is a common species known from the marine littoral and brackish waters all over the Palaearctic and Nearctic regions (De Ridder & Segers, 1997; De Smet, 2009; Wei, De Smet & Xu, 2011; Segers *et al*., 2012). Molecular analyses performed on one population of *T. clypeata* in Sardinia (Italy) revealed the occurrence of at least two cryptic species, with uncorrected genetic distances in their cytochrome oxidase *c* subunit I (the 'barcoding' gene, COI) of *c.* 20% (Curini-Galletti *et al*., 2012). The high genetic distance within this widespread taxon makes it a useful candidate to study adaptations to salinity tolerance. With this rationale, we analysed the presence of cryptic species in *T. clypeata* along the northern European coasts, sampling habitats with diverse salinity from different geographical localities. Our main aims were (1) to test for the presence and extent of genetic diversity in this species complex, and (2) to test whether the resulting entities are ecologically and/or geographically segregated at the analysed spatial and ecological scale; thus, highlighting similarities with and differences from the case of the *B. plicatilis* species complex.

MATERIAL AND METHODS

SAMPLING

During summer 2011, a total of 33 brackish and salt water sites in the southern UK (24), and Sweden (nine) was sampled (Fig. 1A; Table 1). These habitats ranged from littoral beaches and rock pools to retrodunal pools. Samples consisted mostly of algae belonging to *Enteromorpha* sp., in which *T. clypeata* is known to live (De Smet, 2009). For each site geographical coordinates were recorded and salinity was measured with a VWR International Brand Hand Held Refractometer (data summarized in Table 1).

Samples were collected by hand with jars, and taken to the laboratory soon after collection and processed within a few days. Specimens were extracted by siphoning off the water just above the sediment (Willems *et al*., 2009). Live material was studied using dissecting and light microscopes. Live specimens of *T. clypeata* (Fig. 1B, C) were collected from each sample and fixed in 100% ethanol or in Histochoice fixative (Sigma-Aldrich, UK; 50% in distilled H_2O) for subsequent molecular analyses. All the animals used for the morphometric analyses were fixed in Histochoice, an efficient fixative for histological observations (Kacena *et al*., 2004) as it does not contain alcohol so does not dehydrate tissues, and thus does not deform the shape of the lorica (F. Leasi, pers. observ.). Moreover, Histochoice can be efficiently used for PCR gene amplification (Giannella *et al*., 1997).

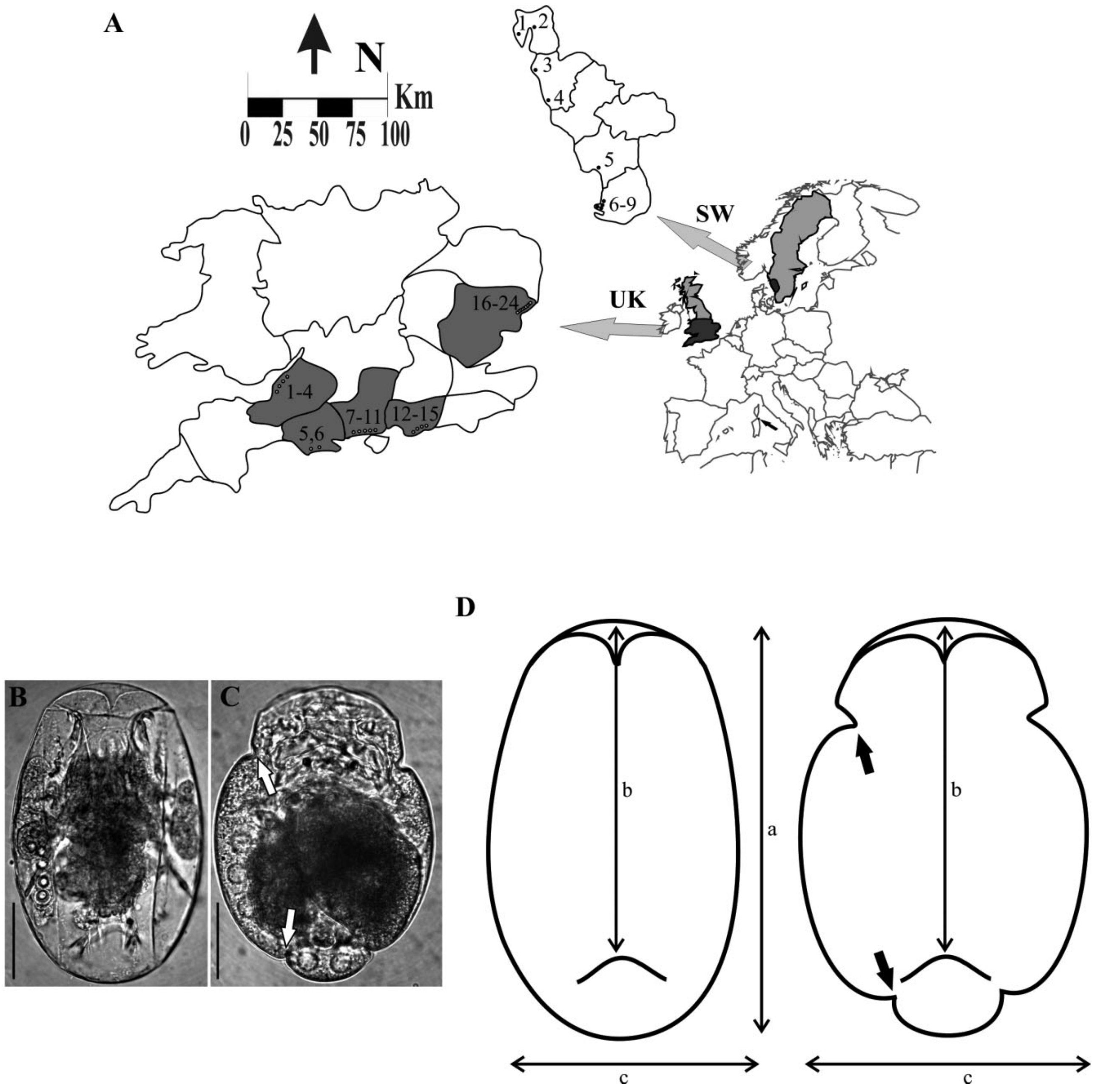

Figure 1. A, location of the 33 sites sampled in the UK (24 sites) and Sweden (nine sites). The UK and Sweden are indicated by the shadowed sampled areas on the map of Europe on the right. The southern area of the UK with shadowed sampled localities (Somerset, Dorset, Hampshire, Sussex, and Essex from western to eastern coast) and the region of Halland (Sweden) are presented in higher magnification on the left. The sampling site from Sardinia is also marked on the map of Europe with an arrow. Filled circles represent sampling sites; numbering is according to Table 1. B, C, *Testudinella clypeata*. Habitus. Head and foot are retracted into the body. B, lorica without any constriction; C, lorica with both anterior and posterior constrictions, indicated by an arrow. D, schematic drawings representing the two different shapes of the lorica in *T. clypeata*. a, maximum length; b, distance between the anterior margin of the lorica and the foot opening; c, maximum width. Bold arrows indicate the constrictions of the lorica. Scale bars: B, C = 50 μm.

Table 1. Locality, coordinates, and salinity value of each sampling site. Cryptic species (number with prefix E), relative haplotypes (numbered with prefix H), and GenBank accession numbers are indicated for each site

Locality	Site	Coordinates	Sampling date (dd/mm/yy)	Salinity (‰)	Cryptic species: haplotype (GenBank code)
Somerset (UK)	UK.01	51°13′N; –03°01′E	11/09/11	10	E.4: H.28 (JN809459, JX404009), H.29 (JN809460)
	UK.02	51°13′N; –03°00′E	11/09/11	30	E.5: H.25 (JN809453, JN809456)
	UK.03	51°13′N; –02°59′E	11/09/11	38	E.5: H.25 (JN809446, JN809449), H.26 (JN809457), H.27 (JN809458)
	UK.04	51°13′N; –02°59′E	11/09/11	36	E.5: H.25 (JN809450, JN809452)
Dorset (UK)	UK.05	50°38′N; –01°55′E	14/08/11	34	E.5: H.10 (JX404012, JX404015)
	UK.06	50°38′N; –01°55′E	14/08/11	39	E.1: H.18 (JN809437, JN809438)
Hampshire (UK)	UK.07	50°46′N; –01°25′E	07/08/11	40	E.5: H.17 (JN809436)
	UK.08	50°43′N; –01°33′E	07/08/11	40	E.4: H.15 (JN809434)
	UK.09	50°46′N; –01°25′E	07/08/11	36	E.5: H.10 (JN809427, JN809429), H.16 (JN809435) E.2: H.04 (JN809398, JN809399)
	UK.10	50°48′N; –01°18′E	31/07/11	34	E.2: H.04 (JN809393, JN809397) E.7: H.05 (JN809405)
	UK.11	50°48′N; –01°18′E	31/07/11	34	E.2: H.04 (JN809390, JN809392) E.5: H.14 (JN809433) E.7: H.05 (JN809400-JN809404, JN809406)
Sussex (UK)	UK.12	50°45′N; 00°09′E	26/04/11	39	E.5: H.07 (JN809419)
	UK.13	50°45′N; 00°09′E	26/04/11	32	E.5: H.07 (JN809421)
	UK.14	50°45′N; 00°08′E	26/04/11	31	E.3: H.09 (JN809422, JN809423) E.5: H.07 (JN809428)
	UK.15	50°45′N; 00°08′E	26/04/11	40	E.3: H.09 (JN809424, JN809426, JX863668)
Essex (UK)	UK.16	51°46′N; 01°04′E	04/09/11	38	E.5:H.07 (JN809414, JN809416)
	UK.17	51°46′N; 01°04′E	04/09/11	46	E.5: H.07 (JN809417)
	UK.18	51°46′N; 01°04′E	04/09/11	40	E.5: H.07 (JN809408), H.19 (JN809439)
	UK.19	51°46′N; 01°04′E	04/09/11	41	E.5: H.07 (JN809412, JN809413), H.34 (JX404010)
	UK.20	51°46′N; 01°04′E	04/09/11	44	E.5: H.07 (JN809418), H.33 (JX404011)
	UK.21	51°46′N; 01°03′E	04/09/11	42	E.5: H.23 (JN809444)
	UK.22	51°46′N; 01°03′E	04/09/11	40	E.5: H.07 (JN809409, JN809410), H.20 (JN809441), H.21 (JN809442)
	UK.23	51°46′N; 01°03′E	04/09/11	44	E.5: H.07 (JN809411), H.22 (JN809443)
	UK.24	51°46′N; 01°03′E	04/09/11	40	E.5: H.07 (JN809420), H.19 (JN809440), H.24 (JN809445)
Halland (Sweden)	SW.01	57°38′N; 11°51′E	08/07/11	22	E.5: H.06 (JN809407), H.11 (JN809430)
	SW.02	57°37′N; 11°55′E	08/07/11	22	E.5: H.12 (JN809431), H13 (JN809432)
	SW.03	56°55′N; 12°21′E	09/07/11	8	E.5: H.32 (JX404007, JX863669, JX863670)
	SW.04	56°27′N; 12°45′E	08/07/11	12	E.5: H.07 (JN809389)
	SW.05	56°45′N; 12°37′E	10/07/11	9	E.5: H.10 (JX404016, JX404017, JX404020), H.32 (JX404025)
	SW.06	56°27′N; 12°40′E	08/07/11	10	E.5: H.10 (JX404005), H.31 (JX404018)
	SW.07	56°27′N; 12°40′E	08/07/11	11	E.5: H.10 (JX404005, JX404006)
	SW.08	56°45′N; 12°37′E	10/07/11	12	E.5: H.10 (JX404019), H.32 (JX404021-JX404024)
	SW.09	56°25′N; 12°37′E	07/07/11	12	E.6: H.30 (JX404008)
Sardinia (Italy)	IT. 01	41°13′N; 09°27′E	12/09/10	35	E.3: H.1 (HQ444166, HQ444167) E.4: H.2 (HQ444168)

DNA TAXONOMY

DNA was extracted from single live or preserved individuals of *T. clypeata* and a fragment of cytochrome *c* oxidase subunit I (COI) mtDNA gene was sequenced according to the protocol for monogonont rotifers (Gómez *et al*., 2002). DNA was extracted in 35 µl of Chelex (InstaGeneMatrix; Bio-Rad, CA, USA); the partial COI gene was PCR amplified using primers LCOI (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCOI (5'-TAA ACT TCA GGG TGA CCA AAAAT CA-3') (Folmer *et al*., 1994). Cycle conditions comprised initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, and a final extension step of 72 °C for 7 min.

We reconstructed phylogenetic trees with both maximum likelihood (ML) and Bayesian inference (BI). As an outgroup, we used the COI sequence (EU499895) of *Floscularia melicerta* (Ehrenberg, 1832), which belongs to the same order, Flosculariacea. The selected model of evolution for the phylogenetic reconstructions was general time reversible plus gamma distribution plus a proportion of invariant sites (GTR + I + G), chosen by hierarchical likelihood ratio tests in ModelGenerator v0.85 (Keane *et al*., 2006). This model was implemented into PhyML 3.0 (Guindon & Gascuel, 2003) for the ML reconstruction, in which 1000 bootstrap replicates were used to evaluate node support. The same model was implemented in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) for the BI reconstruction, in which two parallel runs and four independent Markov chains per run of 5 000 000 generations were used; the first 20% of the trees were discarded to obtain the consensus tree. Ultrametric trees were generated using penalized likelihood in r8s and cross-validation to choose the optimal smoothing parameter (Sanderson, 2002) using the BI tree, which had very similar topology and support values to the ML tree (see Results).

The DNA taxonomy approach that we implemented to evaluate the presence of cryptic species was the generalized mixed Yule coalescent (GMYC) model (Pons *et al*., 2006). The GMYC model was performed on the ultrametric tree in R 2.15.0 (R Development Core Team, 2012) with the *splits* package (http://splits.r-forge.r-project.org/). The GMYC model is a process-based approach for detecting the threshold in a gene tree at which within-species processes, i.e. coalescence, shift to between-species processes, i.e. speciation and extinction (Pons *et al*., 2006). In order to be able to perform this analysis, we sequenced at least ten individuals from different populations, reconstructed the phylogeny, and obtained an ultrametric tree to be used as the input file. Moreover, we tested the reliability of the units of diversity obtained from the GMYC model by looking for congruence with the results from the automatic barcode gap discovery (ABGD) for primary species delimitation (Puillandre *et al*., 2012b), and the K/θ model (formerly known as 4X rule, Birky *et al*., 2005, 2010). The ABGD looks for the best barcoding threshold in the data set, whereas the K/θ model identifies clades that show the phylogenetic signatures of independent evolution. For both methods, the matrix of uncorrected pairwise genetic differences was examined to find putative species that conformed to these criteria.

MORPHOLOGY

In order to identify morphological traits that could be used to discriminate the different cryptic species, we performed measurements and quantitative descriptions of the shape of the same animals, fixed and preserved in Histochoice, used for DNA taxonomy. Using optical microscopy, we obtained digital pictures; to avoid instrumental errors possibly introduced by differential lens distortion, we always positioned each animal in the same direction, centred in the middle of the visible field before taking each picture, and used the same magnification (400 ×). Measures were all taken on the screen of the computer with the software ImageJ (http://rsb.info.nih.gov/ij/).

In order to obtain reliable measurements to describe the shape of the animals, we measured the maximum length and width of the hardened and thickened epidermis, named lorica, and the distance between the anterior margin and the foot opening (Fig. 1B–D). For each animal we evaluated two metrics, the ratio between maximum width ('c' in Fig. 1D) and maximum length ('a'), and the ratio between distance from the anterior margin to the foot opening ('b') and maximum length ('a').

Testudinella clypeata is known to have some degree of variability in the shape of its elliptical lorica, with anterior or posterior constrictions (bold arrows in Fig. 1D), which have been considered to have no taxonomic validity (De Smet, 2009). However, the taxonomic validity of such constrictions has never been tested against the presence of potential cryptic species. Thus, we coded the presence/absence of anterior and posterior constrictions for each individual.

We wanted to test whether different, previously untested morphologies could be used as a diagnostic character to delineate different cryptic taxa within the *T. clypeata* species complex. In order to do so, we checked whether anterior and posterior constrictions were present only in some cryptic species, or if all cryptic species had both forms (with and without constrictions). With the two ratios, we performed explicit tests to look for significant differences in the distribution of the values of the two metrics between cryptic species. We used an analysis of variance (ANOVA) to check for overall differences, and Tukey post hoc tests to disentangle all the pairwise differences between all pairs of comparisons. Linear models were used to test the effect of sample size on the ranges of the values for each cryptic species, using the logarithm of the ratio. All statistical analyses and plots were obtained with R 2.15.0 (R Development Core Team, 2012).

GEOGRAPHY AND ECOLOGY

We evaluated whether cryptic species have a more limited distribution in space and whether they were limited to a lower salinity range compared to the geographical distribution and salinity ranges of the whole species complex. To perform this analysis, we considered only the cryptic species reported for at least three sites, namely E.3, E.4, and E.5 (see Results).

For each cryptic species, we measured the observed geographical range, calculated as the maximum distance between all the populations that we found. We then obtained a random selection of a number of populations equal to the observed number for each cryptic species ($N = 3$ for E.3, $N = 3$ for E.4, and $N = 27$ for E.5), sampled from all the 33 populations that we analysed in the study. We repeated the simulation 1000 times and calculated the maximum distance for each of them. The observed maximum distance was compared to the distribution of the simulated ones; we calculated the proportion of simulations that produced lower values than the observed ones for the cryptic species. The same rationale was used for salinity, but using salinity ranges instead of geographical ranges. To avoid biases in the randomization, if more haplotypes within the same entity were from the same sample, we ran the simulations by randomly including only one representative of each haplotype in the analysis. In this way we controlled for the fact that samples have, by definition, the same geography and ecology. Generalized linear model (GLM) with quasi-Poisson distribution for count data was used to test the effect of sample size, if potentially this could be biasing the results. All simulations and statistical analyses were performed in R.

RESULTS

CRYPTIC SPECIES

We obtained 97 sequences of *T. clypeata* morphospecies, 22 from Sweden and 75 from the UK. Three additional sequences of *T. clypeata* collected in Sardinia (Italy), already available in GenBank (Table 1), were included in the phylogenetic reconstruction. Out of the overall tree with 34 haplotypes from 100 individuals, the GMYC model suggested the presence of seven entities in *T. clypeata* (GMYC model: likelihood of null model = 146.5, likelihood of best model = 162.9, likelihood ratio = 32.9, P-value < 0.0001) as separate cryptic species (Fig. 2). The same seven entities were confirmed by both the ABGD and by K/θ methods with a 95% confidence. Uncorrected genetic distances in COI within the seven putative cryptic species (named E.1 to E.7, Fig. 2) ranged from 0.16 to 4.5% (mean ± standard deviation = 2.7 ± 0.9%), whereas distances between them ranged from 16.7 to 27.7% (mean ± standard deviation = 20.8 ± 2.3%).

We found 53 specimens with a regular elliptical lorica, without any constriction, 18 specimens with a posterior constriction, 15 with both anterior and posterior constrictions, and 11 with an anterior constriction. Both constrictions were present in different individuals in the two cryptic species with more individuals analysed, E.2 ($N = 10$) and E.5 ($N = 67$) (Fig. 3A, B). The other cryptic species found had fewer than eight individuals in our sample; thus, not finding specimens with constrictions in these species cannot be trusted as reliable data for inference on actual morphological differences.

The width to length ratio (c/a in Fig. 1D) ranged between 0.60 and 0.84 (mean ± standard deviation = 0.69 ± 0.04) and was significantly different between cryptic species (ANOVA: $F_{9,60} = 3.54$, $P = 0.003$). However, the ranges in the values of the ratio were nearly significantly related to sample size of each cryptic species (linear model: $F_{1,5} = 5.26$, $P = 0.070$), and the pairs of cryptic species that significantly differed always involved taxa with low sample sizes (Tukey post hoc tests: Appendix S1). Thus, we can infer that such metrics may not be different between cryptic species; they indeed depend on limited sample size for specific cryptic species.

Similarly, the ratio between the distance from the anterior margin of the lorica to the foot opening, and the maximum length of the lorica (b/a in Fig. 1D) ranged between 0.0106 and 0.0127 (mean ± standard deviation = 0.0116 ± 0.0004). The ratio was significantly different between cryptic species (ANOVA: $F_{9,60} = 3.39$, $P = 0.004$). The range in the values of the ratio was related to sample size of each cryptic species (linear model: $F_{1,5} = 14.06$, $P = 0.013$), and the pairs of cryptic species that differed significantly involved taxa with low sample sizes (Tukey post hoc tests: Appendix S1).

GEOGRAPHY

All cryptic species except for E.6 were found in our samples from the UK, whereas in Sweden only two cryptic species (E.5 and E.6) were found. The two

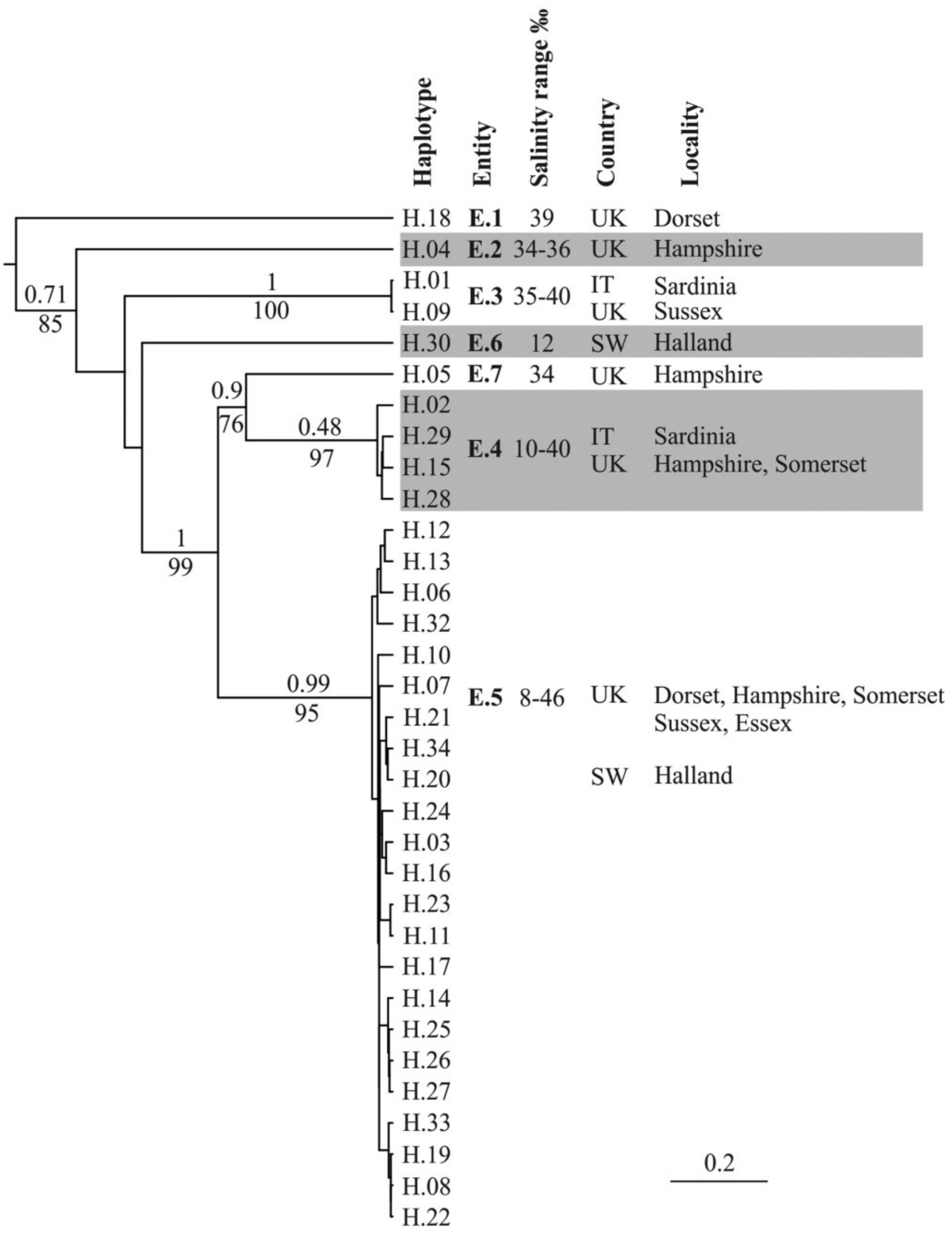

Figure 2. Phylogenetic relationships of the 34 cytochrome *c* oxidase subunit I (COI) haplotypes in the morphological species *Testudinella clypeata* sampled in the UK, Sweden (SW), and Italy (IT). The consensus of 1000 sampled trees from Bayesian analysis of the COI data sets is shown, displaying all compatible groupings and with average branch lengths proportional to numbers of substitutions per site under a general time reversible + I + G substitution model. Posterior probabilities from the Bayesian reconstruction and bootstrap support from the maximum likelihood reconstruction are shown above and below each branch, respectively. Support values are not shown for values below 0.8 for posterior probabilities, 80 for bootstrap support and for within-species short branches.

cryptic species previously reported for Sardinia were also found in Somerset and Hampshire (E.4), and in Sussex (E.3). Different cryptic species were also found in coexistence in three sites in Hampshire and in one site in Sussex. Sampling effort, measured as the number of sites sampled for each locality (Table 1) significantly influenced the number of haplotypes found in each locality (GLM: $P = 0.0101$); for instance, in Dorset only two haplotypes were found but only two sites were examined, whereas in Essex nine haplotypes were found in nine sites (Table 1). Nevertheless, sampling effort did not influence the number

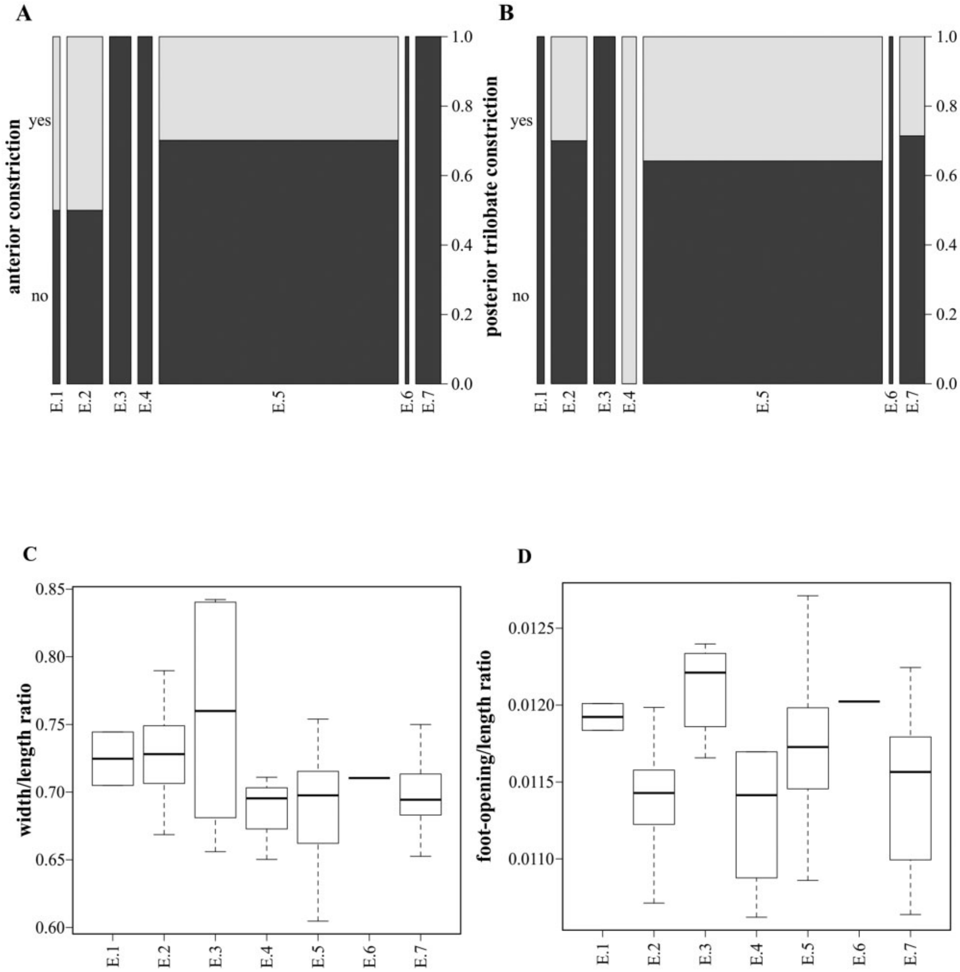

Figure 3. Distribution of the morphological characters in the cryptic species of *Testudinella clypeata*. A, proportion of individuals with and without anterior constriction. B, proportion of individuals with and without posterior constriction. C, boxplot of the distribution of the width to length ratio (c/a in Fig. 1). The horizontal thick bar represents the median value, the box includes 50% of the data, the whiskers extend to cover 95% of the distribution. D, boxplot of the distribution of the ratio between the distance from the anterior margin to the foot opening and the maximum length (b/a in Fig. 1).

of cryptic species per locality (GLM: $P = 0.819$). For example, two cryptic species were found in Dorset, one in each site, but only one was present in all sites collected in Essex, despite the higher number of sampled sites (nine). The highest diversity was found in Hampshire, where four cryptic species (E.2, E.4, E.5, E.7) were detected in five sites.

Common cryptic species had a wide geographical distribution: E.5 was found in almost all sampled locations (27 sites); E.5 was also the only cryptic species occurring in Essex (UK) and in all the sites sampled in Sweden, except for SW.9, where only E.6 was found (Table 1; Figs 1A, 2). Statistical analyses indicated that the geographical range of three of the cryptic species present in at least three sites is not significantly smaller than the range of the species complex at the analysed scale (simulations: E.3, $P = 0.898$; E.4, $P = 0.931$; E.5, $P = 0.297$); one of the cryptic species found in three localities, E.2, has a significantly smaller range than that of the species complex (simulations: $P = 0.002$).

ECOLOGY

Salinity varied from 8‰ in Sweden to 46‰ in Essex and a total of 18 salinity values was recorded. Specimens belonging to E.1, E.2, E.3, and E.7 were found in sites encompassing a salinity range between 31 and 40‰; i.e. in habitats with high salinity values (Table 1; Fig. 2). Cryptic species E.6 was found in brackish water (12‰), whereas E.4 and E.5 were found in both brackish water and salt water (Table 1; Fig. 2).

Statistical analyses indicated that the salinity range of each of the cryptic species present in at least three sites is not significantly smaller than the range of the species complex at the analysed scale (simulations: E.2, $P = 0.174$; E.3, $P = 0.274$; E.4, $P = 0.804$; E.5, $P = 0.999$).

In summary, the most striking results of our phylogenetic and statistical analyses revealed that (1) the morphological species *T. clypeata* is indeed a complex of at least seven cryptic species; (2) and that these cryptic species did not occupy either a significantly smaller range of salinity values or a significantly smaller geographical range than the range of the whole species complex.

DISCUSSION

Cryptic diversity

As expected from previous suggestions (Curini-Galletti *et al*., 2012), DNA taxonomy uncovered a large degree of hidden diversity in the morphological species *T. clypeata*, with potentially seven cryptic species. As suggested by Puillandre *et al*. (2012a), additional studies need to be performed in order to make the species delimitation more reliable (see also De Salle, 2006; Wiemers & Fiedler, 2007), and to be able to formally name the potential cryptic species. Analysis on COI gene should be combined not only with analysis of other independent genes, but also with morphological, geographical, or ecological data to clearly delimit species in what is called an integrative framework (for a review see Puillandre *et al*., 2012a). Notwithstanding this caveat, the barcoding gap defining 'within-species variability' from 'between-species variability' in *T. clypeata* complex, as identified by the ABGD (Puillandre *et al*., 2012b), the K/θ (Birky *et al*., 2005), and GMYC models (Pons *et al*., 2006), is rather wide. The threshold of the barcoding gap in this species complex falls between 4.5 and 16%; this represents a much higher value than the 3% threshold used to delimit units for barcoding (Hebert *et al*., 2004; Smith *et al*., 2007; Tang *et al*., 2012). The barcoding gap in *T. clypeata* is even wider than what is known for the *B. plicatilis* complex, in which the average uncorrected pairwise COI distance is 3.4% (range: 0.1–8.3%) within entities and 18.9% (range: 5.8–23.8%) between entities (Fontaneto *et al*., 2009). Thus, we can be rather confident that the entities identified by the DNA taxonomy approach with COI in *T. clypeata* will represent actual cryptic species.

Taxonomic identification in the genus *Testudinella* is traditionally based on the shape of the lorica and foot opening, and on morphometric ratios (De Smet, 2009). All measures considered in this work were taken from fixed animals. A considerable number of papers on traditional taxonomy of monogononts, especially of loricate species such as *Testudinella*, were performed by observing fixed animals (e.g. Segers & Mertens, 1997; Błędzki & Ellison, 2003; De Smet, 2009). Live animals tend to swim on the slide, making observations and photographing very hard to execute. Moreover, fixed specimens are usually well contracted; therefore, measures of the lorica are facilitated and more accurate.

Statistical analyses revealed that differences in shape and morphometric ratios may not be significantly different between the potential cryptic species of the *T. clypeata* complex. Moreover, the presence of anterior and posterior constrictions in the lorica seems to be a characteristic of all the cryptic species. This supports the suggestions that the constrictions of the lorica have no taxonomic validity (De Smet, 2009). Moreover, they are clearly discernible in live animals and in animals fixed with both kinds of fixative, so are not artefacts resulting from unnatural contraction after fixation.

Our analysis supports the idea that the morphological approach underestimates diversity for ecological analysis involving monogonont rotifers and that a DNA taxonomy approach represents a more accurate technique to estimate the actual diversity of these microscopic, understudied animals (e.g. Suatoni *et al*., 2006; Obertegger *et al*., 2012; Tang *et al*., 2012).

Salinity

The presence of the different cryptic species of *T. clypeata* was not related to narrow salinity ranges. Indeed, at least the four cryptic species reported in at least three sites are tolerant to wide salinity ranges and may be considered truly euryhaline. However, additional physiological studies are needed to reveal the basis of mechanisms mediating physiological responses to wide salinity changes in the cryptic species of *T. clypeata*, and whether this taxon is either an osmoconformer (a change in the concentration of the medium results in a corresponding change in the concentration of the body fluids) or an osmoregulator (cells display mechanisms of osmotic adjustment referred to as intracellular isosmotic regulation). It has been suggested that the response to salinity of an organism mainly depends on its eco/evolutionary origin (e.g. Khlebovich & Aladin, 2010; McNamara & Faria, 2012). In the *B. plicatilis* complex, which belongs to a genus with freshwater origin (Epp & Winston, 1977, 1978), it was demonstrated that cryptic species, even though adapted to limited salinity ranges, are osmoregulators and able to change activity of the plasma membrane-bound Na+/K+ ATPase enzyme in response to a change in salinity (Lowe *et al*., 2007). Further phylogenetic studies, covering additional marine and freshwater species of

Testudinella, are needed to reveal the origin of this genus. If the genus *Testudinella* originated in freshwater habitat, it should be reasonable to hypothesize that *T. clypeata* possesses a physiological membrane system to control osmolarity; moreover, similarly to *B. plicatilis*, costs in regulation are expected.

GEOGRAPHICAL DISTRIBUTION

Cryptic species of *T. clypeata* have been discovered to be potentially ubiquitous in salt water habitats of Europe. Our analyses indicate that the cryptic species E.2 was found in a smaller geographical area than expected by a random simulation of geographical localities; individuals of this cryptic species were only found in three samples in Hampshire, UK. At the moment, we cannot state whether this restricted distribution is the result of sampling bias or to the actual distribution of the cryptic species. All the other cryptic species were indeed as widespread as the whole species complex at the analysed spatial scale. The two cryptic species previously reported for Sardinia by Curini-Galletti *et al*. (2012), here called E.3 and E.4, were both found in our UK sites. Such widespread distribution supports the high dispersal potential of monogonont rotifers and the absence of physical barriers to their dispersal at the analysed spatial scale (see Obertegger *et al*., 2012). We cannot exclude that dispersal, which also involves anthropogenic factors, might take place via current-driven sediment and vegetation transport along the shorelines, a mechanism that is suggested to be involved in dispersal of other microscopic organisms taxa living along marine coasts (Giere, 2009; Kieneke, Martínez Arbizu & Fontaneto, 2012).

In conclusion, *T. clypeata* is confirmed to be a complex of cryptic species. This result agrees with previous analyses on other rotifers (Gómez *et al*., 2002; Gilbert & Walsh, 2005; Schröder & Walsh, 2007; Walsh *et al*., 2009; Obertegger *et al*., 2012), and suggests that this could raise problems in ecological analyses if the correct units of diversity are not adequately recognized.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Results of the Tukey post hoc tests on the metric ratio. The *P*-value of the difference between each pair of cryptic species is reported for the width to length ratio and for the distance of foot opening to total length ratio.