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***Eurytemora carolleeae* in the Laurentian Great Lakes revealed by phylogenetic and morphological analysis**

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Abstract

In the Laurentian Great Lakes, specimens of *Eurytemora* have been reported as *E. affinis* since its invasion in the late 1950s. During an intensive collection of aquatic invertebrates for morphological and molecular identification in Western Lake Erie in 2012–2013, several specimens of *Eurytemora* were collected. Analysis of these specimens identified them as the recently described species *E. carolleeae* Alekseev and Souissi 2011. This result led us to assess *E. carolleeae*'s identifying features, geographic distribution and historical presence in the Laurentian Great Lakes in view of its recent description in 2011. Cytochrome oxidase I (COI) DNA sequences of *Eurytemora* specimens were identified as closer (2 - 4% different) to recently described *E. carolleeae* than to most *Eurytemora affinis* sequences (14% different). *Eurytemora* from other areas of the Great Lakes and from North American rivers as far west as South Dakota (Missouri River) and east to Delaware (Christina River) also keyed to *E. carolleeae*. Morphological analysis of archival specimens from 1962 and from all the Great Lakes were identified as *E. carolleeae*. Additionally, *Eurytemora* drawings in previous publications from studies in the Holarctic region were reassessed to determine if these specimens were *E. carolleeae*. Additional morphological characters that may distinguish the North American *E. carolleeae* from other taxa are also described. We conclude that *E. carolleeae* is the correct name for the species of *Eurytemora* that has inhabited the Great Lakes since its invasion, as established by both morphological and COI sequence comparisons to reference keys and sequence databases in present and archival specimens.

Keywords

Copepod; cytochrome oxidase I (COI); *Eurytemora carolleeae*; biogeography; invasive; taxonomy

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Introduction

In the Laurentian Great Lakes, introductions of non-native copepods have occurred over several decades (Engel, 1962; Horvath et al., 2001; Hudson and Bowen, 2002). However, some publications that list copepods have either mischaracterized their native distribution (Drake and Lodge, 2007a, 2007b; Mills et al., 1993) and/or the taxonomy of the species (Reid and Hudson, 2008). The introduction of the estuarine copepod *Eurytemora* to the Great Lakes was noticed quickly because it is easily distinguished from native calanoid copepods by its long caudal ramus, long pointed metasomal wings, and relatively shorter antennae. *Eurytemora* was likely introduced to the Great Lakes due to the construction and opening of the St. Lawrence Seaway since introductions of many non-native freshwater tolerant marine taxa coincided with the opening of the Seaway or followed shortly thereafter (Mills et al., 1993). *Eurytemora* sp. was first recorded in Lake Ontario at the genus level in 1958 (Anderson and Clayton, 1959) and thereafter reported as *Eurytemora affinis* Poppe 1880 in all the Great Lakes (Mills et al., 1993). However, the recent description (Alekseev and Souissi, 2011) of *Eurytemora carolleeae* Alekseev & Souissi, 2011 raised questions about the appropriate identification of the *Eurytemora* populations in the Great Lakes which the present study seeks to answer.

Significant work has been completed in studying the life history, mechanisms of invasion and biogeography of *Eurytemora* taxa in North America (Cabrol et al., 2015; Dodson et al., 2010; Favier and Winkler, 2014; Lee, 1999; Lee and Frost, 2002; Posavi et al., 2014; Winkler et al., 2008). *Eurytemora*, typically identified as *E. affinis*, has been known to play an important role as a dominant grazer in marine, estuarine, and freshwater systems and is considered to be a cosmopolitan species due to its broad biogeographic range encompassing subtropical to subarctic areas (Lee, 2000; Suarez-Morales et al., 2008). Historically, this coastal-estuarine copepod was considered to be a marine species (Croskery, 1978). Nevertheless, surveys within freshwater systems in North America and Mexico have identified *Eurytemora* clades far from the marine coastline (Lee and Frost, 2002; Suarez-Morales et al., 2008). Evolutionary and physiological osmoregulatory adaptations may have enabled *Eurytemora* taxa to invade freshwater environments from its typical saline habitats (Johnson et al., 2014; Lee, 1999; Posavi et al., 2014).

E. affinis has a geographic range that spans the northern hemisphere and habitat types that range from hypersaline salt marshes to fresh water suggesting a cryptic species complex (Dodson et al., 2010). Sequences of the mitochondrial cytochrome oxidase I (COI) gene have been shown to be very useful for distinguishing calanoid and harpacticoid copepods including cryptic and sibling species in biogeographic studies (Gutierrez-Aguirre et al., 2014; Laakmann et al., 2013; Miracle et al., 2013; Peterson et al., 2013). Previous genetic analyses of the COI gene in *Eurytemora* populations described specimens from the Great Lakes as belonging to an Atlantic clade of *E. affinis* (Lee and Frost, 2002; Winkler et al., 2008). Phylogenetic analysis of North American *Eurytemora* collected from several marine and freshwater sites, including specimens from Lake Michigan and the Detroit River, revealed several distinct clades but did not distinguish any differences in the morphological characters of the specimens associated with the different clades using keys available at that time (Lee and Frost, 2002). Recently, Alekseev and Souissi (2011) identified *E. carolleeae* as

a previously undescribed sibling species to *E. affinis* native to the North American Atlantic coast, with distinct characters to enable its morphological identification. *E. carolleae* was also reported to be a potentially new invasive copepod in the Baltic Sea and European Atlantic coast estuaries first based on COI sequence data and then through taxonomic identification (Alekseev et al., 2009; Sukhikh et al., 2013). *E. carolleae* observations in North America were from the Chesapeake Bay and the St. Lawrence estuary with the possibility of distributions in the inland waters of the Great Lakes to Mexico (Alekseev and Souissi, 2011). COI sequence data was used to corroborate the morphological identification of the *E. carolleae* invasion of the Baltic Sea and European Atlantic coast estuaries (Sukhikh et al., 2013). These recent analyses indicated more than one species of *Eurytemora* contributed to the Great Lakes invasion, which led us to re-examine the classification of *Eurytemora* specimens collected in the Great Lakes.

In order to determine which *Eurytemora* species or clade had invaded the Great Lakes, this present study used morphological and COI molecular barcoding methods to identify the *Eurytemora* taxa. Morphological analysis was carried out for archival specimens from the Great Lakes dating back to 1962, and we reviewed drawings and photographs in past literature. Additionally, this paper describes our analysis of samples collected in 2012-2014 from the Great Lakes and from rivers as far west as South Dakota and east to Delaware to determine the possible distribution and morphological variation associated with this species complex.

Methods

Sampling

Specimens of *Eurytemora* came from various locations in the Great Lakes including western Lake Erie, Detroit River, Lake St. Clair, Lake Huron, Lake Michigan (including from Muskegon Lake, an estuarine lake of Lake Michigan (Weinke et al., 2014)), and two river systems, the Christina River in Delaware and the Missouri River (Lewis and Clark Lake) in South Dakota (Fig. 1). Plankton samples from western Lake Erie were collected using a hand-thrown Wisconsin plankton tow net with a 80 µm mesh (Wildco, USA) during the summer of 2012 and 2013 at sites in and near Toledo Harbor, Ohio USA. Fourteen sites were repeatedly sampled over the 2012 and 2013 summer months beginning in May and ending in August (see Electronic Supplementary Material (ESM) Appendix S1). Samples were split and preserved in 80% ethanol for molecular analysis and in Lugol's solution for morphological analysis. The sample in Lugol's solution was shipped to EcoAnalysts (Moscow, ID) for taxonomic analysis. Sampling during 2014 was limited to spot locations using either a Wisconsin net near shore or in shoreline aquatic vegetation using a bucket and multiple grabs, filtered with an 80 µm sieve and stored in 91% isopropyl alcohol.

Taxonomy

For identifying specimens of *Eurytemora* we used several characters to separate *E. carolleae* from its congener *E. affinis*, including a large outside dent on the mandible and setal segmentation on the caudal rami and swimming legs, which we documented in some of our specimens. However, for routine separation we chose to use the wing-like outgrowths on

the genital double-somite (Fig. 2a) and a small spine near the distal seta insertion point in P5 (Fig. 2b) in females, and the naked caudal rami (Fig. 2c) and cylindrical shape (length/width (L/W) ratio >1.3) of the second segment of the exopod (also known as the basipod) on the left P5 (Fig. 2d) in the male to identify specimens of *E. carolleae* as described in Alekseev and Souissi (2011). These characters were either easily seen under a dissecting microscope or when the P5 was placed under a coverslip on a slide and viewed under a compound microscope at higher magnification. In addition, Great Lakes specimens from the US Geological Survey Great Lake Science Center collections of alcohol preserved plankton samples and specimens archived on microscope slides were examined (Table 1) using the same characteristics. A similar analysis was applied, when possible, to drawings and photographs in descriptions of *Eurytemora* in previous publications from studies in the Holarctic region (see list in Table 2).

To further characterize *E. carolleae* morphologically, the presence/absence and placement of setae on the fifth leg of female and male specimens were analyzed. To supplement this, drawings of the female and male fifth legs of *Eurytemora* in references listed in Table 2 were reviewed for setae presence and placement. Dr. Eduardo Suarez-Morales from El Colegio de la Frontera Sur (ECOSUR), Chetumal, Mexico assisted us by further confirmation of his observations of setae placement and contributed additional morphometrics of the female and male fifth legs of his specimens reported in Suarez-Morales et al. (2008) and evaluated morphological differences. *Eurytemora* specimens analyzed by Dr. Suarez-Morales are deposited in the collection of Zooplankton of ECOSUR under Colina Lake ECO-CHZ-03662, and Balmorhea Lake ECO-CHZ-03440, 03441. Comparisons to the specimens in this study were used to investigate a basis for possible diagnostic characters to further separate the *E. affinis* complex.

DNA Extraction

Individual ethanol-preserved specimens were lysed in ATL lysis buffer (cat. no. 19076, Qiagen, Hilden, Germany), with Proteinase K (cat. no. 19133, Qiagen, Hilden, Germany), followed by DNA purification with the DNAeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and Qiagen Spin Columns according to standard protocols (<https://www.qiagen.com/us/resources/resourcedetail?id=6b09dfb8-6319-464d-996c-79e8c7045a50&lang=en>). Elution with Low TE (Invitrogen, Carlsbad, CA) used a small volume (28 µl) since the resultant purified DNA is from a single microscopic organism.

Polymerase Chain Reaction (PCR)

Purified DNA was amplified by PCR using COI forward primer HCO2198 and reverse primer LCO1490 (Folmer et al., 1994) prepared as stock solutions of 10 pmol/µl. DNA was added to PCR reactions at a quantity of 1.5 µl per 25 µl reaction. PCR master mix contained 0.5 µl of each forward and reverse primer stock solutions, 12.5 µl of SSO Advanced Universal SYBR Green Supermix (BioRad, Irvine, CA), and 10 µl sterile water. Reactions were run on an iCyclerQ Realtime thermocycler (BioRad, Irvine, CA), initiated by heating to 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, and then a final extension of 72 °C for 7 min followed by a hold at 15 °C until further

processing within 3 hours. PCR products were visualized on 1% agarose gels with SYBR Safe DNA Gel Stain (Invitrogen, Grand Island, NY), and images were documented with a DarkReader (Clare Chemical, Dolores, CO). A 100 bp eXact gene DNA ladder (Fisher Scientific, NH) was run alongside the products for size calibration. After purifying PCR amplicons using QIAquick PCR purification columns (Limburg, NL), DNA quality and concentration was measured by spectrophotometry (Tecan US Inc. Infinite F200, Morrisville, NC), and the amplicons were sequenced by Sanger DNA sequencing services of GeneWiz (South Plainfield, NJ).

Sequence and Phylogenetic Analysis

Samples were sequenced bi-directionally and analyzed with DNA Baser software (Heracle BioSoft SRL, Romania) and Mutation Surveyor (Softgenetics, State College, PA) to visualize the chromatogram files to determine quality and accuracy of the sequences. Sequences that were trimmed by the DNA Baser software Trimming Engine to less than 626 bases in length (trimming removed regions where >35% of bases in 16 base windows had quality scores <22) were not used. The remaining sequences were >625 base pairs long and had average quality scores >40. Forward and reverse complement chromatograms were then compared manually to ascertain agreement of base identities bi-directionally and the high quality consensus sequence of each sample was used in subsequent analysis. Sequences were then compared to the existing NCBI GenBank and Barcode of Life Data Systems (BOLD) databases (as of January 5, 2015) focusing particularly on comparisons to sequences uploaded by the laboratory that had identified morphological features that distinguished *E. carolleeae* from other *Eurytemora* species (Sukhikh et al., 2013). MEGA6 was used for phylogenetic analysis, including constructing neighbor-joining trees (Tamura et al., 2013) and, in the ESM Appendix S2, calculations of pairwise differences of nucleotides between sequences.

Results

Toledo Harbor specimens

A neighbor-joining tree examining the relationship of COI sequences from eight Toledo Harbor specimens to reference sequences in the NCBI and BOLD databases revealed that the Toledo Harbor sequences were on a different branch than almost all of the sequences annotated as *E. affinis* (Fig. 3). Branch lengths of the tree and calculations of average pairwise difference (see ESM Appendix S2, Fig. S2) indicated that the Toledo Harbor COI sequences differed from *E. affinis* by an average of 14%. The group of sequences in the NCBI database with the closest similarity to the Toledo Harbor sequences were barcodes of specimens annotated by Sukhikh et al. (2013) as *E. carolleeae*, from which the Toledo Harbor specimens differed by 2 - 4%. The outgroup, *Eurytemora lacustris*, differed from the Toledo Harbor specimens by an average of 18%. Seven of the eight Toledo Harbor specimens as a distinct group within an *E. carolleeae* clade, different from most sequences reported as *E. affinis*. Only one Toledo Harbor specimen (THP2J53012S5) has a sequence that is in the same branch as the type specimens for *E. carolleeae*.

Identification of *Eurytemora* spp. beyond Toledo Harbor

To determine whether the sequences found in Toledo Harbor *Eurytemora* are present over a broad geographic range, we investigated samples from other Great Lakes and elsewhere. Collection sites ranged from the Great Lakes basin (Lake Huron, Lake Michigan, Lake St. Clair and the Detroit River) to Delaware (Christina River) and South Dakota (Missouri River) (Fig. 1).

None of the sequences obtained from more than 25 specimens from these locations were closely related to *E. affinis* or *E. lacustris*, from which they all differed by at least 13% (Fig. 4 and ESM Appendix S2, Fig. S3). In fact, most of the new sequences in Fig. 4 were nearly identical to sequences of previous Toledo Harbor specimens (<2% difference). The added North American sequences are distributed within the branch that includes the Toledo Harbor specimens (Fig. 4). All of the added North American sequences differed from the *E. carolleeae* sequences of Alekseev and Souissi (2011) by 1.5 – 3% (ESM Appendix S2, Fig. S4).

Morphological considerations

Routine review of specimens collected for this study using descriptive morphological features (Fig. 2) clearly identified Great Lakes specimens as *E. carolleeae*. For example, as summarized in Fig. 5, the cylindrical shape of the basipod (arrow in Fig. 2d) of the left P5 leg in males was longer than its width, (in agreement with the L/W ratios of *E. carolleeae* reported by Alekseev and Souissi 2011). The L/W ratio of this segment in 9 mature male North American specimens collected in this study averaged 1.51 ± 0.045 (mean $\pm$ sem, Fig. 5a). A similar analysis for 8 archived male specimens (Table 1) from each of the five Great Lakes and Lake St. Clair averaged 1.47 ± 0.064 (mean $\pm$ sem, Fig. 5b). Examination of drawings in previous research papers listed in Table 2 indicated that L/W ratios of the male P5 basipod segment fell into two distinct clusters, one with averages less than 1.0 and another cluster with L/W ratios >1.3. The summary drawing of the P5 basipod segment of the type material for *E. carolleeae* from Chesapeake Bay in Alekseev and Souissi (2011) had a L/W ratio of 1.44.

Assessment of multiple morphological features in the drawings of male and female *Eurytemora* spp. in papers by Gurney (1931), Busch and Brenning (1992), and Lee and Frost (2002) verified the identification as *E. affinis*. The basipod L/W ratios of the male P5 were < 1.0 in all of these publications (Fig. 5c). The specimens of Davis (1943), Wilson (1959), and Suarez-Morales et al. (2008) are clearly most similar to *E. carolleeae*, with male basipod L/W ratios > 1.3 (Fig. 5c). Katona (1971) drew several specimens and may have been illustrating more than one *Eurytemora* species as the male P5 L/W ratio was 1.0 for Figure 89 and 1.3 for Figure 85. Male and female North American specimens and archived specimens from the US Geological Survey Great Lake Science Center collections (Table 2) had characters that key them more closely to *E. carolleeae* than to *E. affinis*.

Setae size and locations

Drawings representing the typical placement and sizes of setae on the male and female fifth legs (P5) of specimens from the Great Lakes collected for this study are illustrated in Fig.

6a, b. This setation pattern was then compared to relatively complete and detailed drawings of the male P5 in the literature (Table 2) that were identified (see previous paragraph) as either *E. affinis* or *E. carolleae*.

The right P5 in males (Fig. 6a) has a robust basipod (segment A) with a strong inside lateral spine (RA2) plus two strong spines in the middle of the segment centered on each side of the segment (RA1, RA3). The next segment B, or first exopodal, has a strong inside lateral spine (RB3) about 0.7 distance from the base, two setae about midway on the segment (RB1, RB2) and one seta at the distal end of the segment (RB4). The last segment C on our specimens have a seta on the inner surface (RC1) where the segment starts constricting, another one just above and a little further out on the outer surface (RC2) and a peg-like seta on the middle inner surface at the scythe-like end of the segment (RC3). The representative right leg drawn in Fig. 6a is most similar to drawings by Lee and Frost (2002), Busch and Brenning (1992), and Katona (1971) except for the presence of a single setae between RB3 and RB4 seen in Lee and Frost (2002) and Katona (1971).

For the left P5 in males (Fig 6a) the setal patterns of the coxal segment (labeled segment A), the basipod (B) and the two exopodal segments (C and D) were examined. Comparisons could not be made with Busch and Brenning (1992) because they did not include the entire left leg. The coxal segment in Fig. 6a has two to three setae on the outer surface (LA3, LA4, and LA5), one seta on the midline of the segment (LA1) and one seta on the distal inner surface (LA2). The basipod of our specimens have two setae on the outer edge (LB1 and LB2). Segment C (first exopodal segment) in Fig. 6a has two setae midway on the inner surface of the segment (LC2, LC3), a single seta opposite the two on the outer surface (LC1), and setae on the distal end of the segment (LC4, LC5, LC6). The last segment (segment D, the second exopodal) in Fig. 6a has a very strong seta midway on the inner edge (LD1) and a seta on the outer edge about 0.6 distance from the base (LD2) and other features (see below). Segments A and C on the left leg are quite variable, as similarly illustrated by Busch and Brenning (1992). The setal pattern of the left leg (Fig. 6) is identical to those illustrated by Katona (1971).

In the left male exopod, the end of the second segment (Segment D) resembles a “dragon's head” at high power (Fig. 7), but only two of the seven references listed in Table 1 illustrate the complexity present in this structure. A row of short setae covers the inner portion of the bifurcated end of the second exopodal segment and at some positions several spicules come out of small protuberances. The outer surface of the segment has three setae placed as if one were viewing an eye (LD3) and a pair of nostrils (LD4, LD5). Wilson (1959) only showed the row of short setae at the end of the segment but did not show setae LD3-LD5, and Davis (1943) and Katona (1971) both display the row of short setae at the end of the segment but setae LD3-LD5 are not clearly represented. Examination of archived specimens (personal communication, Dr. Eduardo Suarez-Morales, ECOSUR, 2016) described in Suarez-Morales et al. (2008) identified the presence of setae LD4 and LD5; Dr. Suarez Morales also verified (personal communication, Dr. Eduardo Suarez-Morales, ECOSUR, 2016) that the dragon's head structure of segment D in his Balmorhea Lake specimens has a longer “neck” and more bulbous ending illustrated in Fig. 3c of Suarez-Morales et al. (2008), distinctively different from Fig. 7 of this paper.

On the female P5 (Fig. 6b), seta 1 on segment A (designated seta A1) is a strong seta that occurs on the anterior to lateral face of the basipod plus a finer seta occurs in the middle portion of the anterior face (A2). Most authors have not described the finer setae illustrated in the present study. Only the stronger seta (A1) appears in Suarez-Morales et al. (2008), as further confirmed by review of Dr. Suarez-Morales' archived *Eurytemora* from Lake Balmorhea (personal communication, Dr. Eduardo Suarez-Morales, ECOSUR, 2016). In contrast, both setae are illustrated in Katona (1971). In Fig. 6b, the first exopod (segment B) have two fine setae on the central part of the anterior face (B4, B5) which are also drawn or photographed in Suarez-Morales et al. (2008) and Katona (1971). The strong seta 3 on the lateral edge of segment B (B3) also appears to have its counterpart in the lateral position in Fig. 5c and on the posterior face in Fig. 2f of Suarez-Morales et al. (2008). This seta is absent in the Katona (1971) drawing which appears to be a drawing of the anterior view. The drawing by Katona (1971) of the last exopodal segment has three setae. Our specimens have two (C6, C7), but these setae are absent in Suarez-Morales et al. (2008), who further confirmed the fewer number of setae in general on his archived specimens than have been found in this study of Great Lakes *Eurytemora*.

Discussion

***E. carolleeae* as a distinct species in the *Eurytemora* complex**

Previous studies on the morphological and genetic divergence of members of the *Eurytemora* complex from Alaska, Europe and North America demonstrated great diversity and suggested that there were undiscovered clades (Dodson et al., 2010; Lee, 2000; Winkler et al., 2008; Winkler et al., 2011). Mitochondrial DNA analysis performed during the past fifteen years suggested that two clades inhabit the St. Lawrence estuary region, an invasive Atlantic clade and a non-invasive North Atlantic clade (Cabrol et al., 2015; Favier and Winkler, 2014; Lee, 1999; Winkler et al., 2008). Attempts to mate individuals from two populations with each other did not produce viable reproductive adult offspring (Lee, 2000). Alekseev and Souissi (2011) identified the existence of a new species, *E. carolleeae*, and deduced that the Atlantic clade previously referred to as *E. affinis* might be considered the separate species of *E. carolleeae*. Alekseev and Souissi (2011) suggested that a reassessment of the population found in the Great Lakes be done since previous surveys reporting *E. affinis* might actually have been observing *E. carolleeae*. Indeed, the initial reports from EcoAnalysts we received counted all specimens as *E. affinis*, based on the standard taxonomic keys used by many plankton specialists. Nevertheless, the distinct morphological features described by Alekseev and Souissi (2011) and COI sequences differing from most *E. affinis* specimens by a much larger margin than 3% support the identification of *E. carolleeae* as a distinct sibling species within the *Eurytemora* complex.

Small (1.5% - 3%) but consistent differences in the sequences of the Great Lakes specimens in the present study compared to the European sequences by Sukhikh et al. (2013) might indicate further incipient speciation events. To determine if speciation is occurring additional genes should be examined. Such speciation events could occur as a result of geographic separation of populations and subsequent selection or founder effects.

The reanalysis of specimens from the US Geological Survey Great Lake Science Center collection of alcohol preserved plankton samples and microscopic slide collection spanning from 1962 to present identified only *E. carolleae*. These records from all 5 Great Lakes and Lake St. Clair suggest that historically, *E. carolleae* was the species of record, and that *E. affinis* was probably never present in the Great Lakes. The drawings on plate 26 of *E. affinis* in Balcer et al. (1984) which are clearly *E. carolleae*, are further historical evidence. Thus, analysis in the present study of archived, and freshly collected specimens, and literature drawings from Lake Erie, Lake Michigan, Lake Huron, Lake Superior, Lake Ontario, Lake St. Clair and the Detroit River revealed only *E. carolleae*.

Now that *E. carolleae* is considered a distinct species, several previous papers and annotated sequences should be updated to reflect our current understanding of *Eurytemora* diversity and to assist with proper reporting and identification of current populations. Especially relevant to invasion biology are two sequences (Accessions GQ924685 and GQ924686) by Briski et al. (2011) that were uploaded to GenBank in 2009 as *E. affinis* but are 97% and 99% identical to the *E. carolleae* sequences in GenBank. These two specimens were sequenced from diapaused copepod eggs obtained from sediments in ship ballast tanks of transoceanic vessels that had arrived at a Canadian port (Sept-Iles, QC). In additional neighbor-joining tree analysis (ESM Appendix S3) with these shorter sequences (545 and 549 bases, respectively), one of the sequences of Briski et al. (2011) grouped closer to the Sukhikh et al. (2013) sequences than to the Toledo Harbor specimens in this study; the other sequence was closer to the sequences of Toledo Harbor specimens and was 99% identical to specimens collected for the present study from the Detroit River, Lake Huron, and Muskegon Lake. Aside from suggesting that the annotations of GQ924685 and GQ924686 should be updated to reflect their likely re-identification as *E. carolleae*, the presence of these specimens in ballast tank sediment suggests the possibility that *E. carolleae* might invade ports in ship's ballast sediments as suggested by Sukhikh et al. (2013).

Morphological features

Historical comparisons of *Eurytemora* taxa have used various widths, lengths and shapes of various body parts with some parameters involving presence/absence. These comparisons worked well for separating most of the species within the genus as a whole (Dodson et al., 2010) but did not differentiate several subtypes within the *E. affinis* complex (Busch and Brenning, 1992; Dodson et al., 2010; Lee and Frost, 2002). However, Alekseev and Souissi (2011) revealed features that morphologically differentiated the *E. carolleae* subtype from *E. affinis*.

In the present study, the most useful morphological measurement in comparisons to previous *Eurytemora* descriptions was the L/W ratio of the basipod in the left fifth leg of males. Quantitative analysis of this trait was described for *E. affinis* and *E. carolleae* by Alekseev and colleagues (Alekseev and Souissi, 2011; Sukhikh et al., 2013). While the average values for this trait differ significantly (1.43 ± 0.13 [mean $\pm$ SD] in *E. carolleae* v. 0.96 ± 0.05 in *E. affinis* in Alekseev and Souissi (2011)), this measure is not absolute. For example, Figure 5B of Sukhikh et al. (2013), who studied populations from a broader geographic area, illustrated similar averages to Alekseev and Souissi (2011) for the two species but values for

about 12% of the specimens crossed over the value of 1.3 that we have used to differentiate the species. Thus, the L/W ratio of the basipod in the left fifth leg of males represents a useful key feature, but for definitive identification, additional features or DNA sequences are needed.

Setal and exopodal patterns

In an effort to provide a more complete description of the diagnostic fifth legs of males and females and to potentially identify additional key features, this study investigated the pattern of setae on the fifth legs in detail. The illustration by Alekseev and Souissi (2011) of the setal pattern on the male P5 of *E. carolleae* was limited to a few major seta. In reviewing the drawings from past literature, the precision and accuracy of the various artists or the quality of the specimen, the preparation, or the type of microscope used are difficult to assess. For example, Davis (1943) did not illustrate any of the setae on the female P5 and it appears that a number of setae on the male P5 were likely omitted. Busch and Brenning (1992) suggested that the number and positions of setae on the male 5th leg were variable. When we compared the setal patterns of our male specimens with the figures of the authors in Table 2, only the drawings of Katona (1971) matched ours. Because we suspect that he was dealing with two species, this may mean that the male 5th leg setal patterns of *E. affinis* and *E. carolleae* are virtually identical. The drawings from Schmeil (1896) as illustrated in Lee and Frost (2002), which definitely illustrate *E. affinis*, also match ours except for the absence of seta LA1 and an additional seta on segment B on the right side.

In contrast, the drawings of Suarez-Morales et al. (2008) properly characterize the material they studied and they suggest that their specimens may represent the Gulf subclade of *Eurytemora affinis* of Lee and Frost (2002). Interestingly, Segment D (exopod 2) on the left side of the male 5th leg of Suarez-Morales et al. (2008) lacks a few setae (even after review of Suarez-Morales' archived specimens); however, the marked differences in shape of the “dragon's head” ending of Segment D (personal communication, Dr. Suarez-Morales, ECOSUR, 2016) may especially be of some diagnostic value since it is a gross difference in shape and not just setae that may be difficult to see or easily damaged in preparation. Whether the presence or absence or position of any of these setae or the differences in the dragon's head structure has any taxonomic value may come from further detailed morphological studies of the P5 of various clades of the *Eurytemora* complex accompanied by molecular data. This is especially true since the differences in the structure and ornamentation of the male fifth leg is evident in copepods coming from transitional environments such as the Temoridae and Pseudodiaptomidae family as well as from continental waters as in the case of Diaptomidae (personal communication, Dr. Suarez-Morales, ECOSUR, 2016).

An important question is whether the presence of these structures may have any selective role or effect on fitness. As previously pointed out by Lee and Frost (2002), copepods have no image-forming vision. Therefore the reproductive behavior of copepods is unlikely to use fine details of physical structures such as spines and setae, as visual cues. Copepods instead use pheromone trails and olfactory senses to mediate sexual tracking and mating (Seuront, 2013; Yen et al., 2011). Nevertheless, at short range (i.e., once the male tracks down the

female) spines could help as a tactile signal in mate recognition (Holynska, 2000). Future detailed comparisons of reproductive or other behaviors in *E. carolleeae* and *E. affinis* might help determine their adaptive role, if any, and their utility for taxonomy.

Conclusions

Although morphological features that distinguish *E. carolleeae* from *E. affinis* were described more than four years ago, the identification of *E. carolleeae*, rather than *E. affinis*, in the Great Lakes was facilitated by the use of genetic barcoding. The previous observations from Chesapeake Bay and the St. Lawrence estuary apparently did not catch the attention of taxonomists in the Great Lakes, and *E. carolleeae* was not integrated into the commonly used keys for the Great Lakes, such as Dodson et al. (2010), which leads only to *E. affinis*. As a result of the present study, which combined sequence analysis of contemporary specimens of *Eurytemora*, all of which were most similar to sequences of *E. carolleeae*, with morphological analysis of contemporary and historical Great Lakes specimens dating as far back as 1962, we conclude that *Eurytemora carolleeae* is the correct name for the species of *Eurytemora* that invaded the Great Lakes.

As in the present study, the observation of novel COI sequences may alert analysts to examine specimens more closely. Phylogenetic research on the many species of copepods in the Great Lakes should be encouraged in view of their abundance, diversity, and important roles in most freshwater systems, so that routine sequence identification of copepods could be used to determine if rare or non-native species are present.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Alekseev VR, Souissi A. A new species within the *Eurytemora affinis* complex (Copepoda: Calanoida) from the Atlantic Coast of USA, with observations on eight morphologically different European populations. *Zootaxa*. 2011; 2767:41–56.
- Alekseev VR, Abramson NI, Sukhikh NM. Introduction of sibling species to the ecosystem of the Baltic Sea. *Doklady Biological Sciences : Proceedings of the Academy of Sciences of the USSR, Biological sciences sections / translated from Russian*. 2009; 429:544–547.
- Anderson, DV.; Clayton, D. Div Res. Dept. Lands and Forests, Maple; ON, Canada: 1959. Plankton in Lake Ontario.

- Balcer, MD.; Korda, NL.; Dodson, SI. Zooplankton of the Great Lakes : a guide to the identification and ecology of the common crustacean species. University of Wisconsin Press; Madison, Wis: 1984.
- Briski E, Cristescu ME, Bailey SA, MacIsaac HJ. Use of DNA barcoding to detect invertebrate invasive species from diapausing eggs. *Biological Invasions*. 2011; 13:1325–1340.
- Busch A, Brenning U. Studies on the status of *Eurytemora affinis* (Poppe, 1880) (Copepoda, Calanoida). *Crustaceana*. 1992; 62:13–38.
- Cabrol J, Winkler G, Tremblay R. Physiological condition and differential feeding behaviour in the cryptic species complex *Eurytemora affinis* in the St. Lawrence estuary. *Journal of Plankton Research*. 2015; 37:372–387.
- Croskery P. The Freshwater co-occurrence of *Eurytemora affinis* (Copepoda: Calanoida) and *Manayunkia Speciosa* (Annelida: Polychaeta): possible relicts of a marine incursion. *Hydrobiologia*. 1978; 59:237–241.
- Davis CC. The larval stages of the calanoid copepod *Eurytemora hirundoides* (Nordquist). Chesapeake Biological Laboratory. 1943; 58:1–58.
- Dodson SI, Skelly DA, Lee CE. Out of Alaska: morphological diversity within the genus *Eurytemora* from its ancestral Alaskan range (Crustacea, Copepoda). *Hydrobiologia*. 2010; 653:131–148.
- Drake JM, Lodge DM. Hull fouling is a risk factor for intercontinental species exchange in aquatic ecosystems. *Aquatic Invasions*. 2007a; 2:121–131.
- Drake JM, Lodge DM. Rate of species introductions in the Great Lakes via ships' ballast water and sediments. *Canadian Journal of Fisheries and Aquatic Sciences*. 2007b; 64:530–538.
- Engel RA. *Eurytemora affinis* a calanoid copepod new to Lake Erie. *Ohio Journal of Science*. 1962; 62:252.
- Favier JB, Winkler G. Coexistence, distribution patterns and habitat utilization of the sibling species complex *Eurytemora affinis* in the St. Lawrence estuarine transition zone. *Journal of Plankton Research*. 2014; 36:1247–1261.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology Biotechnology*. 1994; 3:294–299. [PubMed: 7881515]
- Gurney, R. British freshwater copepoda. The Ray Society; London: 1931.
- Gutierrez-Aguirre MA, Cervantes-Martinez A, Elias-Gutierrez M. An example of how barcodes can clarify cryptic species: The case of the calanoid copepod *Mastigodiptomus albuquerqueensis* (Herrick). *Plos One*. 2014; 9:1–11.
- Holynska M. Is the spinule pattern on the leg 4 coxopodite a tactile signal in the specific mate recognition system of *Mesocyclops* (Copepoda, Cyclopidae)? *Hydrobiologia*. 2000; 417:11–24.
- Horvath TG, Whitman RL, Last LL. Establishment of two invasive crustaceans (Copepoda : *Harpacticoida*) in the nearshore sands of Lake Michigan. *Canadian Journal of Fisheries and Aquatic Sciences*. 2001; 58:1261–1264.
- Hudson PL, Bowen CA 2nd. First record of *Neoergasilus japonicus* (Poecilostomatoida: *Ergasilidae*), a parasitic copepod new to the Laurentian Great Lakes. *The Journal of Parasitology*. 2002; 88:657–63. [PubMed: 12197110]
- Johnson KE, Perreau L, Charmantier G, Charmantier-Daures M, Lee CE. Without gills: Localization of osmoregulatory function in the copepod *Eurytemora affinis*. *Physiological and Biochemical Zoology*. 2014; 87:310–324. [PubMed: 24642548]
- Katona SK. The developmental stages of *Eurytemora affinis* (Poppe, 1880) (Copepoda, Calanoida) raised in laboratory cultures, including a comparison with the larvae of *Eurytemora americana* Williams, 1906, and *Eurytemora herdmanni* Thompson & Scott, 1897. *Crustaceana*. 1971; 21:5–20.
- Laakmann S, Gerdts G, Erler R, Kneibelsberger T, Arbizu PM, Raupach MJ. Comparison of molecular species identification for North Sea calanoid copepods (Crustacea) using proteome fingerprints and DNA sequences. *Molecular Ecology Resources*. 2013; 13:862–876. [PubMed: 23848968]
- Lee CE. Rapid and repeated invasions of fresh water by the copepod *Eurytemora affinis*. *Evolution*. 1999; 53:1423–1434.
- Lee CE. Global phylogeography of a cryptic copepod species complex and reproductive isolation between genetically proximate “populations. *Evolution*. 2000; 54:2014–2027. [PubMed: 11209778]

- Lee CE, Frost BW. Morphological stasis in the *Eurytemora affinis* species complex (Copepoda: Temoridae). *Hydrobiologia*. 2002; 480:111–128.
- Mills EL, Leach JH, Carlton JT, Secor CL. Exotic species in the Great-Lakes - a history of biotic crises and anthropogenic introductions. *Journal of Great Lakes Research*. 1993; 19:1–54.
- Miracle MR, Alekseev V, Monchenko V, Sentandreu V, Vicente E. Molecular-genetic-based contribution to the taxonomy of the *Acanthocyclops robustus* group. *Journal of Natural History*. 2013; 47:863–888.
- Peterson DL, Kubow KB, Connolly MJ, Kaplan LR, Wetkowski MM, Leong W, Phillips BC, Edmands S. Reproductive and phylogenetic divergence of tidepool copepod populations across a narrow geographical boundary in Baja California. *Journal of Biogeography*. 2013; 40:1664–1675.
- Posavi M, Gelembiuk GW, Larget B, Lee CE. Testing for beneficial reversal of dominance during salinity shifts in the invasive copepod *Eurytemora affinis*, and implications for the maintenance of genetic variation. *Evolution*. 2014; 68:3166–3183. [PubMed: 25135455]
- Reid JW, Hudson PL. Comment on “Rate of species introductions in the Great Lakes via ships' ballast water and sediments. *Canadian Journal of Fisheries and Aquatic Sciences*. 2008; 65:549–553.
- Schmeil, O. Deutschlands freilebende Susswasser-Copepoden. Erwin Nagele, Stuttgart; Germany: 1896.
- Seuront L. Chemical and hydromechanical components of mate-seeking behaviour in the calanoid copepod *Eurytemora affinis*. *Journal of Plankton Research*. 2013; 35:724–743.
- Suarez-Morales E, Rodriguez-Almaraz G, Gutierrez-Aguirre MA, Walsh E. The coastal-estuarine copepod, *Eurytemora affinis* (Poppe) (Calanoida, Temoridae), from arid inland areas of Mexico: An expected occurrence? *Crustaceana*. 2008; 81:679–694.
- Sukhikh N, Souissi A, Souissi S, Alekseev V. Invasion of *Eurytemora* sibling species (Copepoda: Temoridae) from North America into the Baltic Sea and European Atlantic coast estuaries. *Journal of Natural History*. 2013; 47:753–767.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*. 2013; 30:2725–2729. [PubMed: 24132122]
- Weinke AD, Kendall ST, Kroll DJ, Strickler EA, Weinert ME, Holcomb TM, Defore AA, Dila DK, Snider MJ, Gereaux LC, Biddanda BA. Systematically variable planktonic carbon metabolism along a land-to-lake gradient in a Great Lakes coastal zone. *J Plankton Res*. 2014; 36:1528–1542. [PubMed: 25954055]
- Wilson, MS.; Yeatman, HC. Free-living copepoda. John Wiley and Sons; New York: 1959.
- Winkler G, Dodson JJ, Lee CE. Heterogeneity within the native range: population genetic analyses of sympatric invasive and noninvasive clades of the freshwater invading copepod *Eurytemora affinis*. *Molecular Ecology*. 2008; 17:415–430. [PubMed: 17868296]
- Winkler G, Souissi S, Poux C, Castric V. Genetic heterogeneity among *Eurytemora affinis* populations in Western Europe. *Marine Biology*. 2011; 158:1841–1856.
- Yen J, Sehn JK, Catton K, Kramer A, Sarnelle O. Pheromone trail following in three dimensions by the freshwater copepod *Hesperodiaptomus shoshone*. *Journal of Plankton Research*. 2011; 33:907–916.

Fig. 1. Map depicting zooplankton collection sites within the Laurentian Great Lakes system and North American rivers. Circles represent samples collected during 2012, 2013 and 2014. Triangles represent sites where archival samples were collected (see Table 1). Inset in map is of Toledo Harbor and western Lake Erie where *Eurytemora carolleeae* was first found in this study.

Fig. 2. Micrographs of morphological characters used to key *Eurytemora carolleeae* Alekseev and Souissi, (2011). (a) Female, genital somite (arrow); (b) Female fifth leg showing the small spine (arrow) near the distal seta insertion point; (c) Male spineless caudal ramus (arrow); (d) Male fifth leg indicating basipod (arrow).

Fig. 3.

Neighbor-joining tree based on number of base differences per cytochrome oxidase I (COI) sequence for eight specimens from Toledo Harbor (GenBank accession numbers KP792795-KP792802) compared to *Eurytemora carolleae*, *Eurytemora affinis* and *Eurytemora lacustris* sequences from the GenBank database. Sequences were selected from sequences submitted by Victor Alekseev. The analysis of 34 sequences is expressed as percent of 631 nucleotide positions.

Fig. 4. Neighbor-joining tree based on number of base differences per cytochrome oxidase I (COI) sequence for 26 *Eurytemora* specimens collected from various North American sites (GenBank accession numbers KP938969-KP938976, KR611021-KR611038) compared to the eight specimens from Toledo Harbor analyzed in Fig. 3. Also included are the same *Eurytemora carolleae*, *Eurytemora affinis* and *Eurytemora lacustris* GenBank sequences used in Fig. 3. The analysis of 60 sequences is expressed as percent of 626 nucleotide positions.

(a) New specimens (this study)

(b) Archived specimens

(c) Literature drawings

Fig. 5.

Length/width ratios for left basipod in the male fifth leg in (a) North American specimens collected for this study from sites shown by circles shown in Fig. 1, (b) archived specimens from sites shown by triangles in figure 1 and listed in table 1, (c) drawings in the scientific literature. In (c) the abbreviations listed in table 2 are positioned corresponding to their L/W ratios.

Fig. 6. Drawing of *Eurytemora carolleeae* fifth legs in (a) male and (b) female. The segments are lettered from distal to proximal; setae within a pod are numbered from distal to proximal. In the text particular setae are referred to by the designation Side-Podite-Seta # (SPS#). For example RA2 refers to the strong inside lateral spine on the right segment A.

Fig. 7.
“Dragon's head” structures at the end of the left endopod of male fifth leg.

Table 1

Historical biogeography of *Eurytemora carolleeae* using archived specimens and archived slides from the Laurentian Great Lakes. Source and sex of archived specimens from the Great Lakes region are provided.

Lake	Location	Latitude (°)/Longitude (°)	Depth (m)	Date	Sex
Superior	Bibon Lake wetland [#]	46.784/-91.398	1	Aug. 5, 1993	F
Michigan	Milwaukee Harbor [*]	NR	NR	Aug. 27, 1969	M
	Little Bay de Noc [*]	NR	NR	Aug. 31, 1970	M ^θ
	Green Bay [*]	NR	NR	May, 1969	F
Huron	Saginaw Bay [*]	NR	5	July 10, 1974	M
	Saginaw Bay [#]	43.598/-83.664	1	Aug. 17, 1997	M
	Saginaw Bay [#]	43.817/-83.919	1	July 28, 1994	M
	Clinton R. cutoff [*]	42.562/-82.847	NR	July 10, 1973	M, M ^θ
Erie	Sandusky Bay [*]	41.500/-82.702	NR	May 26, 1967	F, M, M
Ontario	Fair Haven, NY [#]	43.428/-76.722	55	Nov. 2, 1992	M

^{*} Specimens on archived slides were collected by John Gannon.

[#] Specimens preserved in alcohol were collected by Patrick Hudson. NR means not recorded.

^θ means only caudal ramus and not basipod was available for morphology. Multiple specimens for a particular collection are indicated by more than one sex designation separated by commas.

Table 2

Summary of illustrations in referenced literature analyzed for presence of *Eurytemora carolleeae* in previously described specimens. “Abbreviation” is the single letter with or without subscript used in an illustration (Fig. 5) in the Results to identify the reference source.

Reference	Figure in Reference	Structures	Abbreviation
Geographic source and date			
Alekseev & Souissi (2011) Chesapeake Bay, USA (2008)	6, 9	♂P5, caudal ramus; ♀P5, genital somite	A
Busch & Brenning (1992) North Sea and Baltic Estuaries (1988)	2, d, e, f, g, m,	♂P5; ♀P5, genital somite	B
Davis (1943) Chesapeake, Bay (1943)	Plate 9: 7	♂P5	D
Gurney (1931) England (prior to 1931)	305	♂P5	G
Katona (1971) Oyster Pond, MA (1969)	88, 89	♂P5	K ₈₈ , K ₈₉
Lee & Frost (2002) Germany (prior to 1896)	2, redrawn from Pl. XI, Fig 10 of Schmeil (1896)	♂P5	L
Suarez-Morales et. al (2008) Presa Falcon, Mexico (2000-2001)	2, 3, 4	♂P5; ♀P5, genital somite	S ₃ , S ₄
Wilson & Yeatman (1959) Lake Providence, Louisiana (prior to 1959)	29.15	♂P5	W