Use of Morphometric Analyses and DNA Barcoding to Distinguish Truncilla donaciformis and Truncilla truncata (Bivalvia: Unionidae)

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REGULAR ARTICLE

USE OF MORPHOMETRIC ANALYSES AND DNA BARCODING TO DISTINGUISH *TRUNCILLA DONACIFORMIS* AND *TRUNCILLA TRUNCATA* (BIVALVIA: UNIONIDAE)

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ABSTRACT

Closely related unionid species often overlap in shell shape and can be difficult to accurately identify in the field. Ambiguity in identification can have serious impacts on conservation efforts and population surveys of threatened and endangered species. *Truncilla donaciformis* and *Truncilla truncata* are sister species that overlap in their distributions and frequently co-occur in central North America. Because *T. donaciformis* is endangered in Canada and imperiled in some US jurisdictions, co-occurrence with the morphologically similar *T. truncata* means that misidentification could seriously impact status assessments and recovery efforts. The objectives of this study were to (1) establish species identifications of specimens using DNA barcoding (COI), (2) determine how well traditional morphometrics and geometric morphometrics accurately discriminate between the two species, and (3) determine the accuracy of field identifications relative to molecular and morphometric identifications. *Truncilla* specimens from four rivers in southern Ontario were photographed and visceral mass swabs were taken. Positive identifications of all specimens were obtained through DNA barcoding and comparison with sequences from GenBank. Traditional and geometric morphometric approaches were used to assign specimens to species. Assignments generated were compared to identifications based on mtDNA barcodes, with traditional and geometric morphometric analyses found to be 90% and 99% accurate in species identifications, respectively. This study confirmed the presence of *T. donaciformis* in Ontario's Thames River, and revealed that all *Truncilla* collected for this study from the other three rivers were *T. truncata*. This study reinforces the utility of combining geometric morphometric analyses and DNA barcoding for identifying problematic unionid specimens.

KEY WORDS: DNA barcoding, Laurentian Great Lakes drainage, morphometric analysis, shell shape, species identification

INTRODUCTION

Early delineations and descriptions of freshwater mussel species (order Unionida) were based on shell morphology (Watters et al. 2009), which can be expressed through coloration, shape, shell sculpture, or size. Some shell-shape characters have a clear genetic basis and are potentially adaptive (Inoue et al. 2013, 2014), but habitat and environment can also have major effects on shell morphology. Use of shell shapes led to overdescription of some species based on sometimes-subtle differences (Haag 2012). More recently, species descriptions have been based on internal soft-tissue anatomy and molecular tools, resulting in the synonymizing of many previously described species (Watters et al. 2009; Haag 2012). Sacrificing animals to examine soft tissues is often not

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an option when dealing with threatened and endangered species, so identifications of live animals are limited to external shell features and genetic methods (e.g., DNA barcoding; Hebert et al. 2003). Because failure to correctly identify species can have important implications for the conservation of unionid diversity, a major challenge in correctly identifying species is dealing with intraspecific variation in shell shape. As shell morphology remains the most common tool used in field survey identifications of freshwater mussels, this intraspecific variation can be problematic when attempting to differentiate among closely related species with similar shell morphologies.

Truncilla donaciformis (Lea, 1828; Fawnsfoot) and *Truncilla truncata* (Rafinesque, 1820; Deertoe) are sister species (Burlakova et al. 2019) with similar shell morphologies (Fig. 1). Both are described mostly using shell morphology that is variable and potentially nondiagnostic. Watters et al. (2009) qualitatively describes *T. donaciformis* as more elongate in shape, whereas *T. truncata* is more triangular and typically exhibits a prominent posterior ridge. Further confounding correct field identifications, both species exhibit some subtle sexual dimorphism, with females being more rounded along the ventral margin (Watters et al. 2009; Burlakova et al. 2019).

Both *T. donaciformis* and *T. truncata* are distributed throughout much of the Mississippi River and Ohio River drainages and parts of the Great Lakes drainage of North America (Watters et al. 2009). In Canada, *T. truncata* is relatively common in southwestern Ontario, while *T. donaciformis* is considered an endangered species (COSEWIC 2008). In the USA, *T. donaciformis* is considered imperiled in several U.S. states and may be declining across its range (NatureServe 2020). The distribution of *T. truncata* is similar to *T. donaciformis*, but it is more widely distributed throughout the USA and is considered less imperiled. As the distributions of *T. donaciformis* and *T. truncata* are mostly sympatric (Watters et al. 2009), and their morphological characters are known to overlap, differentiating between the species can be problematic. This potential ambiguity is even more concerning when one considers the disparity in conservation status of the two species. Misidentifications could lead to misspent resources and incorrect conservation and recovery strategies, resulting in a high cost in terms of both conservation capital and species outcomes (Shea et al. 2011).

A number of practices can be employed to improve the accuracy of identifications based on shell appearance. One such practice is morphometric analysis, which measures and quantifies shape. Traditional morphometrics (TM) in mollusks include the measurement and comparison of ratios between characters such as shell length, width, and height (e.g., Cyr et al. 2007). For some species, once a large sample size of confirmed specimens has been examined, TM can be reasonably accurate in differentiating species, but positive identification cannot be assured due to morphometric variation within populations (Cyr et al. 2007; Inoue et al. 2013, 2014). If the differences are great, traditional morphometrics are usually
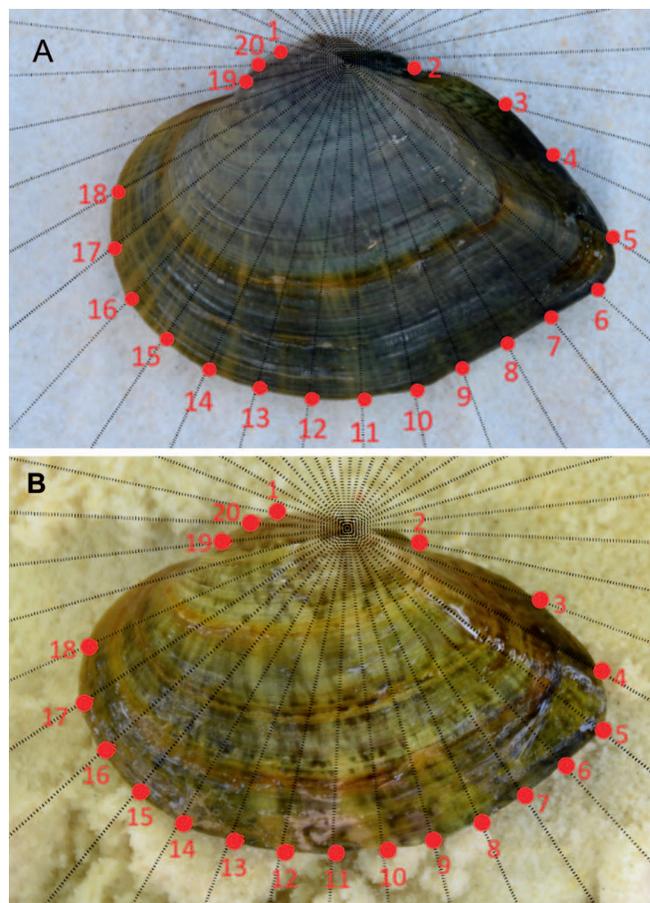

Figure 1. Positions of geometric morphometric type I and type II landmarks on the left valve of A) typical *Truncilla truncata* specimen and B) typical *T. donaciformis* specimen. Type I (anchor) landmarks: LM 1 were placed at the tip of the umbo, LM 2 at the posterior end of the hinge ligament. Type II landmarks: LM 3 to LM 18 were placed where a fan with 40 rays (anchored at midpoint between LM 1 and LM 2) crossed the shell margin; LM 19 and 20 were placed where rays from the fan cross the anterior side of the umbo.

sufficient for differentiating between groups, but if the differences are subtle, they can be confounded easily (Webster and Sheets 2010). Despite these drawbacks, taking shell measurements is quick, convenient, low-cost, and noninvasive, and these measurements are recorded in almost every field survey of unionid mussels. Geometric morphometrics (GM) involves the use of landmarks and pseudo-landmarks to assess the shape of an organism in two or three dimensions (Zelditch et al. 2012). Multivariate statistical analyses of the data are used to compare morphologies of the target specimens (Webster and Sheets 2010). Typically, these analyses are more robust and accurate than TM (Rohlf and Marcus 1993; Adams et al. 2004); their accuracy makes them an ideal method of differentiating between two occasionally ambiguous species. A reliable quantitative approach to species identification using a combination of morphometric analyses and DNA barcoding (see below) is preferable over "best guess" or "expert opinion."

In order to assess the utility and reliability of either

morphometric method, it is important to first independently confirm specimen identifications. In the last two decades, DNA barcoding has become an important tool for species identification (Hebert et al. 2003; Baird and Sweeney 2011). A (~650 bp) fragment of mitochondrial DNA encoding the cytochrome oxidase subunit 1 (COI) gene (Folmer et al. 1994) is often used as a reference sequence to which homologous sequences are compared, and barcoding with this sequence has been employed in the study of many animal groups including unionids (e.g., Inoue et al. 2014, 2018). Previously published *Truncilla* COI sequences (including those from Burlakova et al. 2019) on NCBI GenBank enable the positive identification of *T. donaciformis* and *T. truncata* specimens, which can help establish a group of specimens with confirmed identifications that can be used to discover morphological differences between the two species.

The objectives of this study were to (1) establish species identifications of specimens using DNA barcoding (COI), (2) determine how well TM and GM accurately discriminate between the two species, and (3) determine the accuracy of field identifications relative to molecular and morphometric identifications. We predict that GM will prove more accurate than TM in correctly differentiating between the species and that the combination of these techniques will elucidate areas of difference between the shell morphologies of *T. donaciformis* and *T. truncata*.

METHODS

We collected specimens of *Truncilla donaciformis* and *T. truncata* from the Ausable, Sydenham, Thames, and Welland rivers in Ontario, Canada, during targeted surveys completed in summer 2017 (Table 1 and Fig. 2). Swabs of the foot and mantle were taken from all live specimens and stored in lysis buffer (Sambrook et al. 1989). We photographed all field-collected specimens and returned them to the stream of origin after processing. Additional specimens were added to the morphometric dataset from the University of Michigan Museum of Zoology and the Ohio State University Division of Mollusks. The museum specimens used were lots from the Great Lakes drainage (Ontario, Michigan, and Ohio) and the Ohio River (the type locality for both species; Table 2 and Fig. 2). The left valve of each field- or museum-collected specimen was photographed in the same orientation for geometric morphometric analyses. For photographs, field-collected specimens (all live) were placed on a bed of fine sand, and a digital camera was mounted on a portable copy stand and placed at a 90° angle to the shell valve. Museum specimens (dead shell valves) were photographed using a digital camera mounted in a Stackshot apparatus with the valve placed on a piece of black fabric or modeling clay to ensure it was precisely at a 90° angle to the camera.

A 250 µL aliquot of the swab lysis buffer from each specimen was digested with 15 µL of proteinase K overnight at 56°C, followed by alcohol extraction and purification (Sambrook et al. 1989). Extracted DNA (stained with SYBR

Table 1. Summary of field-collected and identified *Truncilla* specimens from rivers in Ontario, Canada.

Location	*T. truncata*	*T. donaciformis*	Uncertain *Truncilla*	River totals
Welland River	18	0	11	29
Ausable River	2	0	0	2
Thames River	33	28	10	71
Sydenham River	1	0	0	1
Totals	54	28	21	103

Green) was electrophoresed on a 1.5% agarose gel to confirm the success of the extraction and assess DNA quality. The female-lineage cytochrome c oxidase subunit I (COI) region of the mtDNA was amplified using the COI primers described in Campbell et al. (2005). Amplicon aliquots were stained with SYBR Green and electrophoresed in a 1.5% agarose gel to visualize fragment sizes and confirm successful amplifications. Reactions were purified using exonuclease I and shrimp alkaline phosphatase (EXOSAP). Polymerase-chain-reaction products mixed with EXOSAP were incubated at 37°C for 40 min, followed by 80°C for 20 min to denature any enzymes or remaining primers (as in Hewitt et al. 2019). Samples were Sanger sequenced by Eton Biosciences (etonbio.com) on an Applied Biosystems ABI 3730. The generated sequences were compared to those available on GenBank using BLAST (Altschul et al. 1990). The GenBank sequence with the highest percentage identity score resulting from the BLAST search was chosen as the most likely species and identified as such.

Traditional morphometric measurements for each specimen were length (maximum distance anterior to posterior) and height (maximum distance dorsal to ventral) measured to the nearest millimeter using Vernier calipers. For field-collected specimens, shell inflation (= width or maximum distance across left and right valves) was also measured. Length-to-height (L/H), length-to-width (L/W), and height-to-width (H/W) ratios were calculated for all field-collected specimens and then arcsine transformed to control for specimen size (as in Inoue et al. 2014).

For geometric morphometric analyses, an image of the left valve of each specimen was uploaded to the MakeFan program of the Integrated Morphometrics Package 8 (IMP8; Sheets 2014). Anchor (type I) landmarks were placed at the tip of the umbo and the point where the hinge ligament ends on the dorsal-posterior side of the valve (landmarks 1 and 2 on Fig. 1). Using MakeFan8, a 40-ray fan was placed at the midpoint between landmarks 1 and 2. Type II landmarks were placed at the point where each ray transected the edge of the shell, with landmark numbers starting at three and consecutively following the rays clockwise around the shell (Fig. 1). We chose to remove the point along the shell margin following landmark 18 because the curve at this point was highly variable among specimens and resulted in placement of the point at different parts of the shell (at the anterior curve of the shell margin, the anterior slope of the hinge, or the anterior of
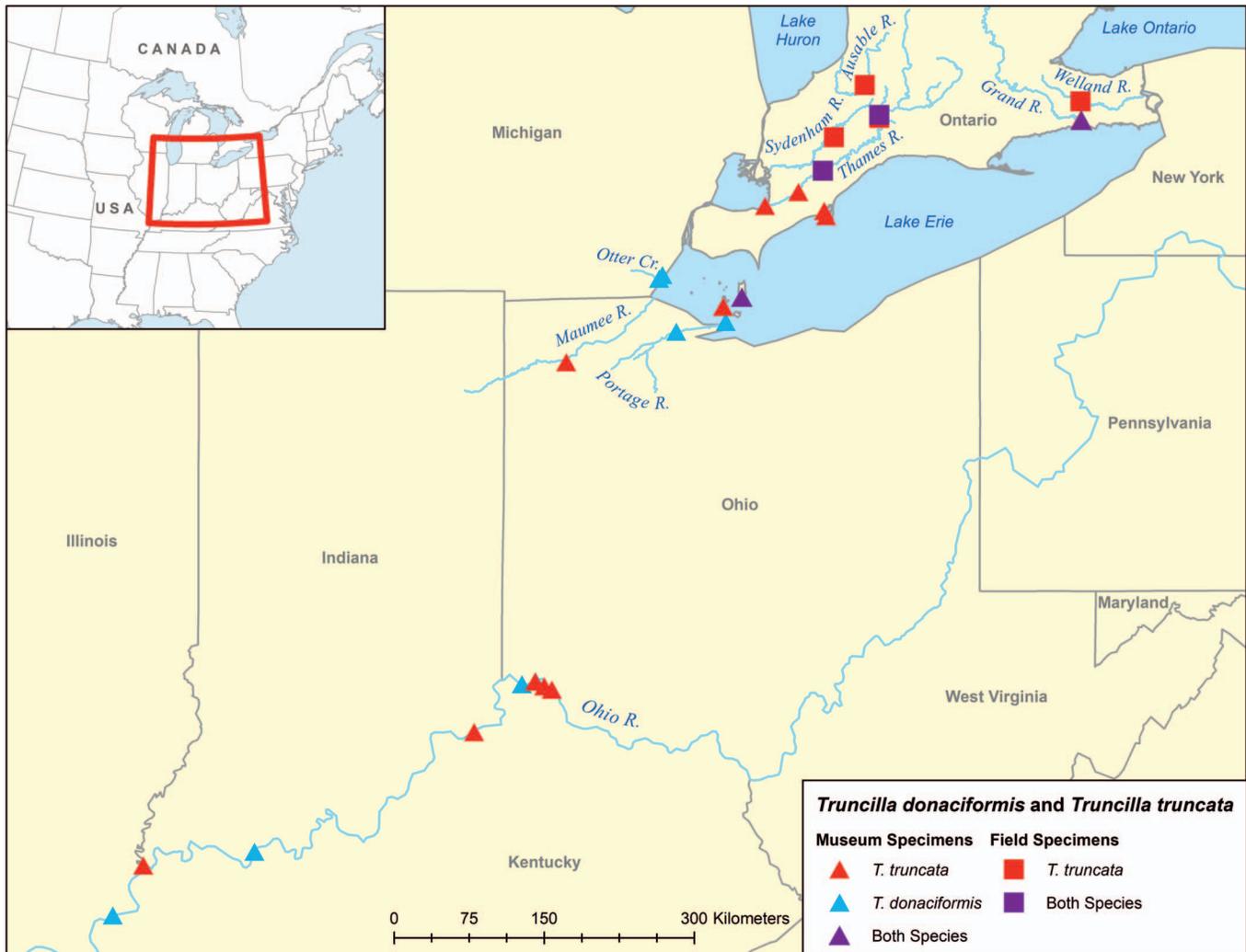
Figure 2. Map of *Truncilla donaciformis* and *T. truncata* field-collection site locations and museum specimen localities.

the raised umbo). Type II landmarks 19 and 20 were placed where the rays cross the anterior side of the umbo. The landmarks for each specimen were digitized using MakeFan8. CoordGen8 was used to implement a generalized Procrustes alignment of the digitized landmarks.

A principal component analysis (PCA) was applied to the transformed TM ratios (L/H, L/W, H/W) using XLSTAT v. 2018.6 (2018). Discriminant analysis (DA), implemented in XLSTAT using field-identified specimens, was employed to assess the utility of traditional morphometric measurements in species identification. The species memberships of unidentified specimens were predicted using the results of the DA and compared to the confirmed species identifications resulting from the NCBI BLAST search of the COI sequences. An alpha of 0.05 was used in all tests of significance.

CVAGen (in IMP8) was used to implement a canonical variates analysis (CVA, equivalent to the DA used with the TM dataset) to determine if and where significant differences in shell shape exist between the species and to visualize and quantify where the differences were greatest. Differences

between shell shapes were visualized using a deformation grid and vectors on landmarks. Museum and field-collected specimens with confirmed identifications (using COI sequences) were used to create the CVA model. The unknown field-collected specimens were used to test the utility of the model using a jackknife assignment test based on the CVA results. We compared the predictions generated by the model to the confirmed species identifications resulting from the NCBI BLAST search of the COI sequences. The species memberships of the unidentified specimens were predicted using a jackknife assignment test based on the CVA results and compared to the confirmed species identifications resulting from the NCBI BLAST search of the COI sequences.

RESULTS

Field collections yielded photographs of 103 individuals: 54 identified as *T. truncata*, 28 as *T. donaciformis*, and 21 unidentified *Truncilla* from the Thames, Welland, Ausable, and Sydenham rivers in Ontario, Canada (Table 1). Museum

Table 2. Summary of *Truncilla donaciformis* ($n = 44$) and *T. truncata* ($n = 42$) specimens examined, photographed, and digitized at the Ohio State University Division of Mollusks and the University of Michigan Museum of Zoology. Museum collection lot numbers and number of specimens are listed for each species.

Ohio State University Division of Mollusks		University of Michigan Museum of Zoology	
T. donaciformis (museum lot–location– no. specimens)	*T. truncata* (museum lot–location– no. specimens)	*T. donaciformis* (museum lot–location– no. specimens)	*T. truncata* (museum lot–location– no. specimens)
8328–Grand R., Ontario–4	*550*–Lake Erie, Ohio–6	*63767*–Grand R., Ontario–1	*164427*–Ohio R., Ohio–3
15831–Lake Erie, Ohio–4	*64392*–Maumee R., Ohio–2	*198126*–Grand R., Ontario–3	*129769*–Ohio R., Illinois–3
66852–Portage R., Ohio–5	*85140*–Grand R., Ontario–2	*128791*–Lake Erie, Ontario–1	*31*–Ohio R.–3
58607–Ohio R., Ohio–4	*14937*–Lake St. Clair, Ontario–2	*70923*–Otter Cr., Michigan–5	*70941*–Ohio R.–1
	6577–Ohio R., Kentucky–5	*45014*–Lake Erie, Michigan–5	*185609*–Grand R., Ontario–4
	1661–Ohio R., Kentucky–5	*232560*–Ohio R., Ohio–1	*198129*–Thames R., Ontario–3
		62218–Ohio R., Kentucky–3	*70991*–Lake Erie, Ontario–1
		70863–Ohio R.–3	*227598*–Lake Erie, Ontario–1
		44852–Ohio R., Illinois–1	*71014*–Lake Erie, Ontario–1
		70915–Ohio R.–2	
		44824–Ohio R., Kentucky–1	
		59–Ohio R.–1	
17–*T. donaciformis*	22–*T. truncata*	27–*T. donaciformis*	20–*T. truncata*

specimens from the Ohio State University Division of Mollusks and University of Michigan Museum of Zoology were examined and photographed for a total of 44 *T. donaciformis* and 42 *T. truncata* specimens (Table 2). Photographs used for morphometric analyses have been submitted to MorphoBank (Project 3457, MorphoBank accession numbers M675689–M675794; http://morphobank. org/permalink/?P3457).

DNA Barcoding

Sequencing resulted in COI amplicons averaging 640 bp in length from all 103 individuals, with six unique haplotypes generated for *T. donaciformis* (GenBank accession nos. MT593033- MT593038) and four unique haplotypes generated for *T. truncata* (GenBank accession nos. MT594464– MT594467). A comparison of the COI sequences to those on GenBank using NCBI BLAST confirmed the identifications of 35 *T. donaciformis* and 68 *T. truncata* specimens. Based on the BLAST search results, one specimen from the Thames River (specimen no. 86; site TR-50) was *T. truncata* based on its COI sequence but was incorrectly identified in the field as *T. donaciformis*. Of the 21 unknown *Truncilla* specimens, 13 were identified as *T. truncata*, and eight as *T. donaciformis*.

Traditional Morphometrics

Principal components analysis of the traditional morphometric ratio data showed that the L/H ratio was the primary driver of differences between species shapes (Fig. 3). A *t*-test of the L/H ratios between species supported the hypothesis that the two groups' values were different ($t = -9.81$, df $= 100$, $P < 0.001$), but there was overlap between the species (Fig. 4).

The H/W ratio explained less differentiation between the species, while the L/W ratio was not a diagnostic character between them.

The DA based on the traditional morphometric ratios revealed significant differences between *T. truncata* and *T. donaciformis* specimens confirmed with the COI sequence data. The DA correctly identified 93 of 103 specimens when compared to BLAST search results of the COI sequences. The misassigned specimens were three *T. truncata* and seven *T. donaciformis*. One of the specimens correctly predicted by the DA was the field-misidentified specimen from the Thames River (specimen no. 86). The DA also was used to assign the unknown *Truncilla* specimens to a species and was correct for 19 of 21 specimens when compared to BLAST search results of the COI sequences. The two misidentified specimens were a COI-identified *T. truncata* from the Welland River (site WLR05 specimen no. 10) and a COI-identified *T. donaciformis* from the Thames River (site TR-24 specimen no. 34). However, the latter specimen had only a 56.1% probability of assignment to the *T. truncata* group.

Geometric Morphometrics

The CVA revealed differences in shape along a single axis between the COI-confirmed species (Fig. 5). Differences in shell shape along CVA axis 1 were visualized using a deformation grid and landmark vectors (Fig. 6). The main differences were in length near the dorsal margin and in height along the ventral margin of the shell. The jackknife assignment test on the landmark-based CVA dataset resulted in 102 of the 103 field-collected specimens and all of the museum specimens being correctly assigned back to the COI-confirmed or expert-identified (for museum specimens) species. Of the
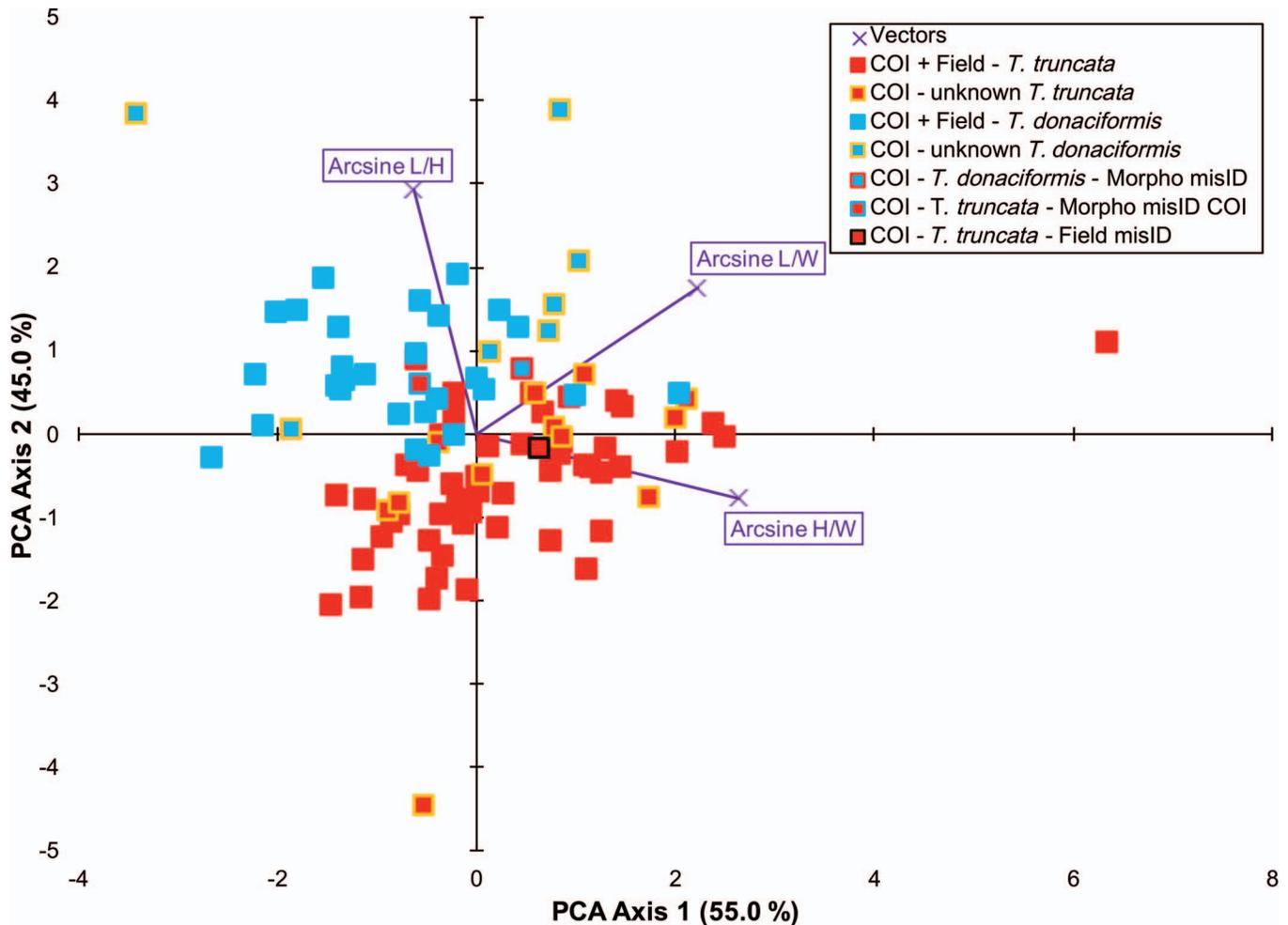
Figure 3. Principal components analysis (PCA) biplot for arcsine-transformed shell length-to-height (L/H), length-to-width (L/W), and height-to-width (H/W) ratios of field-collected *Truncilla* specimens. Species identifications were confirmed by comparing COI sequence data to sequences on GenBank.

specimens of uncertain species membership collected in the field, 20 of 21 were correctly identified based on their COI sequence. The misidentified specimen, from the Thames River (site TR-50 specimen no. 112), was a COI-identified *T. donaciformis* that was grouped with *T. truncata* using geometric morphometrics.

DISCUSSION

In identifying putative *Truncilla donaciformis* and *T. truncata* specimens, both geometric and traditional morphometrics were shown to be useful, providing a high degree of identification certainty when compared to definitive identifications based on DNA barcodes. Traditional morphometrics, paired with multivariate statistical analyses, were found to correctly identify 90% of the field-identified specimens and 90% of unknown specimens when compared to COI barcode identifications. Multivariate statistical analyses of landmark-based morphometric data provided greater identification accuracy with correct identifications of 99% of field- and museum-identified specimens and 95% of unknown specimens

when compared to COI barcode identifications, although in both cases only a single specimen was incorrectly identified.

Shell morphology has long been used to differentiate and identify freshwater mussel species, with varying degrees of reliability (Watters et al. 2009). The utility and accuracy of shell morphology in that capacity has been assessed via mitochondrial DNA barcoding (Hebert et al. 2003; Baird and Sweeney 2011), and recent studies have upheld its merits in assigning individuals to species and groups (Inoue et al. 2013; Guarneri et al. 2014; Inoue et al. 2014; Barreto et al. 2016). However, because freshwater mussel morphology depends, at least partially, on environmental conditions and may vary considerably within species (Hornbach et al. 2010; Suzuki and Nagasawa 2013), care must be taken to systematically quantify the morphological characters being used to identify freshwater mussel specimens. In the sister species *T. donaciformis* and *T. truncata*, applying traditional and geometric morphometrics has provided quantifiable means of differentiating between the two. *Truncilla truncata* is described as being more triangular in shape, while *T. donaciformis* is more elongate following a continuum in the ratio of shell length to shell height (Watters
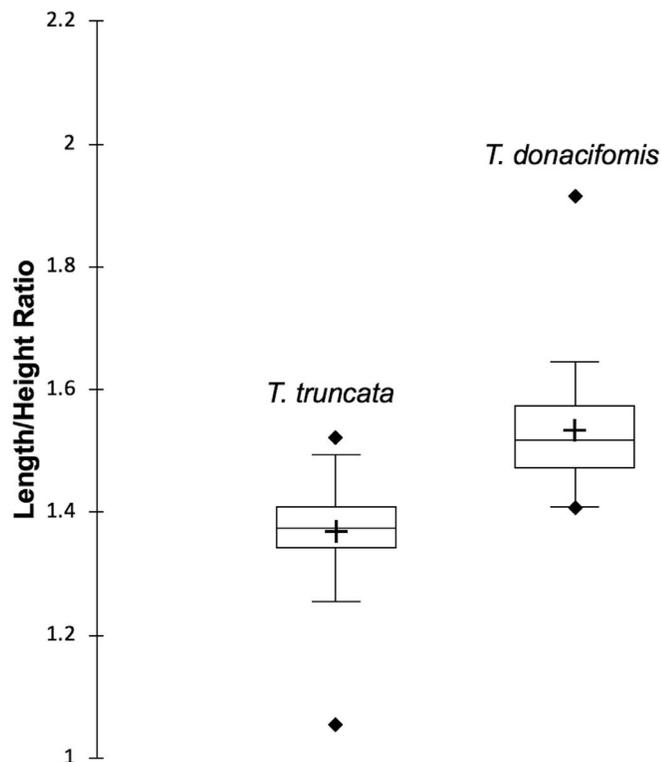
Figure 4. Box plot showing quartiles, means, and outliers of L/H ratios from field-collected *Truncilla* specimens.

et al. 2009). Length-to-height ratios >1.5 were 95% likely to be *T. donaciformis*, and ratios <1.4 were 95% likely to be *T. truncata*, but there was considerable overlap in the L/H ratio between *T. donaciformis* and *T. truncata* specimens. Additionally, the PCA identifies the shell H/W ratio as another important character in differentiating between the species, but this character is difficult to incorporate into field identifications.

Both morphometric techniques were able to differentiate between the two species with somewhat different reliability. Traditional morphometric data applied in a discriminant analysis was able to accurately differentiate between the two *Truncilla* species when compared to DNA barcodes, with an accuracy of 90%. Assignment tests of specimens using the landmark-based geometric morphometric dataset in the CVA provided improved accuracy over traditional morphometrics when compared to the DNA barcodes, with an accuracy of 99% among the specimens analyzed. The analysis of quantifiable morphological characters provided by these two morphometric techniques has the potential to provide researchers and mussel survey teams with greater confidence in assigning specimens to species compared to expert opinion based on qualitative assessment of characters. The general reliability of traditional morphometric techniques in differentiating between *T. truncata* and *T. donaciformis* may be sufficient for most specimens and studies, but we recommend using the more reliable geometric morphometric techniques whenever possible, especially for problematic specimens.

Traditional morphometrics using shell measurements, when applied appropriately, have the potential to provide researchers with a great deal more confidence in field identifications than qualitative assessments of shell characteristics (i.e., expert opinion). Conducted using measurements frequently taken in the field—length, width, and height—traditional morphometric data are relatively simple and cost-effective to analyze. The drawback to this ease is accuracy. Based on our findings, 10% of *Truncilla* specimens identified this way were inaccurately identified. This inaccuracy (i.e., false positives and false negatives) could have some consequences for species conservation efforts in terms of inaccurately estimating the distribution and status of the species, leading to misspent or unnecessary use of resources (Shea et al. 2011).

In contrast to traditional morphometric analyses, landmark-based geometric morphometrics provide a more robust dataset and yield greater accuracy in species identifications (Rohlf and Marcus 1993; Adams et al. 2004; Inoue et al. 2014). However, there is a cost for this accuracy; the methodology requires photos of each specimen, the digitization of landmarks onto those photos, the careful formatting of data, and the use of multivariate statistical analyses. These requirements add a great deal of time and, consequently, higher costs. However, those costs (in time and resources) might be negated by its increased reliability over traditional morphometric analyses; incorrect species identifications made by the latter could result in a waste of time and resources on populations that were erroneously assumed to exist. One relevant example of the implications of misidentification is in the Canadian status assessment of *T. donaciformis* (COSEWIC 2008), which cites historical records of *T. donaciformis* from Lyons Creek in the Welland River drainage, as well as a historical record from the Niagara River near the mouth of the Welland. Some of the specimens collected for the present study were taken from other locales in the Welland River and identified as possible *T. donaciformis*, but mtDNA barcoding and morphometric data both confirmed these as *T. truncata*. Without an examination of the shell specimens, our findings call into question whether *T. donaciformis* was ever really present in the Welland River drainage.

Perhaps the only way to identify a freshwater mussel specimen with near perfect certainty is with genetic techniques, but to do so for every specimen collected in a survey is costly and time-consuming, and it requires finding living animals. DNA barcoding techniques (Hebert et al. 2003) using COI and other mtDNA sequences have proven to be useful for identifying most unionoid species (Campbell et al. 2008; Boyer et al. 2011; Inoue et al. 2013, 2014; Keogh and Simons 2019), but there are some exceptions (i.e., within the genera *Elliptio* and *Pleurobema*; Inoue et al. 2018). Once a morphological database of specimens with confirmed identifications (i.e., using DNA barcodes) is established, geometric morphometric techniques have the potential to provide a relatively cost-effective and less technology-intensive alterna-
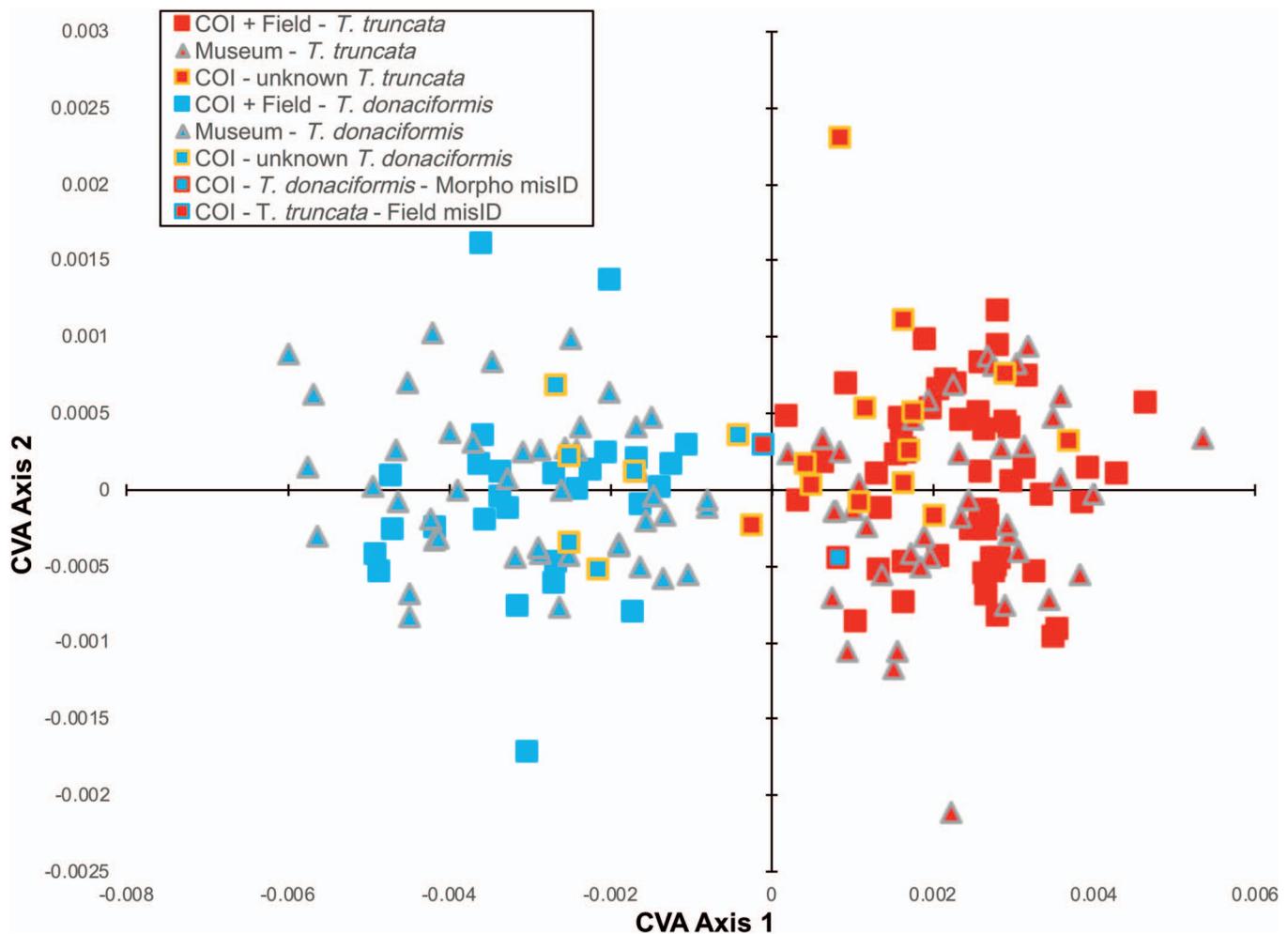
Figure 5. Canonical variates analysis biplot of *Truncilla* specimen shell shape using 20 landmarks. Species identifications were confirmed by comparing COI sequence data to sequences on GenBank or the identification of the museum specimens. The only significant differences in shape between the groups were found along CVA axis 1.

tive to DNA barcoding, with a high degree of accuracy that could be used with less reliance on taxonomic/genetic experts.

Misidentifying endangered *T. donaciformis* in Canada could have negative consequences for efforts directed at conservation of this endangered species, such as in the Welland River (COSEWIC 2008). We used traditional and geometric morphometric techniques, along with COI mtDNA sequencing, to confirm the presence of *T. donaciformis* in select Canadian watersheds presumed to be inhabited by the species. We can confirm definitively the presence of *T. donaciformis* in southwestern Ontario in the Thames River, but none of the specimens examined from the Ausable ($n = 3$), Sydenham ($n = 1$), or Welland rivers were found to be *T. donaciformis* using DNA barcoding or morphometric analyses. However, our sampling was by no means exhaustive, and *T. donaciformis* still may be present in these systems. No *Truncilla* specimens from the lower Grand River in Ontario, where *T. donaciformis* has recently been found alive (COSEWIC 2008), were collected in the field for this study, but Grand River shell specimens from museum collections

(Table 2) did group with DNA barcode-confirmed specimens using landmark-based morphometric analyses, confirming that it was present historically. Museum specimens from Lake Erie grouped with the DNA barcode-confirmed specimens and verify the historical presence of *T. donaciformis* there.

In addition to definitively identifying unionids collected during field surveys in Ontario, our findings could prove useful for further investigations of *T. donaciformis* throughout its distribution. This study provides an accurate morphometric framework and dataset to be used in future surveys; our dataset is publicly available (DRYAD Link: https://doi.org/10.5061/dryad.rn8pk0p6m). Using a similar combination of morphometric and DNA barcoding techniques also could be useful for differentiating among other morphologically similar species. Examples include species in the notoriously difficult-to-identify genera *Pleurobema* and *Fusconaia* (Inoue et al. 2018), *Pyganodon* (Cyr et al. 2007), western North American *Anodonta* (Chong et al. 2008), and some members of the Lampsilini (Keogh and Simons 2019).
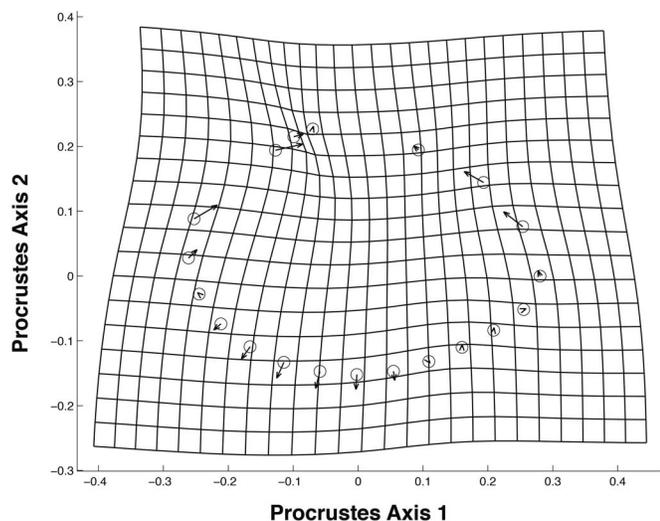
Figure 6. Deformation grid and vectors showing differences in Procrustes-superimposed shell shape along CVA axis 1 (Fig. 5). The differences in shape are denoted by the direction and relative magnitude of vectors and in the areas where the grid is most deformed from a square. The circles represent the mean shape of *T. donaciformis* specimens and the ends of the vectors represent the mean shape of *T. truncata* specimens.

ACKNOWLEDGMENTS

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