Characterization and Analysis of Ctenophore Morphology

Tina Nguyen, Steve Haddock, Shannon Johnson, Lynne Christianson

Monterey Bay Aquarium Research Institute

Abstract:

Despite being one of the oldest metazoans, research regarding ctenophores is relatively lacking. Although scientists are able to distinguish species from each other using gene sequencing, there are currently no quantitative methods for identifying species. Using gene sequencing to identify species can be difficult, as it requires collecting a sample of the organism to analyze. The ctenophore's gelatinous nature often causes the specimen to break apart when brought to the surface, making it difficult to collect them for sequencing. Furthermore, dives containing images and videos of ctenophores without these samples cannot be identified. Even if a sample is collected, researchers must take the time and funding to sequence them. This paper will describe a quantitative method to distinguish ctenophore species based on their morphology using the R package Geomorph. The advantage of using this method is the ability to differentiate species by only using images taken, which can allow researchers to identify ctenophores from dives where samples were not taken. In addition, using this method can prevent future sampling of organisms, which can help preserve their populations and saves the resources needed to perform a gene sequence. Our aim is to use this quantitative method to identify species that are morphologically similar and are often confused with each other. In this Geomorph analysis, we used images collected from MBARI dives of the *Deiopea* and *Kiyohimea* species, two closely-related lobate ctenophores.

Introduction:

Ctenophores are a marine species found in virtually all oceans and at several ocean depths. They are all carnivorous, using either tentacles, auricles, or lobes to feed on prey. Their digestive system is connected by a series of canals, and this arrangement can vary depending on the species. Their tentacles are unique in that they contain colloblasts, sticky cells used to adhere to prey. Ctenophores with lobes often have them lined with mucus to capture prey in a similar manner; auricles, gelatinous projections, help guide the food to their mouths. Their gelatinous form makes it difficult to find preserved specimens, and so little is known about their evolutionary history. Ctenophores were originally grouped with cnidarians due to their similar complexities and morphologies. However, there are many key differences that distinguish ctenophores from cnidarians. Cnidarians use cnidocytes, venomous cells, to sting prey compared to colloblasts, which only stick to their prey. Furthermore, medusae, the most similar group to ctenophores in appearance, swim leading with their aboral end, whereas ctenophores swim leading with their oral end. Gene sequencing also demonstrates that these two groups are genetically distinct.

Deiopea and *Kiyohimea* are two groups of lobate ctenophores that have very similar morphologies, and were even once thought to be the same group at different development stages. The *Kiyohimea* species was first discovered in the 1940's; the *Kiyohimea Usagi* species was characterized based on their lobes and V-shaped bodies that were flattened along one plane. It moves slowly through the water and waits for prey to get trapped in its mucus-lined lobes instead of chasing them. Their comb plates are very closely spaced to each other running down their auricles and substomodaeal and subtentacular comb rows.

Although the *Deiopea* group is very similar in appearance to the *Kiyohimea* family, there are some distinct differences in their morphology that distinguish them. The *Deiopea* genus is smaller overall and more rounded in shape. Its comb row plates are also distinctly less numerous and are much farther apart than in *Kiyohimea*. Gene sequencing demonstrates that these two groups are genetically distinct despite their similar appearances. Although this method can identify different species, it cannot identify specimens that were not sampled. During dives where multiple ctenophores can be observed, it is not realistic to sample all of them, especially due to their fragile nature and the cost to sequence specimens. There are several images of ctenophores that have not been sampled and are difficult to identify based on sight. The R package Geomorph, along with packages Morpho, Shapes, and StereoMorph serve as a practical alternative to gene sequencing. Geomorph uses morphometrics to quantitatively analyze shapes with landmarks, chosen features of a specimen. It is advantageous compared to just analyzing the size and shape of features alone because it can account for body features that can change angles, such as the lobes and auricles in a ctenophore. Furthermore, the software is free to use and contains several online tutorials for beginners. Geomorph can also use the 2D and 3D coordinates of the landmarks to easily perform statistical analyses based on their spatial configurations, such as PCA and mahalanobis tests. Semilandmarks that can be placed along surfaces and curves can also be analyzed, although they have not been used in this project. One aspect of this package that is not ideal for our project is that it cannot take any non-coordinate related data. Thus, the number and spacing of comb rows in *Deiopea* and *Kiyohimea* could not be measured, even though they are very defining features of each group and help with identifying

them. However, analyzing these components can be done separately and later combined with the Geomorph analysis.

Methods:

10 high-quality images of both *Kiyohimea* and *Deiopea* were chosen by sorting through several images collected from dives from the Monterey Bay Aquarium Research Institute. Images were chosen based on their features were clearly visible. The comb rows had to be clear enough to be counted, and most or all of the ctenophore had to be present in the image. Landmarks for each ctenophore were first chosen based on important features present in both species, such as the lobes, mouth, and auricles. The landmarks were placed using StereoMorph, which also saved the 2D coordinates into separate shape files for each image. Curves were also created along both the substomodaeal and subtentacular comb rows using this package and saved into the same file.

Comb row counts, width, and spacing were measured using FIJI. The clearest comb rows of the substomodaeal and subtentacular comb rows were measured on each side for a total of four comb row counts per specimen. Three comb row plates were measured for their width and spacing, and this was similarly repeated for each image. The notes for the StereoMorph and FIJI analysis are below:

Obtain a clear image of a Ctenophore. The individual comb rows should be clear enough that they can be counted. The entire (or at least most of) the individual should also be visible, and each landmark that needs to be marked should be visible as well.

Set your working directory in R to the folder with the image and load the required packages (Geomorph and StereoMorph)

```
library(geomorph)
library(StereoMorph)
```

Load the StereoMorph package and execute the following command:

```
digitizeImage(
    image.file='ctenophore_images',
    shapes.file='ctenophore_shapes',
    landmarks.ref='ctenofeatures-plain.txt',
     curves.ref='ctenofeatures-curves.txt'
)
```

The image.file is the name of the folder (you can also use a file for a single image) with the image.

Shapes.file is the name of the folder that will store all of the coordinate and curve data. When you save coordinates and curves in StereoMorph, files will be created with the same name as the image and will store the corresponding data. Be sure to keep the names of the files in image.file and shapes.file the same as each other, as this is how it knows how to store the data to the correct image. *You do not create these files. StereoMorph will create these once you add coordinates or curves. If you make one before executing this command, the image will not show up and you will get an error message.*

landmarks.ref is a text file with a list of all the landmarks you want to mark on the image. curves.ref is another text file with the list of the bezier curves you want to create. One line will contain the name of the curve itself first, then the start of the curve and the end of the curve. You will have as many rows in this file as the number of curves you want to create. Here is an example:

STCR-left 01-STCR-tip-left 02-STCR-base-left SSCR-left 03-SSCR-tip-left 04-SSCR-base-left STCR-right 05-STCR-tip-right 06-STCR-base-right SSCR-right 07-SSCR-tip-right 08-SSCR-base-right

3. Running the command will open a popup window with the selected image on the left, and on the right there are four tabs.

Landmarks: The second one, called landmarks, is where you will be creating landmarks. Clicking on one of the landmarks names in this tab will make it appear in bold. This means that when you click on an image, it will set whatever point you click on as that landmark. You can always change the landmark by clicking on the landmark name again and selecting another point on the image, or you can even drag the point (double click it to make the normally blue point green, which means it can be changed) to where you want it to be. Clicking on the landmark name again will make it unbold, which can allow you to click on and drag along the picture without creating landmarks.

Curves: The third tab, called curves, is how you will be creating bezier curves.

If creating curves with only two points: Mark the start and end of each curve as designated- this is done the exact same way landmarks are created. Between the names of the landmarks, there should be a blank point without a name- marking an image with this will connect the start and end points of the curve, and moving this point will drag the curve accordingly. If your curve is

near the end of the image, this point unfortunately cannot go past the bounds of the image. To get around this, you can create borders around the image.

If creating curves with three points: StereoMorph has a strange pattern with the unmarked points between the start and end points: every other line will "drag" the curve out as described in the previous paragraph. For example, the first point is the start point, the second point will control the shape/drag the curve, and the third point will be the "end" point of that curve. The third point will also be the start of another curve, then the fourth point will "drag" it and connect it to the final end point. In essence, you create two curves that are connected, which allows you to more precisely fit the curves to the actual image.

Make sure to save the image before moving onto the next one, or StereoMorph will not save your work.

Scale: The fourth tab is the scale tab, which is where you create a scale for the organism. You set the first ruler point to the first point you want to measure, and then set the second point to the end point you want to measure. You then set the ruler interval to the length of this segment (including its units).

4. You can click exit on any of the images to exit the program. If you want to run StereoMorph again, you have reset the kernel because the original link you click on to access the window will not work anymore. You can go back and manually edit the shapes files if you wish to change the coordinates (although this may delete or make your curves or landmarks inaccurate). You can also continue to add more images to the images.file folder to analyze more images.

#Measuring comb row width, spacing, and number

5. To count the number of comb rows in your image, open up Image J/FIJI. Open your image, and use the paint brush (adjust the pixel size so that they can track the individual comb rows), and mark where each comb row is, making sure not to have these marks overlap with each other. FIJI is not very good at letting you undo mistakes: it will allow you to undo the last mark you made, but not any other previous ones, so keep in mind that if you want to delete more than one mark you made, you may have to restart. Make the brush color green by going to edit -> options -> colors, then choosing green as the foreground.
You can create a macro, which will record the upcoming steps so that you will not have to repeat them in the future. Go to Plugins -> Macros -> Record. Then go to Image -> Adjust -> Color Threshold. Set the first one, hue, to approximately 65-100. Saturation should be around 205-255, and brightness should be around 200-255. Set the threshold color to B&W, and then click on the Macro button on the bottom. *Make sure to select Macro after you've made the adjustments, and not before. If you do this before, the Macro will not save these adjustments and it will not work.* Then go to Measure -> Analyze particles and set the minimum size to the approximate area of the brush. Set the "show" option to Outlines, and then click ok. This should have only the dots appear in the popup image in black and white. Other popup windows will show how many dots there are and its measurements such as the areas (which aren't important here).

Here, you should save and name the Macro. This Macro will automatically adjust the color threshold so you don't need to do so next time. To use it, just open it and run it once all the comb rows are marked with the brush. It should also count the number of particles and give you the

measurement popups. Save the measurements as well if you'd like to access them later. The Macro can be somewhat finnicky. Sometimes it will work with one image, but then give an inaccurate measurement for the next time. Sometimes, to get this to work you have to reload the Macro each time you count or analyze an image (even if it's the same image and you're counting or measuring something different). Other times, it may be that the analyze particles function is confused by other marks in the image that are similar in size to the brush, so adjusting the brush size and minimum size of analyze particles may help with this.

Once all of the images were digitized with StereoMorph, the coordinates were read by Geomorph. The landmark coordinates from each image were superimposed, and centroids of each landmark were created as well. An important note about this superimposition is that the y axis will be switched due to the coordinate system used in StereoMorph. StereoMorph's origin is at the top left of the image, and so the farther down and to the right the coordinate is, the higher its x and y values will be. However, Geomorph will plot with the lower y values being at the bottom than at the top. Thus, the superimposition plot will appear to be upside-down. Although rotating it seems like the obvious fix, this will actually switch the left and right sides of the plot as well. Thus, it is better to either switch the signs of all of the coordinates or switch the axis to preserve the correct orientation. The articulation angle of the auricles was also fixed for all of the images using the Geomorph function fixed.angle, allowing for a more accurate analysis. The Mahalanobis distance of the shape data was calculated, demonstrating possible outliers. Mesh plots were also created using the Geomorph function plotreftotarget. The Geomorph code and notes are found below:

```
require(StereoMorph)
require(geomorph)
require(shapes)
require(Morpho)
help(package = "geomorph")
# Convert shape files from StereoMorph into TPS format for import into GeoMorph


if (str_detect(getwd(),"haddock")) {
        basepath = "/Users/haddock/repos/ctenomorph/data/"
}else{
        basepath = "/Users/Tinanguyen/Documents/Ctenophore._image/data"
}
setwd(basepath)
fdir <- paste0(basepath,'ctenophore_shapes')
sh = readShapes(fdir)
ctcurves = sh$curves.control
ctland=sh$landmarks.pixel


# ctgpa = gpagen(ctland)
sliders<-define.sliders(ctland[,,1],nsliders=3)
# define sliders as a list around the perimeter
sliderpoints = c(1,14,13,12,11,23,17,24,5,28,18,27,7,1)
```

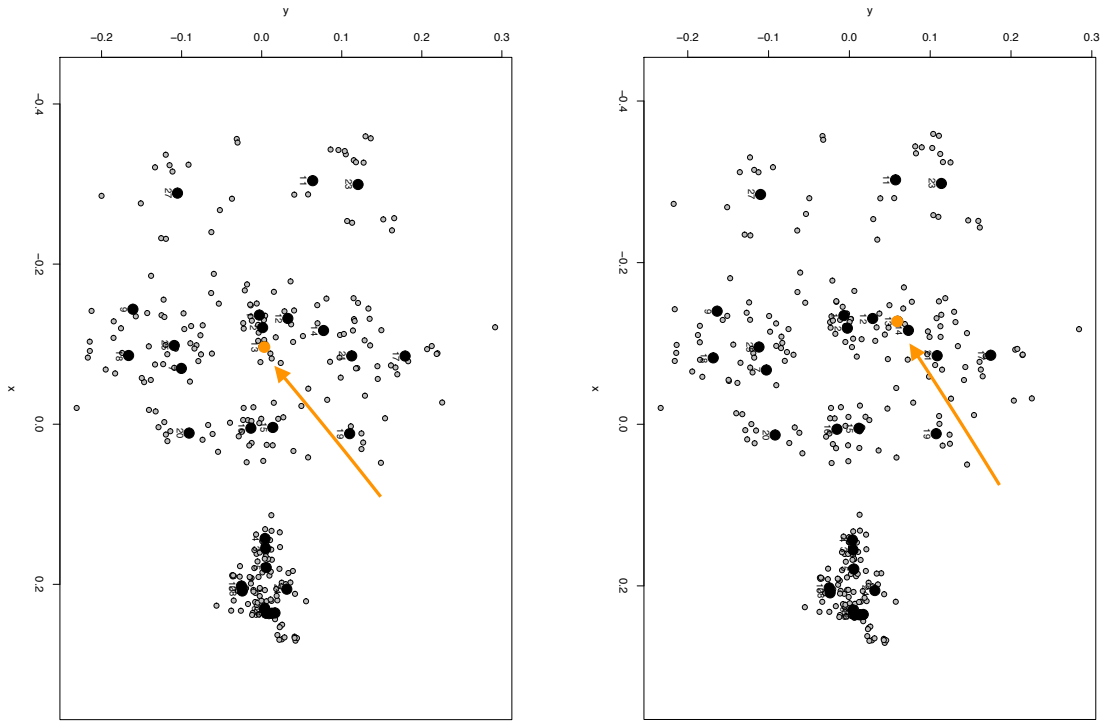
```
# Adjust angle of auricle tip (CHECK THIS!)

ctland_angle = fixed.angle(ctland, art.pt=14, angle.pts.1 = 12,angle.pts.2 = 13, rot.pts = c(13))

plot(ctland_angle[,,3],col="blue",pch=21)
par(new=TRUE)
plot(ctland[,,3],col="red")

# Create superimpositions
super_angle = gpagen(ctland_angle)
super = gpagen(ctland)

# Shape ( $coords) and size ($Csize)

# Each row of the link matrix designates the two landmarks to be connected by that link.
# Should run define.links()

plotOutliers(super$coords)
plotOutliers(super_angle$coords)

plotAllSpecimens(super_angle$coords,label = TRUE)
```

```
#Mahalanobis plot


data <- procSym(ctland)$PCscores[,1:3]
probas <- typprob(data,data,small=TRUE)
maha <- mahalanobis(data,colMeans(data),cov(data))
plot(probas,maha,xlab="Probability",ylab="Mahalanobis D^2")
pos_vector <- rep(4, length(names(maha)))
pos_vector[names(maha) %in% c("KiyohimeaExtended", "border_Kiyohimea_usagi-D1046-
20180730T001002Z", "border_Kiyohimea_usagi-D1047-20180730T180055Z",
"border_Deiopea-D1135-20190311T143432Z")] <- 2
text(probas,maha,xlab="Probability",ylab="Mahalanobis D^2", names(maha), cex=1, pos =
pos_vector,col="blue")


### PLOT EACH SPECIMEN RELATIVE TO TARGET
ref<-mshape(ctland)


pdf("MeshPlots.pdf")
par(mfrow=c(2,5))
myname = names(sh$ruler.pixel)
for (i in seq(10)){
        plotRefToTarget(ref,ctland[,,i])
        title(str_remove(myname[i],"border_"))
        }
```

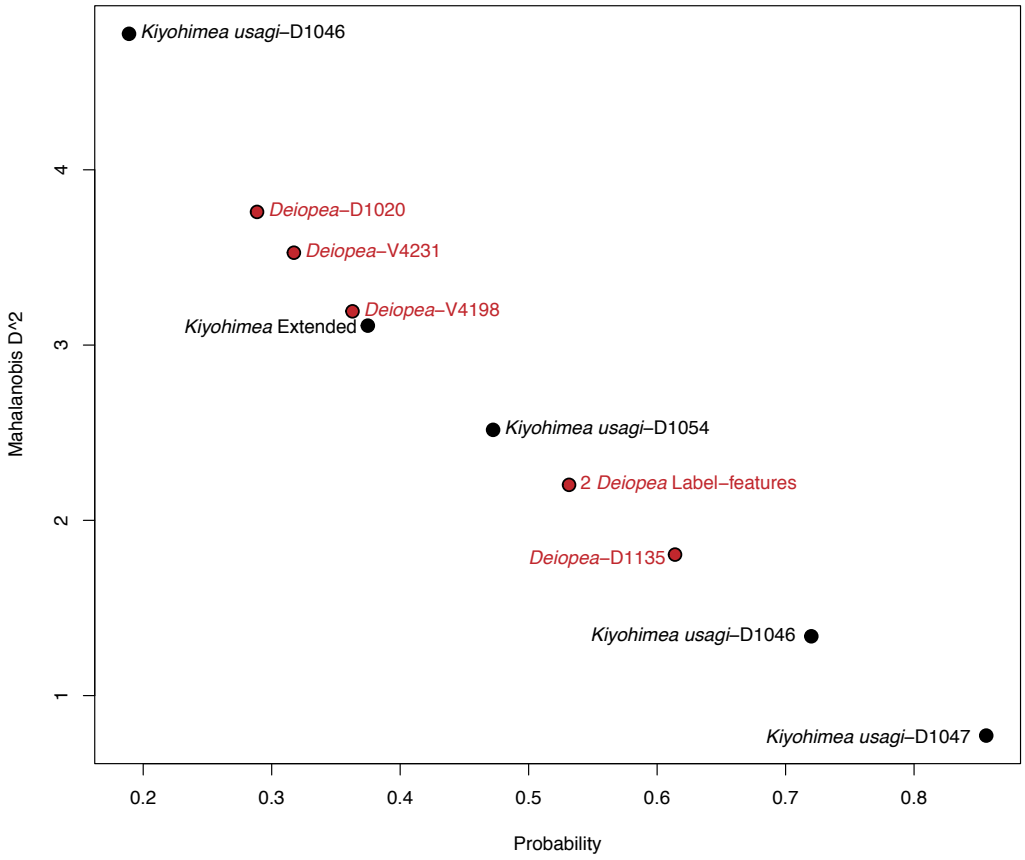
```
dev.off()# plotTangentSpace has been removed (gm.prcomp, set)
# gm.prcomp plot

PCA <- gm.prcomp(ctland)
pca2 <- prcomp(ctland)
summary(PCA)
plot(PCA, main = "PCA",labels=rownames(PCA$x))
```

Results:

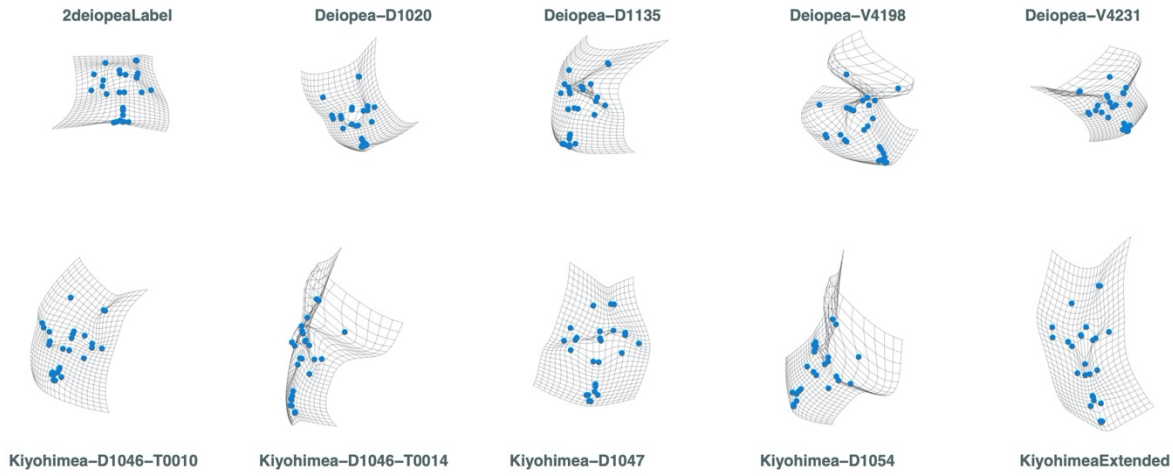
The fixed.angle function is demonstrated in the following two figures, with the first one being the original superimposition and the second having the fixed angle. The orange dot marked as the auricle landmark clearly moves positions, demonstrating the difference this can make in the analysis.



One outlier from the Malahanobis distance plot is an image where the ctenophore is at a different angle from the rest of the other specimens (the top left point labeled D1046). This may impact the shape analysis comparing the two species and identifying them, and so more images from different angles may be necessary in future datasets to get a wholistic view of what the entire organism looks like. During dives, it may be difficult to capture an image of a specimen from the same angle every time, and so it is important that the Geomorph analysis can still identify specimens from multiple angles.



The warp mesh plots demonstrated how different each shape of the ctenophore differed from the mean of the landmarks from each image. The outlier described has twisted in the angle of the ctenophore in the image, and Deiopea-V4198 has twisted completely in the middle. Although it not clear as to why this occurred, a potential reason could be that the landmarks may have been switched in StereoMorph. Further analysis of this plot is required to assess the differences in shape between *Deiopea* and *Kiyohimea*.



Discussion:

Much of this project focused on developing the methods for the Geomorph and StereoMorph analysis. Online Geomorph and StereoMorph tutorials were used as a baseline for our procedure. It is important to note that many of these tutorials rely on earlier versions of Geomorph, and so certain functions, such as plottangentspace, are no longer used in the current version. Geomorph is also capable of analyzing semilandmarks that add a three-dimensional function to the datasets and also allows for a more detailed analysis of shapes. These semilandmarks need to be defined and "slid" using the sliders function in Geomorph. Although semilandmarks have not been used in this analysis, it is something we plan to implement.

These preliminary results, especially the mesh warp plot, can inform us of key differences that may be causing each image to be different from another. Investigating these plots may help identify what variables to focus on in future analyses.

Future directions:

We would like to strengthen our dataset by adding more images of both *Deiopea* and *Kiyohimea*, which can make the process of identifying them more accurate. Since we currently cannot incorporate comb row count, spacing, or width into the Geomorph analysis, we plan to either analyze these separately and then combine it with the Geomorph data or develop a method to analyze them both using the R package Morpho.

Once we have developed quantitative rules to accurately identify *Deiopea* and *Kiyohimea*, we plan to use this method to identify other pairs of ctenophore species with similar morphologies. For example, the *Lampocteis cruentiventer* looks very similar to unidentified lobate ctenophore species, and so differentiating these groups would be useful.

References

Adams, D.C., E. Otarola-Castillo and E. Sherratt. 2014 geomorph: Software for geometric morphometric analyses. R package version 3.3.1. http://cran.r-project.org/web/packages/geomorph/index.html.

Adams, D.C., and E. Otarola-Castillo. 2013. geomorph: an R package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution. 4:393-399.

Haddock, Steven. (2007). Comparative feeding behavior of planktonic ctenophores. Integrative and comparative biology. 47. 847-53. 10.1093/icb/icm088.

Matsumoto, George & Robison, B.H.. (1992). Kiyohimea Usagi, a New Species of Lobate Ctenophore from the Monterey Submarine Canyon. Bulletin of Marine Science. 51. 19-29.

Theska, T., Sieriebriennikov, B., Wighard, S.S. et al. Geometric morphometrics of microscopic animals as exemplified by model nematodes. Nat Protoc 15, 2611–2644 (2020). https://doi.org/10.1038/s41596-020-0347-z

Zelditch, Miriam & Swiderski, Donald. (2018). A Practical Companion to Geometric Morphometrics for Biologists: Running analyses in freely-available software (Second Edition).