Angel Flowers
Major in Biology
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Population Distribution Analysis of Tardigrades found on *Quercus virginiana* (Southern Live Oak)

Introduction

Tardigrades are bilateral, eight legged lobopods of the Panarthropoda clade best known for their ability to survive extreme environmental conditions, and even space, while in a cryptobiotic state (Kinchen, 1994; Jönsson et al., 2008; Nelson et al., 2010). These aquatic microinvertebrates can be found in every biome on earth living in the water trapped in the interstitial spaces of moss, lichen, detritus, and sediment of various environments (Nelson et al., 2018). Despite this, ecological studies are scarce, and the phylum remains relatively understudied (Nelson et al., 2018). Tardigrades, like other microorganisms, are indicators of the health of the larger ecosystem. Understanding their inner workings and role they play in the big picture is extremely important for future conservation efforts. As of July 2021, only 1,380 species have been described by science (Degma et al., 2021). Previous studies have focused on the identification of species on specific substrates and habitats, but there is little evidence to explain why or how these species exist within their preferred microhabitats. Tardigrades require only a thin film of water to be active, and those found in terrestrial environments generally survive in moist microhabitats of moss and lichens (Møbjerg et al., 2018). Formerly, tardigrades found in moss samples were collected within reach at ground level, however, studies of the canopy in the late 1960’s revealed their presence well above ground level (Chang et al., 2015; Kimmel and Meglitsch, 1969; Erwin, 1982; Counts et al, 2001). Studies of tardigrade distribution patterns in trees have identified
substrate (tree) selection, with some trees demonstrating a denser, more diverse population at the top of the tree (Miller et al., 2013; Chang et al., 2015; Mitchell et al., 2009).

*Quercus virginiana*, the Southern Live Oak, is easily identifiable by a massive, short trunk, buttressed base, and long sprawling branches (National Wildlife Federation, 2008). The trees offer collection points for tardigrades transmitted on the winds as they blow past, as well as for tardigrades hitching rides on the feathers and feet of birds (Nelson et al., 2010; Ramazzotti & Maucci, 1983; Mogle et al., 2018). To date there is no publication on tardigrade population distributions in the *Q. virginiana* on the Gulf coast, USA. This research contributes to filling this knowledge gap by defining the distribution, density and diversity of tardigrade population of epiphytic moss on five tree features, roots, trunk, crotches, and limbs. The study site is a small 27-acre stand of *Q. virginiana* managed by the Texas Ornithological Society known as Sabine Woods Bird Sanctuary and located a few miles west of Sabine Pass, Texas, on the Gulf of Mexico. Its position on a chenier ridge dominated by coastal live oak trees surrounded by saltmarsh makes it a critical stopover for annual avian migrations including neotropical species (Port Arthur Convention and Visitors Bureau, 2022; Beaumont Convention and Visitors Bureau, 2022). This location was partly chosen because of the potential for migratory birds from North and Central America to deposit various species of tardigrades from afar during their stopovers. It was also chosen for the fact that it is barraged with warm, moist, Southern winds for much of the year, again believed to increase the chances for finding various tardigrade species.

**Materials and Methods**

Five of the oldest, largest live oak trees were chosen for sampling in Sabine Woods Bird Sanctuary. A total of 12 moss samples were collected around each tree from each of four features: limbs, crotches, trunk, and roots. Sample was collected by scraping the moss off the tree bark with a knife into a labeled paper bag (Tibbs et al., 2016; Kim-Koutsis and Miller, 2019; Villella et al., 2020). Distance from the ground and distance from the base of the tree were recorded for each sample, as well as directionality of the sample (N, S, E, W). The paper bags were stored in the field lab with a dehumidifier and moss samples were allowed to dry over the weekend. Once dry, one gram of moss was weighed and placed into a labeled 2oz cup with a lid (Tibbs et al., 2016; Kim-Koutsis and Miller, 2019; Villella et al., 2020). Samples were prepared for sorting by adding 20 mL of DI
water and soaking for 24 hours (Tibbs et al., 2016; Kim-Koutsis and Miller, 2019; Villella et al., 2020).

From each sample cup, a 1 mL disposable pipet was used to place 1 mL of water and debris drawn from the bottom of the cup into each of three small petri dishes (Tibbs et al., 2016; Kim-Koutsis and Miller, 2019; Villella et al., 2020). Another 10 mL of water from each sample was placed into a labeled tube for testing pH and conductivity. 5 uL of Bioquip double stain was added to each 1 mL subsample. This would stain the tardigrades and their eggs pink, making them much easier to find under the dissecting scope. Each 1 mL subsample was then searched thoroughly under 40x magnification, and any tardigrades or eggs found were extracted with an Irwin loop (Schram and Davison, 2012) and mounted in PVA mounting medium on a glass slide labeled with the sample number and either A, B, or C to indicate the subsample (Tibbs et al., 2016; Kim-Koutsis and Miller, 2019; Villella et al., 2020). A cover slip was applied, and after a few days of allowing the mounting medium to dry, slides were sealed with clear nail polish to preserve the specimen morphology (Tibbs et al., 2016; Kim-Koutsis and Miller, 2019; Villella et al., 2020). Mounted tardigrades were viewed with an inverted microscope equipped with phase contrast and differential interference contrast optics at 40x and 200x and 1000x with oil immersion. Images were capture digitally for claws and buccal-apparatus to identify and document morphological variations. Using the characteristics of the cuticle, claws, and buccal-pharyngeal apparatus, identification of species was made using the key by Ramazzotti and Maucci (1983) and Pilato & Binda (2010). Molecular analysis of 18SrRNA and COI gene sequences were performed on the morphological variations (see Broussard et al. poster at this EXPO 2022) in conjunction with visual identification to confirm identification (Bertolani et al., 2010; Nelson et al., 2010; Boothby et al., 2018). The tardigrade counts from the three 1 mL pipetted aliquots of each sample were combined for relative density of the sample, and the number of species per sample is referred to as the sample richness (Kim-Koutsis and Miller, 2019; Villella et al., 2020).
Results

Out of 60 moss samples, 321 tardigrades and six eggs were found. All total there were six species belonging to four genera of the tardigrade super-family Macrobiotidae: *Macrobiotus echinogenitus*, *Macrobiotus evelinae*, *Mesobiotus harmsworthi*, *Minibiotus intermedius*, *Paramacrobiotus areolates*, and *Paramacrobiotus tonollii*. Collectively the identifiable tardigrade specimens were dominated by *Mesobiotus harmsworthi* (55%) followed by *Minibiotus intermedius* (36%; Fig. 1). Single-factor ANOVA demonstrated that four tree features drives significant variation in both relative density (p = 0.00849) and species richness (p = 0.03186). A two-factor ANOVA tests the importance of individual trees, features, and their interaction on each parameter, relative diversity and species richness. Neither trees, or their interaction with features explained significant variation for either relative density (p > 0.05) or species richness (p > 0.05). Sample relative density positively correlated with species richness (p < 0.01; R² = 0.45; Fig. 2A). Mean relative density (± 95% CI) was not significantly different among most feature type, except the branches had significantly greater mean density than in the roots (Fig. 2B). Mean species richness (± 95% CI) in branches was significantly greater than in the crotches, but there was no significant difference between the other tree features (Fig. 2B).
Figure 2. Relative density versus species richness of all sample from the four features (A) and means (± 95% CI error bars) of relative density and species richness for each feature type.
Figure 3.  *M. evelinae* images from this study (A-D): 100x oil immersion of buccal-pharyngeal apparatus (A); 100x oil immersion of claws (B); 20x image of *M. evelinae* body (C); and 100x oil immersion of eggs of similar type of *M. evelinae* (D). Description of *M. evelinae* from Ramazzotti and Maucci, 1983 (E).
**Discussion**

Distribution analysis of the tardigrades found on the southern live oak reveal that their presence greatly increased in epiphytic moss of the limbs or branches, which have greater height from the ground and distance from the tree center. Branches were more heavily populated and there was a greater species richness moving from the tree center outward. Most interesting of note was the discovery of *Macrobiotus evelinae* (Fig. 3A). This tardigrade was last described by de Barros in 1938 in Brazil (Fig. 3B; Ramazzotti and Maucci, 1983). It was listed in the 2021 edition of *Actual Checklist of Tardigrada Species* (Degma et al., 2021) as *nomen inquendrum* by Stec et al. (2021). This discovery of *M. evelinae* is the first in North America (Miller and Perry, 2019).

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**Bibliography**


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