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Michael E. Wheeler

FWC-FWRI, mike.wheeler@myfwc.com

Bradley T. Furman

FWC-FWRI, brad.furman@myfwc.com

Margaret O. Hall

FWC-FWRI, penny.hall@myfwc.com

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SHORT COMMUNICATION

LEAF—TIP MORPHOLOGY DOES NOT SUPPORT SPECIES STATUS FOR THE SEAGRASS, *HALODULE BEAUDETTEI* IN FLORIDA, USA

Michael E. Wheeler, Bradley T. Furman*, and Margaret O. Hall

Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, 100 Eighth Avenue, Southeast, St. Petersburg, FL 33701, USA; *Corresponding author, email: brad.furman@myfwc.com

KEY WORDS: *Halodule wrightii*, morphospecies, phenotypic plasticity, tridentate, bicuspidate

INTRODUCTION

Seagrasses in the genus *Halodule* are pioneering species that inhabit shallow tropical and subtropical waters around the world (Zieman 1982, Short et al. 2007). Originally comprised of two species (*H. wrightii* and *H. uninervis*) based largely on geographic distribution (i.e., Atlantic vs. Pacific, respectively; Sauvageau 1890, Ascherson and Graebner 1907, den Hartog 1964), *Halodule* has grown to include 7 recognized species (den Hartog 1964, 1970) with *H. wrightii* now considered one of the rare pantropical seagrasses (Green and Short 2003). In the Gulf of Mexico (GOM), 2 species have been reported: *H. wrightii* and *H. beaudettei* (den Hartog and Kuo 2006).

Mirroring taxonomic confusion elsewhere in the genus (Ito and Tanaka 2011, Wagey and Calumpang 2013), species status and distribution in the GOM has long been the subject of debate due to phenotypic variation in leaf morphology and the apparent scarcity of reproductive structures, generally the preferred diagnostic for differentiating morphospecies in angiosperms (den Hartog 1970). Unfortunately, observations of *H. wrightii* or *H. beaudettei* fruits, flowers, and seeds remain rare and little is known of their respective reproductive ecologies. At present, it is unclear whether diminutive short-lived floral structures, geocarpic fruit development, or paradigmatic focus on asexual reproduction have limited floral observations or whether sexual reproductive effort is indeed absent in most places (den Hartog 1964, Phillips and Meñez 1988).

Without detailed descriptions of floral morphology, species status here and elsewhere in the genus has been defined by vegetative characters: mature leaf—tip shape, leaf width, and the presence of midvein lacunae (Sauvageau 1890, Phillips 1967). Using these characters, along with occasional references to leaf width, den Hartog (1964) proposed 7 species of *Halodule*: *H. beaudettei*, *H. wrightii*, *H. bermudensis*, *H. uninervis*, *H. tridentata*, *H. ciliata*, and *H. pinifolia*, and later added 2 additional candidates from Brazil: *H. emarginata* and *H. lilianeae* (den Hartog 1972). Of particular relevance to *H. wrightii* and *H. beaudettei* is the mature leaf—tip shape, which has been relied upon to identify individuals in the field (Magalhães and Barros 2017). In these species, leaf tips vary due to extension of lateral vascular bundles and/or the midvein resulting in 1–3 angular protrusions or ‘teeth’ in bicuspidate (lateral teeth only; *H. wrightii*) or

tridentate (2 laterals, one larger median tooth; *H. beaudettei*) arrangement, each with variation in tooth length, margin serration, and pointedness (den Hartog 1964).

Many of the morphospecies in *Halodule* have gained acceptance by seagrass ecologists and practitioners; however, recent molecular work questions their validity (Ito and Tanaka 2011; but see Wagey and Calumpang 2013). Earlier rebuttals to taxonomic splitting argued that variation in leaf morphology resulted from environmentally driven phenotypic plasticity and that one species, *H. wrightii*, had taxonomic priority in the GOM (Phillips and Meñez 1988). Precedence for the name *wrightii* derives from Ascherson’s original description of the then pan-Atlantic species, *Diplanthera wrightii*, from 2 Cuban type—specimens (Ascherson and Graebner 1907). Later, after correction of *Diplanthera* to *Halodule*, den Hartog (1964) split the species into *H. wrightii* and *H. beaudettei* based on leaf morphology, placed *H. wrightii* after *H. beaudettei* in evolutionary sequence, and all but excluded *H. wrightii* from the GOM. Phillips rejected this view and asserted that both species should be re-merged under *H. wrightii* (Phillips 1967, Phillips et al. 1974).

To test the hypothesis that differences in leaf—tip morphology were a product of phenotypic plasticity, Phillips (1967) collected *Halodule* spp. from 3 tidal zones in Point Pinellas, Florida, USA. He concluded that variation was sufficient, using the morphological descriptions provided by den Hartog (1964), to identify both *H. wrightii* and *H. beaudettei* within connected shoots (i.e., within a single genet; Phillips, 1967). These results call into question leaf—tip morphology as a species—specific characteristic for *Halodule*, and although *H. wrightii* has proven the more common identification by investigators working in Florida and much of the GOM, debate remains regarding the existence and distribution of *H. beaudettei* in the region.

One reason for continued ambiguity might be that the Phillips—den Hartog dichotomy was argued without quantitative methods, relying on careful albeit subjective interpretations of public herbaria and published drawings (den Hartog 1964). To apply a quantitative approach, we re-sampled the site visited by Phillips (1967) and used digital image analysis and decision tree learning to probe natural variation in leaf morphology

within the *Halodule* genus in Florida, USA. Specifically, we asked: (1) can the morphospecies descriptions used to distinguish *H. wrightii* from *H. beaudettei* be validated using literature line drawings and photographs, and (2) does significant variation exist that would lead to identification of both *H. wrightii* and *H. beaudettei* within a genet? Finally, we summarize our results in light of recent molecular work.

MATERIALS AND METHODS

We used Web of Science searches to obtain digitized line drawings and published photographs of *Halodule* leaf tips from the literature. We chose to focus on *H. beaudettei* and *H. wrightii*, as these were the 2 species documented in the GOM and debated in the Phillips–den Hartog exchange.

In June 2019, we re-visited the Phillips (1967) study site (27.703455°N, 82.640867°W) in Point Pinellas, FL to collect *Halodule* rhizome sections from a shore–perpendicular transect through the fringing *Halodule* bed (35 m; see Phillips 1967 for site description). Three genets, defined as rhizome–connected shoots, were collected every 5 m along the transect. Genets were carefully excavated using a submersible hand pump (see Figure S1) to remove surficial sediments. Undamaged tips of each leaf on each ramet along every genet were organized by ramet and leaf rank, placed under a dissecting microscope (Leica/Wild M3Z stereo microscope with a Leica IC80 HD camera) at 10–16x magnification, and photographed using Leica LAS EZ imaging software. Ramets were rank–ordered from the apical meristem, if present, and leaves from youngest to oldest. All images were saved using JPEG compression at a size of 96 x 96 pixels.

To quantify leaf–tip characteristics, we developed a set of 15 binary questions regarding the presence, size and shapes of the median and lateral teeth, the degree of midrib projection, and the presence of tooth serration (Table S1). These questions were specifically developed to differentiate between *H. wrightii* and *H. beaudettei* based on literature descriptions of leaf–tip morphology. The question set was then applied to: (1) literature line drawings and photographs of the species in question, (2) the Phillips (1967) images and (3) our field–collected samples, providing 3 binary datasets (hereafter, ‘literature’, ‘Phillips’ and ‘Point Pinellas’), each comprised of 15 descriptive variables.

To test how well published accounts of leaf–tip morphology resolved 2 distinct morphospecies, *H. wrightii* and *H. beaudettei*, we attempted a classification and regression tree (CART) model of the literature dataset using the package ‘rpart’ (Therneau and Atkinson 2019) in the statistical computing software R (R Core Team 2019). The saturated model was assessed for accuracy using a confusion matrix. Based on empirical descriptions, we reasoned that plants included in the literature dataset would cluster by species in multivariate space and that the Phillips dataset would span both species clusters. ANOSIM was used to test for significant differences between these 3 groups of samples: the 2 literature–defined species within the literature dataset and the Phillips dataset treated as unknowns. Multivariate patterns were visualized with non–metric multi–

dimensional scaling (nMDS) ordination of Euclidean distance matrices, implemented by the ‘vegan’ (Oksanen et al. 2019) package in R.

The CART model was then used to predict species identity for each leaf in our 2019 Point Pinellas dataset, providing empirical estimates of species identity based solely on leaf–tip shape. Patterns in species identity by distance from shore, genet, and ramet were assessed using ANOSIM and nMDS ordination.

RESULTS AND DISCUSSION

A total of 194 line–drawings and photographs of 7 *Halodule* species were obtained from 9 publications (Table S2). Publications spanned 127 years (i.e., 1890 to 2017) with 9 lead authors, including Phillips and den Hartog. For subsequent analyses, the 22 images from Phillips (1967), presented entirely as *H. wrightii* by the author, were treated as unknowns. We restricted the use of literature line–drawings in our analysis to only those documented as *H. wrightii* (56 images) or *H. beaudettei* (15 images).

The CART model of species identity based on the literature dataset accurately distinguished *H. wrightii* from *H. beaudettei* in all but one case (98.5% or 70 of 71 images). Of the 15 questions, the most influential to the model were: (1) are there 3 teeth? (2) is the median tooth longer than both lateral teeth? (3) does the midrib project beyond the leaf tip? and (4) does the midrib widen or split at the tip? The majority of *H. wrightii* predictions (49 out of 56 images) were identified based on the first question alone, which was unsurprising, given that the main diagnostic character in the literature has been the bidentate or tridentate form of the leaf tip. Ultimately, the CART model resulted in 9 branches with most terminal groupings smaller than 5 images. Importantly, the model was still able to discriminate *H. beaudettei* from *H. wrightii* not differentiated by the first question, indicating that authors were identifying leaf–tip shapes based on a variety of primary and secondary characteristics. The single incorrect prediction was a *H. beaudettei* image from den Hartog’s 1970 publication that was seemingly identical to an image of *H. wrightii* from Eiseman’s 1980 publication. Both leaves had 3 distinct teeth with the lateral teeth being similar in height to the median tooth, as well as midrib projections that widened or split at the leaf apex. Our CART model results provide clear evidence that species definitions based on leaf–tip morphology are in fact distinct in the literature, and have remained so despite many decades, locations, and investigators. It also suggests that the empirical relationships identified by the model can be used to assign species identity to line drawings and digital images of unknown samples, such as the Phillips (1967) drawings and our own 2019 images.

Consistent with the findings of Phillips (1967), nMDS ordination of the combined literature and Phillips datasets showed 2 distinct clusters corresponding to either *H. wrightii* or *H. beaudettei* from the literature dataset, with the Phillips samples clearly interspersed among them (Figure 1). ANOSIM revealed significant global differences ($R = 0.348$, $p = 0.03$) with *H. wrightii* different from *H. beaudettei* (pairwise $R = 0.563$), but

Figure 1. Non-metric multi-dimensional scaling (nMDS) ordination of literature-derived leaf-tip line drawings and images identified as *Halodule wrightii* or *Halodule beaudettei*, along with leaf-tip drawings reported as unknowns in Phillips (1967).

neither different from Phillips (pairwise $R = 0.252$ and $R = 0.107$, respectively). This would at first seem contradictory, lending support to both the morphospecies concept of den Hartog and the degree of plasticity reported by Phillips. However, for the leaf-tip definitions to represent true species, they must be reasonably stable within ramets and, at a minimum, consistent within genets.

Our Point Pinellas data allowed for an explicit test of this by examining intra- and inter-genet patterns in predicted species identity. We collected 25 *Halodule* genets each containing between 3–12 ramets (137 ramets total) with 1–4 leaves for a

total of 225 leaf-tip images (Figure 2). No fruits or flowers were found during the survey. Application of the CART model to these unknowns yielded predictions of both *H. wrightii* and *H. beaudettei* at every station along the depth gradient, among leaves within single ramets and, importantly, within genets. We found evidence for both species in 100% of genets tested, and in 48% of all ramets (shoots) that had more than one leaf.

den Hartog (1964) and Phillips (1960, 1967) acknowledged the role that leaf age might play in the diagnostic value of leaf shape, both favoring mature leaves for analysis. Phillips (1967) reported that most immature leaves were tridentate, which would have most likely resulted in a prediction of *H. beaudettei* by our model. Analysis of our field data, however, could not corroborate this pattern. We found that 52% of all shoots with more than one leaf ($n = 85$) were all single species (27 *H. wrightii*, 17 *H. beaudettei*, or 44 out of 85). Of the remaining 41 mixed species shoots (i.e., a shoot for which both species were predicted), 39 switched shape once during shoot development (12 from *H. beaudettei* to *H. wrightii* and 27 from *H. wrightii* to *H. beaudettei*). Of the 2 remaining mixed-species shoots, both switched shape twice during shoot development, one beginning as *H. wrightii* and the other as *H. beaudettei*. Thus, no consistent developmental shift in leaf shape or corresponding species prediction was seen. Among first- and second-rank leaves within mixed-species ramets ($n = 65$ leaves), 60% were *H. wrightii*, while for third- and fourth-rank leaves ($n = 20$), this fell to 20% *H. wrightii*. Although the bicuspidate-tridentate pattern within selected shoots might indicate leaf development, a similar pattern was also seen at the shoot level among single-species ramets, where first- and second-rank ramets were also predominantly *H. wrightii* (73%). Mixed-species first- and second-rank ramets were nearly evenly split (49% *H. wrightii* and 51% *H. beaudettei*). Again, no consistent relationship between leaf-tip shape – here interpreted through model-predicted species – and leaf age could be found.

None of the 25 genets we collected from Point Pinellas yielded leaves that were all predicted to be the same species. Considering only mature leaves (third and fourth-rank leaves, $n = 20$ genets), the majority of genets still had predictions of both species (11 of 20). In multivariate space, 7 of 25 genets spanned literature-defined *H. wrightii* and *H. beaudettei* clusters (Figure 3). Because mixed-species genets were not isolated to the boundaries of species-specific clusters, we can disregard the notion that they are merely artifacts of model performance. Rather, leaves characteristic of both *H. wrightii* and *H. beaudettei*, predicted using stable literature-derived empirical relationships among leaf-tip attributes, were common within individual shoots and within single genets. Our modeling results therefore provide strong evidence against the use of leaf-tip

Figure 2. Examples of *Halodule* leaf tips collected from Point Pinellas in St. Petersburg, FL. Panels (A–D) display all analyzed leaves from a respective shoot or ramet. The model-predicted species abbreviations (Hw = *H. wrightii* and Hb = *H. beaudettei*) are shown in white text. The far-right leaf in panel D was not used in the analysis due to a broken lateral tooth.

FIGURE 3. Non-metric multi-dimensional scaling (nMDS) ordination of the 2019 Point Pinellas, FL field data. Black line connects leaves from a single genet. Labels denote the ramet from which each leaf was taken, numbered from the apical. Species were identified based upon the CART model.

morphology as a sole indicator of species status in *Halodule*.

Recent molecular studies on the phylogeny of *Halodule* have begun to test the current taxonomy; however, their findings, based on *rbcL* and *phyB* sequences, have been inconsistent with regard to *H. wrightii* and *H. beaudettei* (Ito and Tanaka 2011, Wagey and Calumpong, 2013). The reasons for continued ambiguity are unclear, but because a genus-wide phylogeny has not yet been attempted, our understanding has undoubtedly been limited by insufficient sampling intensity or extent, and a reliance on the few published sequences currently available for analysis. More targeted work will be needed to assess whether inferred phylogenetic history and present genetic variation warrant the rank of species for *H. wrightii* and *H. beaudettei*.

Early in the study of the genus, Ostenfeld (den Hartog 1964), Bernatowicz (1952) and Phillips (1967) all argued against the use of leaf-tip shape for identifying and defining *Halodule* species. Leaf width and presence of midvein lacunae have also been refuted as indicators of species status (Phillips 1960, den Hartog 1970, Phillips and Meñez 1988). Nevertheless, the modern taxonomy, built largely by den Hartog, has been successful in the literature, efficient for the purposes of species identification, and relatively unambiguous at the site and regional scales. Perhaps owing to the local nature of most seagrass studies, the ever-present conflict between lumpers and splitters has remained muted. Yet, even den Hartog (1964) acknowledged the problem of plasticity, questioning the role of sexual dimorphism in the morphological patterns he documented. Now, with renewed interest in *Halodule* reproductive ecology (Darnell et al. 2020) and population genetics (Reynolds et al. 2019), there is a clear need to better understand its systematics and taxonomy (Magalhães and Barros 2017).

Based on our quantitative assessment of leaf plasticity in *Halodule* spp. specimens from the Florida Gulf coast, several conclusions can be drawn. First, literature line drawings and photographs support the morphological descriptions of den Hartog. His morphospecies did indeed form distinct groups that were consistent among published reports. Second, Phillips did observe enough leaf plasticity to warrant identification as both *H. wrightii* and *H. beaudettei*. Third, from our own 2019 field samples collected from the same location as Phillips (1967), we observed substantial variation at both the ramet and genet levels. It is elemental that a properly defined morphospecies be conserved within genets. This has been qualitatively (Phillips 1967) and quantitatively (present study) invalidated for *H. beaudettei* from *H. wrightii* in the GOM. It is clear that the use of leaf-tip morphology to resolve these 2 species is insufficient and requires revision. We suggest tentatively consolidating *H. wrightii*, followed by further work to reassess species status within the genus. It is time for the taxonomy of *Halodule* to move from a morphospecies to a phylogenetic species concept.

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