Genetic identification and hybridization in the seagrass genus *Halophila* (Hydrocharitaceae) in Sri Lankan waters

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Seagrasses, as marine angiosperms, play important roles in coastal ecosystems. With increasing anthropogenic impacts, they are facing dramatic declines on a global scale. *Halophila* is well-known as a complex taxonomic challenge mainly due to high morphological plasticity. By using only a morphological approach, the genus could be over-split or similar species could be erroneously lumped, thus masking its true biodiversity. In the present study, we incorporated genetic identification with morphological examination to reveal the identity of *Halophila* plants in southern and northwestern Sri Lankan waters. The nuclear ribosomal internal transcribed spacer (ITS) region and chloroplast ribulose-bisphosphate carboxylase gene (rbcL) were used to identify plants collected from the Gulf of Mannar, Puttalam Lagoon, and Matara, Sri Lanka. Based on genetic identification, *H. major* (Zoll.) Miquel is reported for the first time from Sri Lanka, which might have been misidentified as *H. ovalis* in previous literature based on morphology alone. We also observed a first hybridization case of *Halophila* cross between *H. ovalis* and *H. major*. Two potential cryptic species were found, herein designated *Halophila* sp. 1 (allied to H. minor) and *Halophila* sp. 2 (closely related to *H. decipiens*). In order to clarify taxonomic ambiguity caused by morphological plasticity and the low resolution of genetic markers, further comparative phylogenomic approaches might be needed to solve species boundary issues in this genus.

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Abstract

Seagrasses, as marine angiosperms, play important roles in coastal ecosystems. With increasing anthropogenic impacts, they are facing dramatic declines on a global scale. *Halophila* is well-known as a complex taxonomic challenge mainly due to high morphological plasticity. By using only a morphological approach, the genus could be over-split or similar species could be erroneously lumped, thus masking its true biodiversity. In the present study, we incorporated genetic identification with morphological examination to reveal the identity of *Halophila* plants in southern and northwestern Sri Lankan waters. The nuclear ribosomal internal transcribed spacer (ITS) region and chloroplast ribulose-bisphosphate carboxylase gene (rbcL) were used to identify plants collected from the Gulf of Mannar, Puttalam Lagoon, and Matara, Sri Lanka. Based on genetic identification, *H. major* (Zoll.) Miquel is reported for the first time from Sri Lanka, which might have been misidentified as *H. ovalis* in previous literature based on morphology alone. We also observed a first hybridization case of *Halophila* cross between *H. ovalis* and *H. major*. Two potential cryptic species were found, herein designated *Halophila* sp. 1 (allied to *H. minor*) and *Halophila* sp. 2 (closely related to *H. decipiens*). In order to clarify taxonomic ambiguity caused by morphological plasticity and the low resolution of genetic markers, further comparative phylogenomic approaches might be needed to solve species boundary issues in this genus.

Introduction

Seagrasses, a functional group of marine flowering plants found in coastal areas of the world's oceans, provide essential habitat for many coastal species and support marine food webs, playing critical roles in the balance of coastal ecosystems and human livelihoods (*Mtwana Nordlund et al. 2016*). It has been shown that seagrass habitat declined worldwide at a rate of 110 km^2 per year between 1980 and 2006 (*Waycott et al., 2009*). Short et al. (2011) suggested 72 seagrass species needed to be listed in the Red List of International Union for the Conservation of Nature (IUCN) based on global population status. Therefore, there is an urgent need to conduct baseline studies (*i.e.*, diversity, abundance, and distribution) for establishing conservation plans in the future. However, identification based on morphological traits in the genus *Halophila* is considered to be very challenging since few morphological differences or characteristics exist among closely related species (*Kuo et al. 2006*). Field ecologists without taxonomic knowledge of this genus may either overestimate or underestimate its true biodiversity (*Shimada et al., 2012; Tuntiprapas*

61 *et al., 2015; Kurniawan et al., 2020*).

62 The genus *Halophila* comprises approximately 20 species within five sections based on

63 morphological differences (*Den Hartog & Kuo 2006; Kuo et al. 2006*). Most species in the genus

64 are in section *Halophila*, which contains species with a pair of petiolate leaves borne on short,

65 erect lateral shoots (*Den Hartog & Kuo 2006; Kuo et al. 2006*). All other species are in sections

66 *Microhalophila* (*H. beccarii*), *Spinulosae* (*H. spinulosa*), *Tricostata* (*H. tricostata*), and

67 *Americanae* (*H. engelmannii and H. baillonis*). However, molecular genetic studies propose that

68 *H. hawaiiana* and *H. johnsonii* should be treated as conspecific with *H. ovalis* (*Short et al. 2010*).

69 The ITS (internal transcribed spacer) sequence is proven to have great resolution for acting as a

70 genetic barcode for the genus *Halophila* (*Kim et al. 2017*). Kim et al. (2017) showed that five

71 major clades can be identified in section *Halophila,* which has relatively simple phyllotaxy

72 compared to other sections. However, five morphologically similar species cannot be

73 distinguished in the *Halophila ovalis* complex with ITS: *H. ovalis*, *H. minor*, *H. hawaiiana*, *H.*

74 *johnsonii,* and *H. ovata*. On the other hand, that section's ITS region is capable of identifying *H.*

75 *decipiens*, *H. major*, *H. nipponica*, *H. okinawensis*, *H. guidichudii,* and *H. stipulacea*. On the

76 other hand, the rbcL (ribulose-bisphosphate carboxylase) gene of the chloroplast is suggested as

77 a potential barcode region for land plants since it can discriminate among species in

78 approximately 85% of congeneric pair-wise comparisons (*Newmaster et al. 2006*). For

79 seagrasses, the combined use of rbcL and matK (maturase K) genes is recommended by the

80 Consortium for the Barcoding of Life (CBOL). However, neither the resolution of rbcL alone or

81 the combination of rbcL and matK can well resolve the phylogenetic relationship of closely

82 related species within the genus *Halophila* (Nguyen *et al. 2015*). Since *Halophila* species have

83 notoriously great morphological plasticity, ITS and rbcL resolution incorporated with detailed

84 morphological examination should provide valuable insight on the biodiversity of the genus in

85 Sri Lanka.

86 Fourteen species belonging to six genera (60% of Indo-Pacific bioregion seagrasses) have been

87 recorded in Sri Lanka: *Enhalus acoroides*, *H. beccarii*, *H. decipiens*, *H. ovalis*, *H. ovata*, *H.*

88 *minor*, *H. stipulacea*, *Thalassia hemprichii*, *Cymodocea rotundata*, *C. serrulata*, *Halodule*

89 *uninervis*, *H. pinifolia*, *Ruppia maritima,* and *Syringodium isoetifolium* (*Udagedara et al. 2017*).

90 Among these, *H. ovalis*, *H. ovata*, and *H. minor* belong to the *H. ovalis* complex, which is very

91 difficult to differentiate by morphological traits. Udagedare et al. (2017) have also mentioned

92   that the distribution records of seagrasses in the Sri Lankan coast are extremely limited due to

93   three decades of civil conflict ending in 2009 and a concurrent severe decline in seagrasses. A

94   recent study showed that species composition changed and biodiversity decreased from 1991 to

95   2013 at Puttalam Lagoon in association with human activities (Ranahewa et al. 2018). Therefore,

96   there is an urgent need to understand the distribution and diversity of seagrasses in Sri Lankan

97   waters before local extinctions occur.

98   In the present study, we incorporate morphological and genetic analyses on plants belonging to

99   the genus *Halophila* collected from Sri Lankan waters that are difficult to identify by using either

100   approach alone. With this integration, we attempt to reveal potentially overlooked biodiversity

101   and species distribution.

102

Materials & Methods

104   **Sampling and phylogenetic analyses**

105   *Halophila* samples were collected by either snorkeling or sampling from the shoreline in early

106   December 2018 under the permission No. NIC:196931003193 from three sites at depths from 0.5

107   m to 2 m based on occurrence data in previous literature and information from local fishermen:

108   the Gulf of Mannar (N = 32; GPS: 8.9753739N, 79.9224197E), Puttalam Lagoon, Kalpitiya (N =

109   32; GPS: 8.3671739N, 79.785565E), and Matara (N = 33; GPS: 5.9474017N, 80.6349298E)

110   (Figure 1). The former two locations are within a lagoon system having very low visibility (< 20

111   cm). *Halophila* plants collected from Puttalam were scattered below a huge meadow of

112   *Thalassia hemprichii* that occurred along the lagoonal side of a sandbar. We collected *Halophila*

113   plants only from the shoreline at Mannar, since the water is highly polluted by households.

114   Conversely, the site in Matara where we collected *Halophila* plants faces a coastal ocean area

115   dominated by a 10 x 50 m *H. major* meadow having > 5 m visibility. Leaves were preserved in

116   silica gel for further DNA extraction. Genomic DNA were extracted from leaves using a Plant

117   Genomic DNA Mini Kit (Geneaid Biotech, Taipei, Taiwan). The two markers used in the present

118   study were the rbcL gene from chloroplast DNA and nuclear ITS1-5.8S-ITS2. Primer pairs used

119   in this study were P609 5'- GTAAAATCAAGTCCACCRCG -3'and P610 5'-

120   ATGTCACCACAAACAGAGACTAAAGC -3' for rbcL (*Lucas et al. 2012*) and ITS5a 5'-

121   CCTTATCATTTAGAGGAAGGAG-3'and ITS4 5'-TCCTCCGCTTATTGATATGC-3' for

122   ITS1-5.8S-ITS2 (*Nguyen et al. 2014*).

123 Two loci were amplified in 25 μL reactions in a gradient thermocycler (Veriti 96-well thermal

124 cycler, Thermo Fisher Scientific) over an initial denaturation step at 95 °C for 3 min, 30 cycles

125 of denaturation at 94 °C for 30 s, annealing at 50 °C (ITS1-5.8S-ITS2) and 57 °C (rbcL) for 30 s,

126 extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Each reaction

127 contained 30 ng template DNA, 12.5 ul 2X master Mix RED (15 mM MgCl, 0.4 mM each

128 dNTPs, 200 nM of each primer, 0.2 unit of Ampliqon DNA polymerase (Ampliqon, Denmark)

129 and dd water was added to make a final volume of 25 ul. PCR products were sent to Genomics

130 (New Taipei City, Taiwan) for sequencing by an ABI 377 automated sequencer (Carlsbad CA,

131 U.S.A.).

132 Known ITS sequences and rbcL from other *Halophila* species were added to the dataset for

133 comparison (Table S.1). Sequences obtained in the present study were aligned with reference

134 sequences, including those within sections Spinulosae, Tricostata, Microhalophila, and

135 Americanae as outgroups by MEGA 7 (Tamura *et al., 2013*) to visually inspect all alignments as

136 well as search for the best nucleotide mutation model. Phylogenetic analyses were performed to

137 reveal genetic divergences among *Halophila* plants collected from different geographic locations,

138 with Bayesian inference assessments through Mr

139 Bayes (MB) version 3.2.2 (Ronquist *et al., 2012*) and maximum likelihood (ML) being

140 performed by CIPRES Science Gateway (Miller et al., 2015). The former implemented two

141 parallel runs of four simultaneous Markov chains for 10 million generations, sampling every

142 1000 generations and using default parameters. We discarded the first million generations (10%)

143 as burn-in, based on the stationarity of log-likelihood tree scores. ML analyses were conducted in

144 RAxML version 8.1.24 (*Stamatakis, 2014*) on CIPRES Science Gateway with default settings.

145 Supporting value on the branches were evaluated by non-parametric bootstrapping with the

146 automatically halt bootstrapping option by RAxML (ML).

147 **Molecular cloning**

148 Among the three sites, a majority of the plants (22/32) collected from Puttalam Lagoon,

149 Kalpitiya, failed to sequence on the ITS1-5.8S-ITS2 region due to multiple templates. We then

150 obtained pure ITS1-5.8S-ITS2 sequences by using molecular cloning.

151 ITS1-5.8S-ITS2 PCR products amplified from SB21, SB22, SB23, and SB 24 were ligated into

152 pJET1.2/blunt vector and cloned using a CloneJet PCR cloning kit (Thermo Scientific, U.S.A.).

153 In total, 17 positive clones were selected for further PCR reaction. The final cloned PCR

154   fragments were sequenced by Genomics (New Taipei City, Taiwan) using pJET1.2 forward and

155   reverse primers. All sequences derived from the present study were submitted to GenBank under

156   accession Nos. MT347850-MT347937 (ITS) and MT422621-MT422718 (rbcL).

157

158   **Morphological analyses**

159

160   One mature leaf was taken from 48 different plants in each category, comprising *H. major*, *H.* sp.

161   1 (allied to *H. minor*), *H. ovalis*, hybrids, potential hybrids, and *Halophila* sp. 2 (*H. decipiens*

162   like) (Table 1), for morphological measurements consisting of lamina width, lamina length,

163   distance from intramarginal vein to lamina margin, cross-vein angle, and number of cross-veins

164   (Figure S. 1). We calculated the ratio between intramarginal veins to the edge and the half-length

165   of the width, and the ratio between lamina width and length. Specimens were identified using the

166   keys of Den Hartog and Kuo (2007) and Kuo et al. (2006). Morphological data were transformed

167   (x-mean/standard deviation) and subjected to PCA to find out the variation among categories

168   using Past3 software (*Hammer et al.*, *2001*).

169

170   **Results**

171   **Phylogenetic analyses**

172   For the ITS1-5.8S-ITS2 region, only 59 of 97 plants were successfully sequenced, 38 failing to

173   achieve consensus sequencing due to the multiple template effect. We subsequently selected four

174   samples for molecular cloning and obtained 17 sequences of the ITS1-5.8S-ITS2 region. In total,

175   with 24 sequences obtained from GenBank and two sequences from *H. decipiens* collected from

176   southern Taiwan, 14 valid species of *Halophila* were represented. In total 112 sequences were

177   used for alignment and phylogenetic analyses. The length of aligned sequences is 615 bp, with

178   157 parsimony informative sites. Based on the ITS phylogenetic tree, sequences in the present

179   study can be divided into three highly supported clades, which are *H. major*, the *H. ovalis*

180   complex, and a potential new species (*Halophila* sp. 2) that is closely related to *H. decipiens* in

181   terms of anatomic structure as seen under a SEM (J Kuo & S Liu, 2020, unpublished data).

182   Interestingly, fresh plants of *Halophila* sp. 2 are very similar to *H. stipulacea* (Figure S. 2).

183   Outgroups are in sections Microhalophila (*H. beccarii*), Spinulosae (*H. spinulosa*), Tricostata (*H.*

184   *tricostata*), and Americanae (*H. engelmannii*), which have complicated phyllotaxy and can

185   clearly be separated from species having simple phyllotaxy in section Halophila, except *H.*

186   *australis*. The basal clades of the ingroup comprised three species having leaf edge serrations,

187   including *H. stipulacea*, *H. decipiens,* and *Halophila* sp. (*H. stipulacea* like clade). Sequences

188   derived from plants collected from all three Sri Lankan sites fell into two main clades: the *H.*

189   *ovalis* complex clade and *H. major* clade. Sequences clustering in the *H. major* clade were

190   mainly from Mannar and Matara, whereas sequences clustering in the *H. ovalis* complex clade

191   were from Mannar and Puttalam Lagoon. Interestingly, sequences derived from molecular

192   cloning showed that any given plant contained ITS sequences of both *H. ovalis* and *H. major,* as

193   shown in Figure 2. For example, six sequences were obtained from SB24, two of them belonging

194   to the *H. major* clade and the remainder to the *H. ovalis* complex clade. This may indicate a

195   possible hybrid cross between *H. ovalis* and *H. major*.

196   Only one of the 97 samples failed to amplify rbcL. With eight sequences downloaded from

197   GenBank representing seven species of *Halophila*, the length of the final alignment was 440 bp

198   with only six parsimony informative sites. *H. beccarii* and *H. engelmannii* served as outgroups.

199   At the basal-most position of the ingroup, *Halophila* sp. 2 clustered with a sequence derived

200   from a sample collected from India that was identified as *H. stipulacea* and sister to *H. decipiens*.

201   However, sequences derived from most Sri Lankan samples formed a monophyletic clade along

202   with references identified as *H. ovalis*, *H. major,* and *H. minor* (Figure 3). Since rbcL lacked

203   genetic variation among the different *Halophila* species, the unresolved phylogeny indicates that

204   rbcL cannot resolve species boundaries in *Halophila*.

205   **Morphological analyses**

206   We were unable to measure all the plants that we collected in the present study because we did

207   not collect enough shoots for both DNA extraction and morphological examination during

208   sampling. After DNA extraction, we were able to measure 48 plants, which were further defined

209   into six categories comprised of potential hybrid (failure to sequence without further cloning due

210   to multiple templates), hybrid, *H. ovalis*, *Halophila* sp. 1 (allied to *H. minor*), *H. major,* and

211   *Halophila* sp. 2 (*H. decipiens* like) (Figure 4). The number of plants examined and results of five

212   measurements and two ratios are given in Table 1. Raw data were transformed and a principal

213   component analysis (PCA) was performed. Variance explained by the first two PCA components

214   (PCA1 and PCA2) is 79.576%. The majority of the variance (95%) of PCA-1 and PCA-2 was

215   explained by lamina length and lamina width:lamina length, respectively. The result of the PCA

216 plot shows that *Halophila* sp. 1 (allied to *H. minor*) and *Halophila* sp. 2 can be distinguished

217 from other categories by smaller lamina length and smaller lamina width:lamina length,

218 respectively. *H. major* had the widest distribution in the PCA plot, and the hybrid and potential

219 hybrid fell within the range of *H. ovalis* and *H. major* (Figure 5).

220

Discussion

221

222 The literature related to seagrass communities and biodiversity in the Sri Lanka is scarce.

223 Jayasuriya (1991) mentioned that there are 12 species among nine genera recorded from Sri

224 Lanka, the number being increased to 15 in 2007 (*De Silva & Amarasinghe, 2007*). The latest

225 report showed 14 species as of 2017 (*Udagedara et al.*, *2017*), with the complete list being

226 *Enhalus acoroides*, *Halophila beccarii*, *H. decipiens*, *H. ovalis*, *H. ovata*, *H. minor*, *H.*

227 *stipulacea*, *Thalassia hemprichii*, *Cymodocea rotundata*, *C. serrulata*, *Halodule uninervis*, *H.*

228 *pinifolia*, *Ruppia maritima*, and *Syringodium isoetifolium*. In the present study, *H. major*

229 (Zollinger) Miquel (1855) is a new species record for Sri Lanka based on genetic analyses. *H.*

230 *major* was previously treated as a synonym of *H. ovalis* by den Hartog (1970), and in 2006 Kuo

231 (2006) examined global type materials and concluded that reinstating the taxon status of *H.*

232 *major* was warranted. Additionally, further phylogenetic studies (*Uchimura et al.*, *2008; Nguyen*

233 *et al.*, *2013*) showed molecular evidence that *H. major* can also be separated from *H. ovalis* by

234 using the ITS region. Kuo (2006) suggested that *H. major* and *H. ovalis* can be distinguished by

235 a ratio of 1/2 of the lamina width to the distance between the intramarginal veins and lamina

236 margin, and the number of cross-veins. However, most of our measurements of *H. ovalis* and *H.*

237 *major* overlap (Table 1). This morphological plasticity could lead to underestimating seagrass

238 biodiversity as mentioned in Nguyen et al. (2014). In the recent survey of the Gulf of Mannar,

239 Puttalum Lagoon, and southern Sri Lanka (*Ranahewa et al., 2018*; *Ranatunga & Pethiyagoda,*

240 *2015*; *Gunasekara, 2017*), *H. major* may mistakenly be identified as *H. ovalis*. In addition, our

241 phylogenetic analysis also points out that *H. major* could be widely distributed in Sri Lanka,

242 since plants collected from Mannar and Matara are identified as *H. major*. Potential hybrids from

243 Puttalum Lagoon have both *H. ovalis* and *H. major* ITS sequences, which may indicate the

244 presence of *H. major* actually occurring where we failed to collect it. The ITS phylogenetic tree

245 also showed that the *H. decipiens* like plants (*Halophila* sp. 2) collected from the Gulf of Mannar

246 are distinct from *H. decipiens* in Taiwan. Conversely, rbcL sequences derived from *H. decipiens*

247 like plants cluster with *H. stipulacea* collected from India. This incongruence could possibly

248 result in erroneous identifications since *Halophila* sp. 2 resembles *H. stipulacea* in the field.

249 Another possible explanation is the lack of genetic variation on the rbcL chloroplast gene, which

250 failed to resolve species boundaries in the genus *Halophila* (Lucas *et al., 2012*). The present

251 study also shows that there are only six parsimony informative sites across seven *Halophila*

252 species among 440 bp.

253 PCA analyses based on morphology show that *Halophila* sp.1 (allied to *H. minor*) and *Halophila*

254 sp. 2 (*H. decipiens* like) can be separated from other categories. Although *Halophila* sp. 2 is very

255 similar to *H. stipulacea* in appearance, most measurements are smaller than plants in the

256 Mediterranean Sea (*Procaccini et al., 1999*) as well as those described as *H. decipiens* in Kuo et

257 al (2006). As mentioned by Den Hartog (1970), Indian Ocean *H. stipulacea* plants often have

258 delicate and membranous but never bullate leaves, and more or less deciduous stipules. These

259 plants were initially collected by Isaac Bailey Balfour during the Transit of Venus expedition at

260 Rodrigues Island in 1874 (*Balfour, 1879*), and later described as *Halophila balfourii* Soler.

261 (*Solereder, 1913*). Currently, it is treated as a synonym of *Halophila stipulacea* (Forsskål)

262 Ascherson. Therefore, the plants collected from the Gulf of Mannar could be *H. balfourii*.

263 However, further genetic analyses from a broad sampling across its current distribution,

264 including the population from the type locality of *H. stipulacea* and *H. decipiens* in the Red Sea,

265 is needed to clarify the identity of *Halophila* sp 2. Most *Halophila* sp.1 (allied to *H. minor*)

266 measurements overlap with *H. minor* or *H. ovate* (*Kuo & Den Hartog, 2001*), except that lamina

267 width (1.15-1.89 mm) is smaller compared to these two species (*H. minor*: 3.5-6 mm; *H. ovata*:

268 4-8 mm). Unfortunately, even by using combination loci including matK, rbcL, and trnH-psbA

269 (Lucas et al. 2012) or ITS, rbcL, and matK (*Nguyen et al.*, *2015*), there was a failure to resolve

270 species boundaries in the *H. ovalis* complex. Further comparative phylogenomic approaches (Liu

271 *et al., 2017*; Yu *et al., 2018*) may be useful in resolving *H. ovalis* complex species boundaries.

272 Soltis & Soltis (2009) suggested that natural hybridization could be an important creative force

273 and evolutionary process responsible for the increasing of angiosperm species diversity. The

274 incongruence between phylogenetic relationships constructed based on different markers can be

275 considered a signature of hybridization, as well as two divergent alleles of a single locus found in

276 one individual. Intra-species variation in ITS have been identified in many different plant groups,

277 which may hamper attempts to uncover accurate phylogenetic species relationships (*Poczai &*

278   *Hyvönen, 2010*). Meanwhile, the high intraspecific variation in ITS is considered as incomplete

279   concerted evolution driven by hybridization (*Xu et al., 2017*). Additionally, the maternal

280   inheritance of the chloroplast gene tree (*i.e.*, rbcL tree in the present study) reflects only the

281   evolutionary processes of maternal lineages, which may mask genetic evidence of hybridization

282   (*Okuyama et al., 2005; Soltis & Soltis, 2009*). Either of these reasons may cause incongruence

283   between ITS and plastid phylogenies. Among marine angiosperms, natural hybridization has

284   been observed in only four genera (*Halodule, Ruppia, Posidonia,* and *Zostera*) (*Ito & Tanaka,*

285   *2011; Coyer et al., 2008; Martínez-Garrido et al., 2016; Sinclair et al. 2019*). Ito & Tanaka

286   (2011) found sympatric *Halodule uninervis* and *H. pinifolia* hybridizing in the waters of

287   Okinawa by reconstructing their phylogenetic relationship with rbcL and psbA-trnH loci. The

288   congruent pattern between morphological traits and nuclear loci was also observed in two

289   sympatric species of *Posidonia* (*P. australis* and *P. coriacea*) in Australia that show signs of

290   hybridization (*Sinclair et al., 2019*). In the present study, the majority of *Halophila* samples

291   collected from Kapitya failed to sequence due to multiple templates found in single plants, but 17

292   pure sequences in the ITS region were obtained with further cloning. Phylogenetic analyses

293   showed that a single plant contained ITS sequences clustered with both *H. ovalis* and *H. major*

294   (Figure 2). The percentage of sequencing failure due to multiple templates varied among the

295   three sites (22/32 at Kalpitiya, 1/35 at Mannar, and 5/33 at Matara), indicating that hybridization

296   may be common at these three sites, especially Kalpitiya. However, the PCA plot based on

297   morphology showed that most traits overlap among hybrid, *H. ovalis,* and *H. major*. This may be

298   due to the morphological plasticity found in *Halophila* (*Hartog & Kuo, 2006; Kuo et al., 2006;*

299   *Singh et al., 2019*).

300

301   **Conclusions**

302   In conclusion, *Halophila* plants collected from Sri Lanka cluster into three clades by the ITS tree,

303   represented as *H. major*, *H. ovalis* complex, and *Halophila* sp. 2 clade. *H. major* is recorded as a

304   new species of the genus *Halophila* in Sri Lanka, and may have a wide distribution and possibly

305   be misidentified as *H. ovalis* in the previous literature. Meanwhile, *H. decipiens* like plants

306   collected from Mannar may represent a cryptic species of either *H. stipulacea* or *H. decipiens*

307   based on phylogenetic relationship traits shared among them. Surprisingly, we found the first

308   case of hybridization in the genus *Halophila,* which may be a cross between *H. ovalis* and *H.*

309   *major.* Further phylogeographic study with a broader sampling scheme that includes plants from

310   type localities and applying methods based on massive parallel sequencing (*i.e.*, Hyb-Seq, review

311   in *Yu et al., 2018*) that can obtain genome wide genetic variation is needed to clarify the

312   taxonomic status of *Halophila* sp. 1 and sp. 2.

313

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439 Figure 1. Sample collection sites coded with different colors (blue: Mannar, gray: Kalpitiya, and

440 black: Matara) for *Halophila* surveys in Sri Lanka. Numbers in brackets indicate sample size.

441 Figure 2. Comparison of leaf morphology of *H. major*, *H. ovalis,* and *H. stipulacea* like

442 specimens collected in Sri Lanka. Samples displayed in this figure were included and showed in

443 ITS tree (Figure 3).

444 Figure 3. Phylogeny of *Halophila* inferred from maximum likelihood and Bayesian analysis

445 based on 615 bp (including gaps) of nrDNA sequences comprising ITS-1, 5.8S rDNA and ITS-2.

446 Nodes are presented only for those with bootstrap scores > 90% majority rule for maximum

447 likelihood and > 90% majority probabilities for Bayesian probability values (ML /BI). Sequences

448 are color-coded based on different sampling locations as in Figure 1. Sample names with shading

449 are sequences derived from molecular cloning.

450 Figure 4. Phylogeny of *Halophila* inferred from maximum likelihood and Bayesian analysis

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455 Figure 5. Component loadings for the first of two principal components of the PCA of

456 morphological traits with convex hull of different sample groups (LL: lamina length, LW: lamina

457 width, VA: crossed vein angle, DE: distance between marginal vein and lamina edge, LW / LL:

458 lamina width / lamina length, and HLW/DE: half lamina width/distance between marginal vein

459 and lamina edge).

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Sample collection sites coded with different colors (blue: Mannar, green: Kalpitiya, and red: Matara) for *Halophila* surveys in Sri Lanka. Numbers in brackets indicate sample size.

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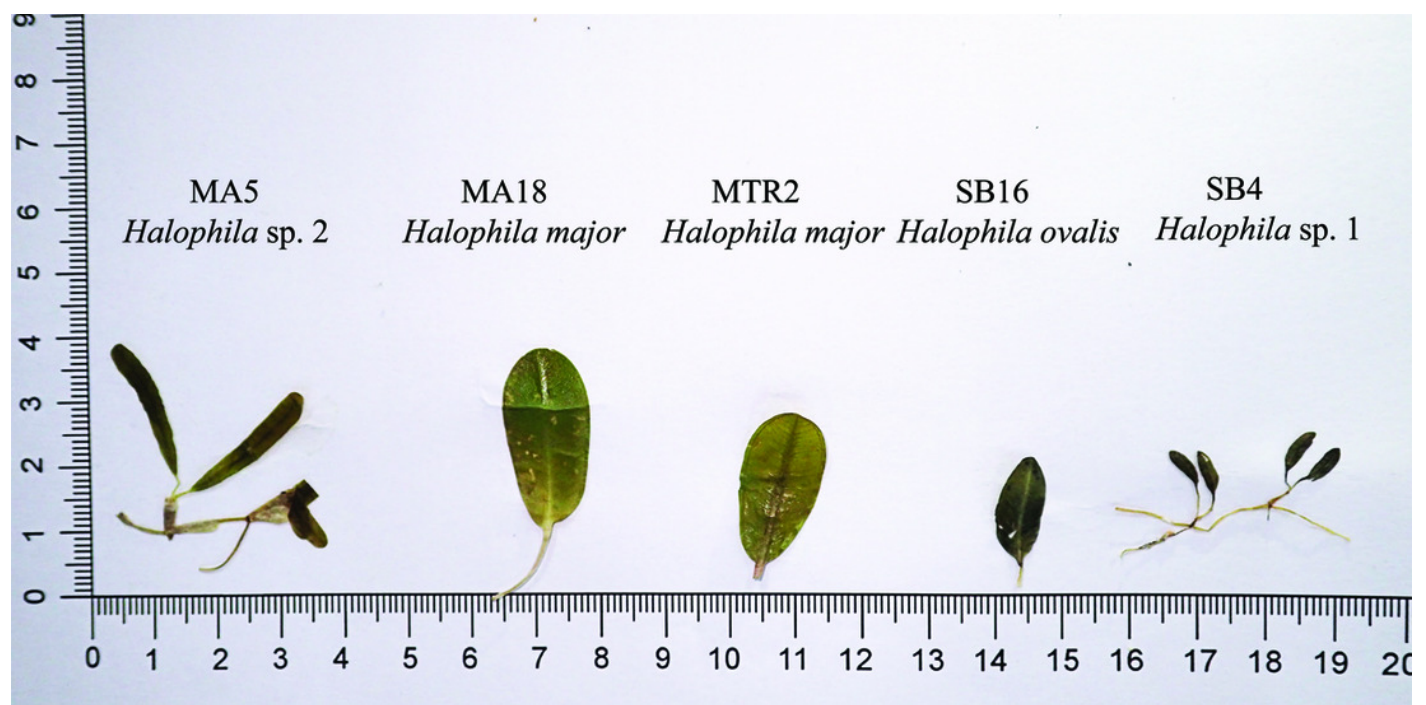
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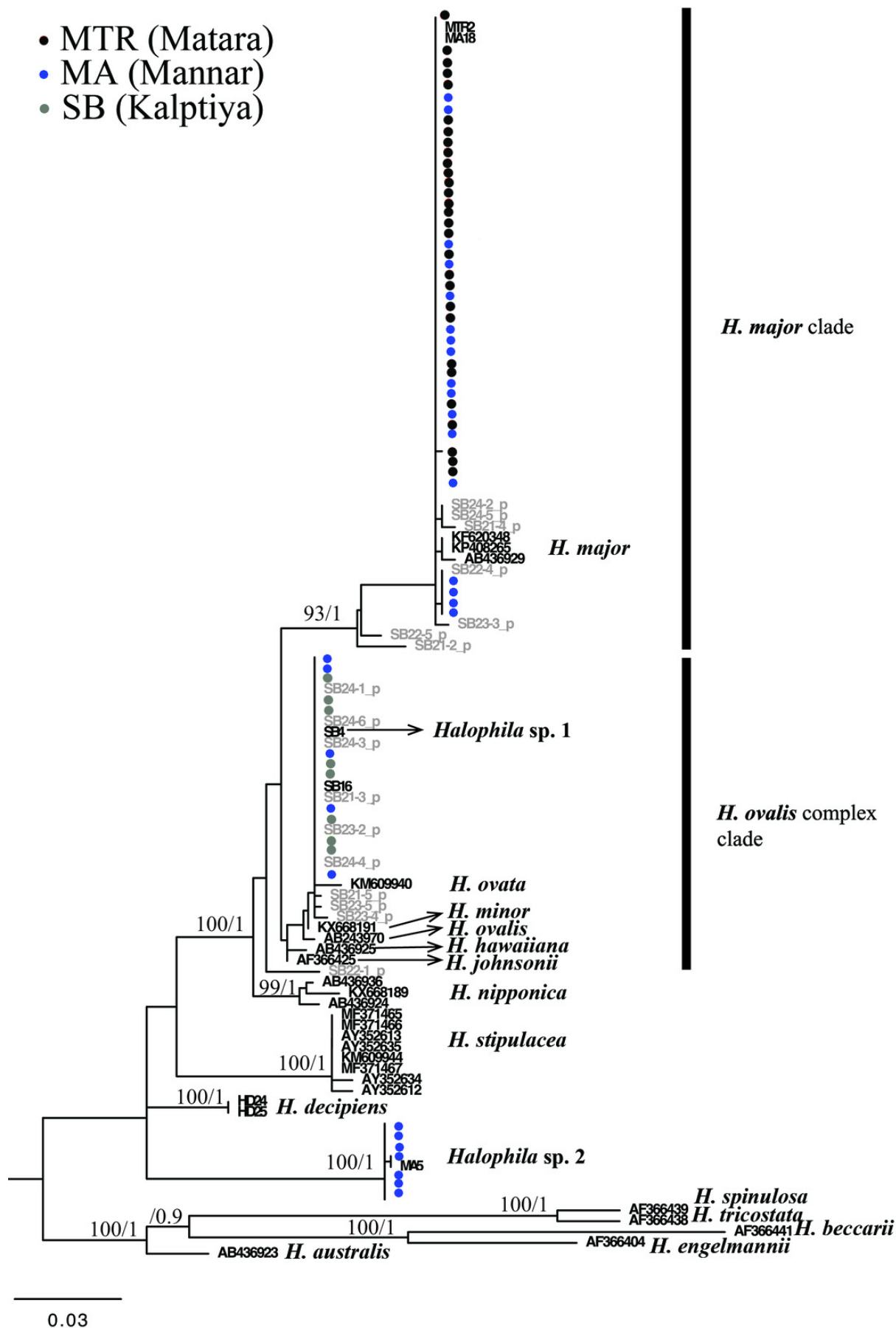
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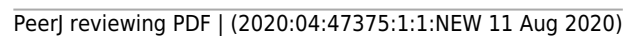
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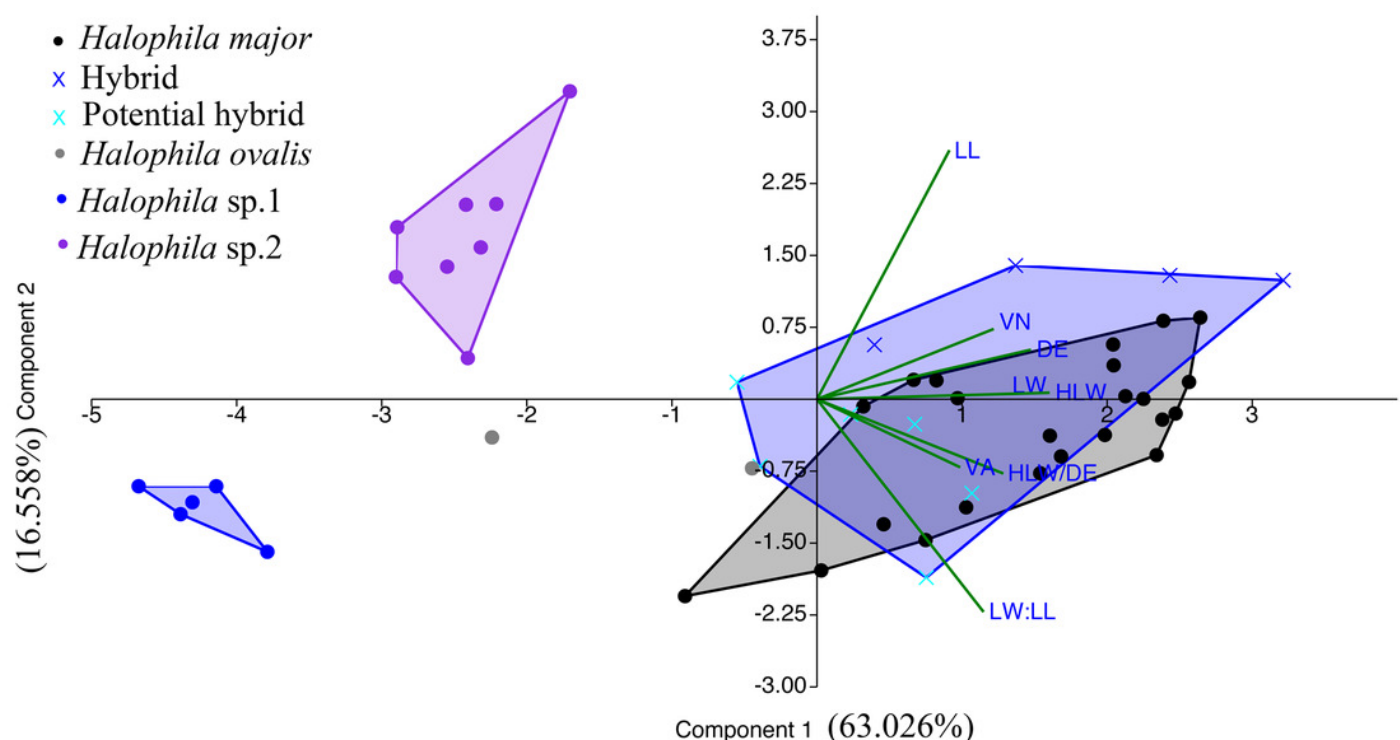
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1 Table 1: Comparisons of leaf morphology of *H. major*, *H. ovalis*, and *H. stipulacea* like species collected in Sri Lanka and previous

2 studies.

Characteristic	Species										
	H. ovalis					*H. major*			*H. stipulacea*	*H. decipiens.*	*Halop*
	den Hartog (1970)	*Halophila* sp. 1 (SB)	*H. ovalis* (SB)	Potential hybrid (SB)	Hybrid (SB)	Kuo et al. (2006)	*H. major* (MTR)	*H. major* (MA)	Procaccini et al. 1999, Vera et al. 2014	Kuo et al. (2006)	*H. de like (*
Number of samples		5	2	6	4			13	10		
Lamina length (mm)	10-40	4.42-6.61	11.00-16.00	12.00-20.00	23.80-33.00	15-25	19.80-31.00	8.05-31.13	63.8-84.3	20	17.1
Lamina width (mm)	5-20	1.15-1.89	3.90-6.89	6.55-9.90	8.60-14.13	9-11	10.50-13.75	4.72-14.63	6.5-8.43	4-6	2.1
No. of pair cross veins	10-25	3-4	13	11-17	18-27	14-17	11-17	12-18	11-18	6-9	
Space between intramarginal veins (mm)	0.1-0.3	0.09-0.17	0.29-0.33	0.34-0.51	0.39-0.54	0.2	0.50-0.72	0.29-0.66	0.5 (Ruiz & Ballantine, 2004)	0.25-3	0.2
Cross-vein	45-60∘	46-68∘	54∘	58-82∘	57-62∘	45-60∘	59-78∘	57-80∘	45-60∘	n/a	5

angles

LW:LL	n/a	0.25-0.35	0.43-0.35	0.33-0.59	0.33-0.43	n/a	0.44-0.56	0.40-0.59	n/a	0.2-0.33	0.0
HLW:DE	0.5-0.63	4.95-10.03	10.51-6.75	7.90-12.94	10.29-13.25	0.71-0.33	9.01-12.69	8.27-6.32	n/a	2-3	4.8

7   n/a: not available