Genetic diversity and structure of the critically endangered *Artocarpus annulatus,* a crop wild relative of jackfruit (*A. heterophyllus*)

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Limestone karsts of Southeast Asia can harbor high levels of endemism, but are highly fragmented, increasingly threatened, and their biodiversity is often poorly studied. This is true of the Padawan Limestone Area of Sarawak, Malaysia, home to the endemic *Artocarpus annulatus,* the closest known wild relative of two important and underutilized fruit tree crops, jackfruit (*A. heterophyllus*) and cempedak (*A. integer*). Identifying and conserving crop wild relatives is critical for the conservation of crop genetic diversity and breeding. In 2016 and 2017, five *A. annulatus* populations were located, and leaf material, locality information, and demographic data were collected. Microsatellite markers were used to assess genetic diversity and structure among populations, and to compare levels of genetic diversity to closely related congeneric species. Results indicate no evidence of inbreeding in *A. annulatus*, and there is no genetic structure among the five populations. However, diversity measures trended lower in seedlings compared to mature trees, suggesting allelic diversity may be under threat in the youngest generation of plants. Also, genetic diversity is lower in *A. annulatus* compared to closely related congeners. The present study provides a baseline estimate of *A. annulatus* genetic diversity that can be used for comparison in future studies and to other species in the unique limestone karst ecosystems. Considerations for *in situ* and *ex situ* conservation approaches are discussed.

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3 **wild relative of jackfruit (*A. heterophyllus*)**

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23 **Abstract**

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25 fragmented, increasingly threatened, and their biodiversity is often poorly studied. This is true of

26 the Padawan Limestone Area of Sarawak, Malaysia, home to the endemic *Artocarpus annulatus,*

27 the closest known wild relative of two important and underutilized fruit tree crops, jackfruit (*A.*

28 *heterophyllus*) and cempedak (*A. integer*). Identifying and conserving crop wild relatives is

29 critical for the conservation of crop genetic diversity and breeding. In 2016 and 2017, five *A.*

30 *annulatus* populations were located, and leaf material, locality information, and demographic

31 data were collected. Microsatellite markers were used to assess genetic diversity and structure

32 among populations, and to compare levels of genetic diversity to closely related congeneric

33 species. Results indicate no evidence of inbreeding in *A. annulatus*, and there is no genetic

34 structure among the five populations. However, diversity measures trended lower in seedlings

35   compared to mature trees, suggesting allelic diversity may be under threat in the youngest

36   generation of plants. Also, genetic diversity is lower in *A. annulatus* compared to closely related

37   congeners. The present study provides a baseline estimate of *A. annulatus* genetic diversity that

38   can be used for comparison in future studies and to other species in the unique limestone karst

39   ecosystems. Considerations for *in situ* and *ex situ* conservation approaches are discussed.

41   **Introduction**

42   The karst limestone formations of Southeast Asia are dramatic, rugged landscapes that are home

43   to some of the most unique and understudied flora and fauna in the world (Tuyet 2001; Clements

44   et al. 2006; Chung et al. 2014; Hughes 2017). There are approximately 800,000 km^2 of karst

45   ecosystems in tropical Southeast Asia and southern China, and they include several UNESCO

46   World Heritage sites (Day and Urich 2000; Williams 2008). Most karsts were formed millions of

47   years ago by calcium-secreting marine organisms, and the current landscapes are the result of

48   geomorphological processes that have formed complex terrains such as fissured and precipitous

49   cliffs, jagged towers, sinkholes, extensive cave systems, and subterranean rivers, harboring a

50   range of ecological niches (Clements et al. 2006; Williams 2008). The resulting landscapes are

51   naturally fragmented mosaics of karst and ordinary terrain, promoting edaphic isolation and high

52   levels of endemism (Kruckeberg and Rabinowitz 1985; Farjon et al. 2002; Schilthuizen 2004;

53   Musser et al. 2005; Williams 2008). Healthy karst habitats provide tangible benefits to humans,

54   such as water from below-ground aquifers, as well as a range of ecosystem services (Beynan

55   2011), some of which are due to their unique biodiversity (Schilthuizen 2004; Clements et al.

56   2006). However, due to human practices, the karst ecosystems and the species inhabiting them

57   are themselves increasingly at risk of further fragmentation and degradation (Clements et al.

58   2006, Clements et al. 2008, Struebig et al. 2009, Latinne et al. 2011, Hughes et al. 2017). The

59   most immediate and direct threat is overexploitation of resource extraction, especially limestone

60   and mineral mining (Kiew et al. 2017; Beynan 2011, Hughes et al. 2017). These extremely

61   lucrative but destructive extractive practices can have cascading effects of severe fragmentation

62   and habitat degradation, leading to serious negative impacts on flora, fauna, and humans.

63   In Sarawak, Malaysia, the Padawan limestone formation (in the southwest part of Kuching

64   Division) is the largest and one of the oldest (dating from about 163 to 100 million years ago)

65   outcrops among the six limestone biodistricts there (Lim 2008) (Cranbrook 2004). One of the

66   species endemic to this habitat is the critically endangered tree, *Artocarpus annulatus* Jarrett,

67   known only from the Padawan-Serian-Tebedu karst forests (Fig. 1). This species is a member of

68   the mulberry (Moraceae) family and the closest wild relative of the important tropical food crops

69   jackfruit (*A. heterophyllus* Lam.) and cempedak (*A. integer* (Thunb.) Merr.) (Zerega et al. 2010;

70   Williams et al. 2017, Zerega et al. 2019). *Artocarpus annulatus* was first described nearly 45

71   years ago (Jarrett 1975) but remains poorly understood in terms of its biology and distribution.

72   As of 2015, the species was known from only nine herbarium specimens. They were all collected

73   between 1960-1999 and came from just four limestone hill forest outcrops within the Padawan-

74   Serian-Tebedu limestone karst formation in Kuching (Gunung Mentawa, Gunung Gayu, and

75   Gunung Teng Bukap) and Serian (Gunung Payang) Divisions (Fig. 2). According to Zerega et al.

76   (2019), there are thought to be fewer than 50 mature individuals in an area of occupancy (AOO)

77   of 32 km^2.

78   The importance of studying and conserving *A. annulatus* has broad implications beyond karst

79   systems, as the genetic diversity of crop wild relatives can prove a useful source of novel traits

80   (Lobell et al. 2008). The economic and food value of its cultivated close relatives, cempedak and

81   jackfruit, are likely to increase, as they grow in tropical regions where food insecurity is high,

82 and provide greater local autonomy in food production systems (Ford-Lloyd 2011; Jones et al.

83 2011, Wang et al. 2018, Witehrup et al. 2019). As agriculture adapts to climate change, long-

84 lived tree crops may play an increasingly important role in the global diet. It is known that

85 jackfruit and cempedak are interfertile, because hybrid cultivars exist (Wang et al. 2018). Given

86 that *A. annulatus* is the only other member of the lineage including jackfruit and cempedak

87 (Williams et al. 2017), it is possible that they could all be interfertile and traditional plant

88 breeding could incorporate desirable traits, such as expanding the edaphic conditions of the crop

89 species.

90   Depending on what we know about the genetic diversity and structure of endangered species,

91 different approaches to conservation may be warranted (Widener and Fant 2018, Münzbergová

92 2005, Crozier 1997). Due to the unique karst environment, it is possible that *A. annulatus*

93 populations have long been isolated and small. However, human-caused fragmentation may have

94 further reduced genetic diversity, making it harder for the species to rebound and recover (Leimu

95 et al. 2006). While there are limited studies examining population genetics of endangered flora in

96 limestone karst systems of Southeast Asia, the unique habitat and fragmented nature of karst

97 systems have made them of interest for understanding species diversification through isolation.

98 Recent studies of common karst endemic plant genera (*Begonia* and *Primulina*) found substantial

99 differentiation and significant isolation by distance across populations (Gao et al. 2015, Tseng et

100 al. 2019). Understanding genetic patterns and diversity in *A. annulatus* is an important step

101 towards developing conservation strategies for this crop wild relative. This study uses

102 microsatellite and demographic data to assess the genetic diversity and structure of *A. annulatus,*

103 an endemic tree of the Padawan-Serian-Tebedu karst forests of Sarawak, Malaysia. Comparisons

104 of genetic diversity are also made to its two closest relatives, which are commonly occurring

105   underutilized crops. Specifically, we test the following hypotheses: Given the relatively recent

106   (in decades) destruction of *A. annulatus* habitat, the known populations of *A. annulatus* will

107   show recent evidence of gene flow and as there will not be population specific alleles; due to

108   pressures of increased fragmentation and habitat loss, recruitment of younger individuals may be

109   more tenuous and lead to decreased genetic diversity in younger individuals compared to mature

110   trees; and as a critically endangered species with restricted distribution, *A. annulatus* will harbor

111   less genetic diversity than its widely distributed congeners. It is hoped that the results can inform

112   management plans for *in situ* and *ex situ* conservation efforts and provide much needed

113   information about the unique biota of the Southeast Asian karst formations. Understanding how

114   diversity is distributed among populations will provide insight into the importance of whether *in*

115   *situ* or *ex situ* conservation efforts should be targeted toward all or some populations to capture

116   the existing diversity of this critically endangered species.

117

118   **Materials & Methods**

119   **Species information**

120   *Artocarpus annulatus* is a small to mid-sized evergreen tree, growing up to 10 m tall on karst

121   outcrops in Sarawak, Malaysia. It is monoecious, with separate male and female inflorescences

122   occurring on the same individual tree. The male inflorescences have rings around them, for

123   which the specific epithet "*annulatus*" is named (Fig. 1). Once fertilized, the female

124   inflorescence develops into a multiple fruit structure (called a syncarp) that is cauliflorous (i.e.

125   grows directly from the tree trunk) (Fig. 1). Like all members of the genus *Artocarpus,* it

126   produces copious white exudate from all parts when cut. The closest relatives of *A. annulatus* are

127   two important crop species: *A. heterophyllus* (jackfruit) and *A. integer* (cempedak). They also

128   bear cauliflorous fruit structures, which are edible and much larger than *A. annulatus* (reaching

129   up to 45 kg in *A. heterophyllus*). Jackfruit is thought to be native to the western Ghats of India

130   (with a possible secondary center of diversity in Bangladesh) (Witherup et al. 2019). It is grown

131   throughout much of the tropics today. Cempedak (*A. integer* var. *integer*) is thought to be native

132   to peninsular Malaysia and possibly Borneo; bangkong (*A. integer* var. *silvestris*) is a close wild

133   relative native to peninsular Malaysia (Wang et al. 2018).

134   **Site information**

135   *Artocarpus annulatus* is known only from the Padawan limestone area in Kuching District,

136   Sarawak, Malaysia (Fig. 2). This area consists of small mountains with tower karst formations.

137   The landscape is dominated by very steep, or even vertical sided limestone "towers," known as

138   tower karst formations. It is the result of once continuous limestone rock being eroded (Brenda et

139   al, 2004), The towers are sculptured by chemical and mechanical processes forming a terrain

140   covered by jagged pinnacles and deep pits and crevices. *Artocarpus annulatus* can be found at

141   the base of the karst forest mountains on relatively flat ground, as well as at higher elevations

142   among the karst towers and growing sideways from outcrops, with roots clinging to the limetone

143   rock (Fig. 1).

144   **Sampling**

145   In 2016 and 2017, fieldwork was undertaken in the Padawan-Serian-Tebedu karst limestone

146   forests in Sarawak, Malaysia for collection and observation of *A. annulatus* (Zerega et al. 2019).

147   Research permits were approved by the Ibu Pejabat Jabatan Hutan Wisma Sumber Alam

148   (National Parks and Nature Reserves, Forest Department), Sarawak for 2016 (Permit no.

149   NCCD.907.4.4(JLD.13)-195) and renewed for 2017 (Permit no. NPW.907.4.4(JLD.14)-137). All

150   known sites from which *A. annulatus* had previously been recorded (Gunung Gayu, Gunung

151   Mentawa, Gunung Teng Bukap, and Gunung Payang), as well as four additional sites in

152   surrounding areas of limestone hill forest (Gunung Manok, Gunung Bedoh, Gunung Jambusan,

153   and Fairy Caves) were visited to search for *A. annulatus* individuals (Zerega et al. 2019).

154   Individuals of *A. annulatus* were found in five sites (Gunung Mentawa, Gunung Gayu, Gunung

155   Teng Bukap, Gunung Manok, and Gunung Bedoh) (Fig. 2). Representative collections were

156   made at each site, and the number and age class of individuals from each population varied

157   (Table S1). At one of the sites where collections had previously been made in 1999 (Gunung

158   Payang), the area was found in 2017 to have a small area of limestone hill forest remaining, and

159   a nearby rubber plantation. However, no *A. annulatus* was found (pers. obs. Zakaria). For all

160   sites where *A. annulatus* was found, GPS coordinates, images, and herbarium collections were

161   taken for representative samples. In addition, leaf tissue samples (dried in silica), for 131 *A*

162   *annulatus* trees were taken across the five sites (Table S1). For most of these 131 trees, diameter

163   at breast height (dbh) measurements, and notes about the age class of the samples were also

164   taken. The trees sampled were of varying maturity (seedling, sapling, and reproductively

165   mature). Seedlings were defined as having a height less than 1 meter, while mature plants were

166   defined as having a dbh of 5.5 cm or more, because plants that met this criterion showed signs of

167   past reproduction. Anything taller than 1 meter and with a dbh less than 5.5 cm was considered a

168   sapling. The dried leaf material was shipped to the Chicago Botanic Garden where it was stored

169   at -20°C. Addititional data for comparison to closely related congeners of *A. annulatus* came

170   from jackfruit (*A. heterophyllus,* Witherup et al. 2019, n=373 from Bangladesh; and Melhem

171   2015, n=373 from India), cempedak (*A. integer* var. *integer*, n=344), and *A. integer* var. *silvestris*

172   (n=187, bangkong, a wild relative of cempedak) (Wang et al. 2018).

173   **Genetic data collection**

174   Genomic DNA was extracted from 1 cm^2 dried leaf using either the Qiagen DNeasy kits (Hilden,

175   Germany) following standard protocol or a modified cetyltrimethylammonium bromide (CTAB)

176   method (Doyle and Doyle 1987). Nine different microsatellite primers developed for *A. altilis*

177   (Witherup et al. 2013) were used following the methods of Witherup et al. (2013). These loci

178   previously revealed diversity in the two most closely relatives of *A. annulatus*: jackfruit (*A.*

179   *heterophyllus*) and cempedak (*A. integer*) (Wang et al. 2018, Witherup et al. 2019). The

180   microsatellite regions amplified in this study were all dinucleotide repeats: MAA26 (black D2

181   dye), MAA54 (blue D4 dye), MAA105 (black D2 dye), MAA122 (green D3 dye), MAA140

182   (blue D4 dye), MAA156 (green D3 dye), MAA182 (blue D4 dye), MAA178 (green D3 dye), and

183   MAA196 (black D2 dye). The forward primers were labelled with WellRed fluorescent dyes,

184   shown in parentheses above.

185   The PCR reactions contained 5 μL of 2x myTaq Mix (Bioline, Taunton, Massachusetts,

186   USA), 0.15 μL 20 mg/ml BSA (bovine serum albumin), 0.25 μL each of labelled forward and

187   reverse primers (10 μm concentration), 3.35 μL of DNA-free water, and 1 μL of DNA (with

188   concentrations between 10 and 80 ng/μL). The PCR cycles were: 2 minutes at 94°C for initial

189   denaturization, followed by 34 cycles of 94°C for 30 seconds of denaturation, 52°C for 1 minute

190   of annealing, then 72°C for 2 minutes of extension; the 34 cycles were then followed by 72°C for

191   10 minutes of final extension (Wang et al. 2018).

192   The PCR products were run on a Beckman Coulter CEQ 8000 Genetic Analysis System

193   (Beckman Coulter, Brea, CA) following the methods of Witherup et al. (2013) and multiplexed

194   via the same groupings of Wang et al. (2018). The samples were scored alongside a GenomeLab

195   400 bp internal size standard ladder that was added to HiDi formamide (20 μL ladder per 1.5 mL

196   of HiDi) (Azco Biotech., San Diego, CA, USA). In each well, 30 μL of the HiDi /ladder mixture

197 were added to between 1.0 and 2.0 µL of PCR product, depending on the strength of the

198 fluorescence color. These concentrations were determined by running a few different

199 combinations and dilutions of primers and determining which resulted in the clearest peaks.

200   The resulting peaks were scored manually using Beckman Coulter CEQ 8000 Software 9.0.

201 Allele sizes are recorded based on the size of the ladder and electrophoresis rates as described in

202 Wang et al. (2018). *Artocarpus annulatus* is thought to be functionally diploid, and as such each

203 region should display either one (homozygous) or two (heterozygous) alleles. Previous studies

204 have revealed that some primers (including primers MAA196 and MAA156) may amplify two

205 regions, and up to four alleles can be present (Wang et al. 2018; Witherup et al. 2013; Witherup

206 et al. 2019). This was rarely observed in *A. annulatus.* In a few samples when more than three

207 strong peaks were observed, the rarest allele was omitted (allele size 268 for primer MAA196;

208 allele size 170 for primer MAA54) (Wang et al. 2018).

209 **Analysis of genetic data**

210 Microchecker analysis (Van Oosterhout et al. 2004), using the Oosterhout method was run to

211 check for null alleles. The microsatellite data were analyzed using GenAlEx v.6.5 (Peakall and

212 Smouse 2012) to calculate diversity measures, including average number of alleles per locus

213 (N_a), number of effective alleles (N_e), observed and expected heterozygosities (H_o and H_e),

214 number of private alleles (P_a), inbreeding coefficient (F_{IS}) and fixation index (F_{ST}). Allelic

215 richness was calculated in the R package Hierfstat (Goudet 2005). To test if diversity measures

216 were significantly different across populations and age classes, single factor ANOVAs were

217 conducted in Excel v. 16.16.14.

218 Genetic structure among populations was visualized using STRUCTURE v.2.3.4 (Pritchard,

219 Stephens, and Donnelly 2000). Twenty independent runs per *K* were carried out with a burn-in

220 period of 10,000 and 10,000 MCMC iterations for $K = 1 - 20$. Structure Harvester (Earl and

221 vonHoldt 2012) was used to determine the most likely value of genetic clusters (K)

222 implementing the Evanno method (Evanno et al. 2005). Spatial clustering of populations was

223 assessed using Principle Coordinate Analysis (PCoA) of Nei's genetic distance in GeneAlEx

224 (Peakall and Smouse 2012). Additionally, GEneAlEx v.6.5 (Peakall and Smouse 2012) was used

225 to assess genetic differentiation among populations AMOVA. To assess whether there was any

226 genetic differentiation among age classes, PCoA, F_{ST}, and AMOVA analyses were also run for

227 the three age classes in GeneAlEx v.6.5 (Peakall and Smouse 2012). For PCoA, the two principle

228 coordinates were plotted with 95% confidence ellipse in R (ggplot) (Wickham 2016).

229 **Results**

230 **Microsatellite loci**

231 Of the nine different microsatellite primers that were tested, one (MAA178) consistently

232 amplified two readily distinguishable regions, which were scored as separate loci and

233 differentiated as primer MAA178A and primer MAA178B (following Wang et al. 2018). Across

234 the resulting 10 loci, there were 44 detected alleles, however, two loci were excluded from

235 further analyses as they were monomorphic across all samples (MAA122 and MAA140) (Table

236 S2). Another locus (MAA54) was excluded from further analyses as it was functionally

237 monomorphic, in that nearly all individuals were heterozygotes with the same two alleles,

238 suggesting that this primer may amplify two fixed homozygote loci (MAA54 has been shown to

239 amplify two loci in other *Artocarpus* species) (Wang et al. 2018). Although these three

240 monomorphic loci were excluded from subsequent analyses, the fact that the same loci were

241 found to be polymorphic (with $3 - 18$ alleles) in the two closest relatives to *A. annulatus* (Wang

242 et al. 2018, Witherup et al. 2019), suggests that *A. annulauts* is more depauperate in genetic

243   diversity. Finally, MAA105 was excluded from further analysis due to a null allele frequency

244   value > 0.20, which can cause significant underestimation of population differentiation (Table

245   S2). This threshold value has been used in other studies to exclude primers that may significantly

246   affect heterozygosity measures (Chapuis and Estoup, 2007; Minn et al., 2014; Muzzalupo et al.,

247   2014). The following loci were included in subsequent genetic diversity and structure analyses:

248   MAA26, MAA156, MAA182, MAA178 A, MAA178 B, MAA196.

249   **Genetic diversity and structure**

250   Analysis of the polymorphic loci with no evidence of null alleles revealed no significant

251   differences in diversity measures across populations (Table 1). The Fixation Index of individuals

252   (F_{IS}) for all populations was negative (indicating no signs of inbreeding), or in the case of

253   Gunung Mentawa it was barely positive (0.05). The population at Gunung Mentawa had no

254   private alleles, while the other four populations had between one (Gunung Manok) and six

255   (Gunung Bedoh) private alleles. Pairwise comparisons of F_{ST} showed limited levels of genetic

256   differentiation across populations (Table 2). Results of AMOVA among and within populations

257   showed that only 8% of diversity can be accounted for among populations, and 92% is within

258   populations (Table 3).

259   All samples were sorted into age classes as described in the methods. Analysis of the six

260   microsatellite loci revealed no significant differences in diversity measures across size classes,

261   except in the case of private alleles; seedlings had significantly fewer private alleles than

262   saplings or mature trees (Table 4). Pairwise comparisons of F_{ST} across tree size class showed no

263   signs of genetic differentiation across size classes (Table 2). Results of AMOVA among and

264   within tree size classes indicated that only 6% of diversity can be accounted for among

265   populations, and 94% is within populations (Table 3).

266   The first two principle coordinates of the PCoA account for 25% and 10%, respectively, of

267   the variability in the dataset, and there are no clear clusters based on populations or tree size

268   class (Fig. 3). The population at Gunung Mentawa occupies the smallest space, as does the

269   mature tree size class. STRUCTURE was run on all samples with six loci, and results were

270   analyzed using the Evanno method in Structure Harvester (Earl and vonHoldt 2012) to determine

271   the most probable number of genetic clusters (K). The most likely number was $K = 3$. The

272   structure plot shows that the genetic clusters are restricted to neither distinct geographic

273   populations nor size classes of trees, with all three clusters being fairly equally present in each

274   population and each size class (Fig. S1).

275   **Genetic diversity comparisons with closely related species**

276   Expected heterozygosities (H_e) and inbreeding coefficients (F_{IS}), based on the same six

277   microsatellite loci, were calculated for *A. annulatus* and its closest relatives: *A. integer* var.

278   *integer* (cempedak)*, A. integer* var. *silvestris* (bangkong, a wild relative of cempedak) (Data from

279   Wang et al. 2018)*,* and *A. heterophyllus* (jackfruit) (Data from Witherup et al. 2019)*.* A one-way

280   ANOVA found no significant difference (p=0.113) across the species for H_e, though *A.*

281   *annulatus* tended to have the lowest values (Table 5). A one-way ANOVA did find significant

282   difference (p=0.020) across species for F_{IS}, and a posthoc Tukey HSD test then found significant

283   differences between *A. annulatus* and the other three taxa (*A. heterophyllus,* p<0.01; *A. integer*

284   var. *integer,* p=0.013; *A. integer* var. *silvestris*, p=0.039)

285

286   **Discussion**

287   **Genetic diversity of closely related *Artocarpus* species**

288   Understanding genetic diversity and its spatial and temporal structure within endangered species

289   and populations is important in order to make sound conservation management plans, especially

290   for imperiled species with very restricted habitats. When starting with zero information about the

291   population genetics of a species, assessing standard measures of genetic diversity is important to

292   establish a baseline of information, but it is also valuable to consider those measures in a broader

293   context. Looking at closely related, more common congeners allows for an evolutionary,

294   comparative perspective. For example, Widener and Fant (2018) found higher levels of

295   inbreeding in an endangered edaphic specialist compared to its more common congener. In the

296   case of *A. annulatus* and its closest relatives, when considering measures of diversity (H_e) of its

297   most closely related congeners (*A. heterophyllus, A. integer* var. *integer*, and *A. integer* var.

298   *silvestris*) using the same six microsatellite markers, *A. annulatus* tends to have lower measures,

299   but there is no significant difference (p = 0.597) (Table 5). We also considered broader

300   comparisons with other long-lived, outcrossing wild trees and closely related domesticates.

301   Miller and Gross (2011) reviewed genetic diversity in perennial crops and their wild relatives

302   and reported that based on neutral codominant markers (SSRs and allozymes) cultivated

303   perennial fruit crops retain on average 95% (from 65% - 127%) of the neutral variation found in

304   wild populations. Cultivated *Artocarpus* crops had elevated levels of genetic variation compared

305   to the closely related *A. annulatus* (Table 5). This could be an artifact of smaller sampling size in

306   *A. annulatus*, or it could reflect loss of diversity due to habitat destruction. Another possible

307   explanation is that the cultivated populations may represent descendants of crosses between

308   geographically and genetically distinct individuals, giving rise to novel diversity. Studies have

309   demonstrated that allelic diversity in small populations are likely to be very low simply by

310   chance (Cole 2003), which may be the case for *A. annulatus*.

311   Despite the lower than expected levels of diversity, *A. annulatus* does not show signs of

312   inbreeding, as evidenced by the negative value of the F_{IS} compared to its three relatives—

313   displaying F_{IS} values between 0.149 and 0.314 (Table 5). The results might seem counter-

314   intuitive for such small and restricted populations. However, since most of the variation present

315   in *A. annulatus* is distributed within populations (93%), and because *A. annulatus* is a long-lived,

316   likely outcrossing tree (as are other members of the genus), these results are put into perspective.

317   Genetic diversity levels could be remnant from when the population of *A. annulatus* may have

318   been larger and may have had higher levels of diversity, and the remaining individuals still retain

319   this because of their lifespan (Guries and Ledig 1979). Additionally, it is possible that *A.*

320   *annulatus* shows no signs of inbreeding, because long-lived perennials often exhibit stronger

321   selection than non-perennials against inbred plants. Indeed, most woody perennials exhibit high

322   outcrossing rates to maintain greater levels of diversity (Duminil, Hardy, and Petit 2009).

323   Meanwhile, crops like jackfruit (*A. heterophyllus*) are under human selection and are frequently

324   vegetatively propagated (i.e. grafting) to maintain desired traits (Witherup et al. 2019).

325   **Genetic structure within and across *Artocarpus annulatus* populations**

326   The results of several analyses indicate that there is little to no geographical genetic structure

327   exhibited across the five populations of *A. annulatus*. Principle Coordinate Analysis and

328   Structure analyses indicate there is no genetic differentiation based on the geography of the

329   populations (Fig. 3, Fig. S1). The results from the AMOVA test revealed that only 8% of

330   diversity was found among populations (Table 3), and the F_{ST} values demonstrated low genetic

331   differentiation among populations (Table 2). Values below 0.05 are considered to represent very

332   low to no genetic differentiation (Frankham et al. 2002), and the values in this study ranged from

333   0.028 to 0.089. The five populations have an area of occupancy of approximately 32 km² (Zerega

334   et al. 2019). Three of the populations (Gunung Gayu, Gunung Mentawa, and Gunung Teng

335   Bukap) are geographically closely clustered, each being ca. 0.3 km apart. Gunung Manok is the

336   most isolated, situated ca. 6 km from the cluster; and Gunung Bedoh is in the middle, ca. 3 km

337   away from both Gunung Manok and the cluster of three (Fig. 2). Not surprisingly, Gunung

338   Manok is the most differentiated from the others, being the least differentiated from the closest

339   population (Gunung Bedoh). These results, taken in aggregate, suggest that gene flow has

340   occurred among the populations, but that it may be limited across greater distances. The five *A.*

341   *annulatus* populations may be part of one largely intermixing population across which genetic

342   material is shared via gene flow or may represent remnants of populations that were once larger

343   and less fragmented. Because it is a long-lived tree, the effects of fragmentation and isolation

344   may be slower manifested in the genetic structure.

345   It is of interest to consider methods of gene flow to understand what might be causing low

346   levels of genetic differentiation across populations in such a dramatic landscape as karst forests,

347   where dispersal ability of inhabitants may be limited (Hughes 2017). The two ways for plants to

348   spread genes across space are via pollen dispersal (pollination) and fruit/seed dispersal. While we

349   know virtually nothing about this in *A. annulatus,* we can turn to its closest relatives to consider

350   possibilities. Recent work has been conducted on elucidating pollination mechanisms in its two

351   closet relatives, jackfruit (*A. heterophyllus*) and cempedak (*A. integer*). Both of these

352   monoecious species (separate male and female inflorescences present on the same individual

353   tree) are thought to be pollinated via a three-way mutualistic relationship between the plant itself,

354   gall midges (genus: *Clinodiplosis*, Family: Cecidomyiidae, Order: Diptera), and a fungus (yet to

355   be identified, but possibly Genus: *Choanephora,* Family: Choanephoraceae, Order: Mucorales)

356   that grows on the male inflorescences (Gardner et al. 2018; Sakai 2000). The adult gall midges

357   are thought to be attracted to the same volatile compounds that are similarly emitted by both

358   male and female inflorescences. The midges feed on the pollen of male flowers, and at the same

359   time a fungus (which does not appear to infest other parts of the tree) gradually grows over the

360   length of the male inflorescence. Gravid female midges lay their eggs on the fungus and the

361   young larvae feed on the liquid exuded by the fungal mycelia. As the midges seek out male

362   inflorescences for feeding and egg-laying, they sometimes mistakenly visit female flowers

363   (which offer no apparent reward for the midges but mimic the scent of the male flowers)

364   (Gardner et al. 2018), and in the process may transfer pollen to effect pollination. Given that its

365   closest relatives are both pollinated by gall midges, it is hypothesized that *A. annulatus* may

366   share a similar mechanism (Gardner et al. 2018). Gall midges are tiny insects, about 1 mm in

367   length, and have short lifespans (adults live between a few hours to a few days), so they are

368   likely limited in the distances they can travel to disperse pollen (Gardner et al. 2018; Skuhrava

369   1991). Some of the *A. annulatus* populations in this study are found within just 0.25 km of each

370   other, while the most isolated population (Gunung Manok) is about 3 km to the closet population

371   (Gunung Bedoh) and about 6 km to the next closet populations. Most of what is known about

372   flight range of members of the gall midge family comes from studies of pests. For example,

373   studies of Hessian flies (*Mayetiola destructor, Cecidomyiidae)* suggest that they choose when,

374   where, and how far to move based on cues from the environment, and are also affected by wind.

375   They can disperse from a few meters to a few kilometers and exhibit nonrandom movement

376   directed by things like scents or certain wavelengths of color (Harris and Rose 1990, Withers et

377   al. 1997, Anderson et al. 2012). Additionally, female midges are more likely to stay in an area

378   where their plant of interest is located as opposed to moving to an area with less attractive plants

379   (Withers and Harris 1996). We still need to learn more about pollination in *A. annulatus,* but it

380   seems possible that if it is similar to its closest relatives, gall midges do not typically travel long

381   distances, and the more fragmented *A. annulatus* populations become over time, the more greatly

382   genetically differentiated they could become. This may explain why the most geographically

383   distant population (Gunung Manok) is also the most genetically differentiated from the others.

384   However, none of the populations are very highly differentiated from one another, and gene

385   flow appears to be occurring (or has recently occurred) among them (Table 2, Fig. S1). This

386   brings us to the other mechanism for gene flow: fruit (i.e. seed) dispersal. The fruits of *A.*

387   *annulatus* are large (ca. 6 x 8 cm) and distinctive with bumpy or spiky skin and a strong

388   fragrance (Jarrett 1975). Just like its crop relatives, jackfruit and cempedak, the fruit structure

389   grows from the trunk of the tree (Fig. 1), but the wild *A. annulatus* has much smaller and less

390   fleshy fruits than its crop relatives (Jarrett 1975). While research is limited on fruit dispersal in

391   *Artocarpus,* there are studies that suggest fruits are spread by large mammals, and this may also

392   be the case in *A. annulatus.* In India, the related *A. chaplasha* depends on large mammals such as

393   the Asian elephant (*Elephas maximus*), domestic cows (*Bos primigenius*), buffalo (*Bubalus*

394   *bubalis*), and rhesus macaques (*Macaca mulatta*) as seed dispersers (Sekar 2014). The study

395   species, *A. annulatus,* shares its habitat with a few sizeable mammals, including François's leaf

396   monkey (*Trachypithecus francoisi*) and the serow (*Capricornis sumatraensis*) (Kiew et al. 2017).

397   While anecdotal information from fieldwork suggests that many of the seeds simply germinate

398   under the mother tree, where the fruits drop (i.e. numerous saplings were observed growing in

399   clusters beneath mature trees), it is possible that birds or mammals disperse some *A. annulatus*

400   seeds, accounting for gene flow and low genetic differentiation across sites (Zerega et al. 2019).

401   Future research could focus on better understanding the contributions of these two modes of gene

402   flow by studying the distribution of genetic structure of seed (maternal chloroplast loci) vs.

403   pollen dispersed alleles.

404   **Genetic structure across age classes of *Artocarpus annulatus***

405   When an endangered species occurs in habitats that are under threat, it is valuable to know if

406   genetic diversity is being lost over time, such that older, mature individuals harbor greater or

407   unique genetic diversity compared to young seedlings and saplings. As the highly specialized

408   karst habitat becomes increasingly smaller and fragmented due to human pressures, it might be

409   expected that the *A. annulatus* gene pool could be reduced due to random, stochastic loss of

410   individuals, leading to a decreased level of diversity in younger generations and a genetic

411   bottleneck. When *A. annulatus* individuals were analyzed by size class (seedling, sapling, or

412   reproductively mature), diversity measures tended to be lower in seedlings compared to saplings

413   and mature trees. However, only the number of private alleles was significantly less in seedlings

414   (Table 4). Additionally, PCoA clustering analysis shows that the age classes do not cluster

415   separately (Fig. 3), and AMOVA (Table 3) suggests that only 6% of diversity was found within

416   age classes. This all suggests that there is no genetic differentiation across size classes, but that

417   allelic diversity may be under threat in the youngest generation of plants. To better understand

418   possible temporal changes, it would be useful to look at a much longer trajectory of time and

419   over a larger space. Assessing the genetic diversity of older herbarium specimens would be one

420   way to help determine if diversity has been lost. Unfortunately, there are only nine historical

421   collections known of *A. annulatus,* the oldest only dates back to the 1960s, and due to its

422   critically endangered status, sampling of herbarium specimens for DNA was not possible.

423   However, the present study provides a baseline estimate of genetic diversity that can be used for

424   comparison of future studies.

425 **Informing conservation strategies**

426 Understanding *A. annulatus* genetic diversity can inform conservation management, including *ex*

427 *situ* conservation. In the case of *A. annulatus,* different populations were not all strongly

428 genetically differentiated from one another, so there may be little justification for collecting

429 germplasm from multiple individuals from all populations for *ex situ* conservation in local

430 arboreta and conservation collections. Instead, seeds or seedlings from populations with the most

431 individuals or the most accessible population could be targeted for conservation. For *in situ*

432 conservation efforts, all sites should ideally be protected (Crozier 1997), however, focusing

433 conservation efforts on the largest population would likely yield similar results in terms of

434 conserving the breadth of *A. annulatus* genetic diversity. In many cases, *in situ* conservation may

435 be the preferred approach. A recent meta-analysis of 32 plant species from various habitats

436 compared how well plants grew in their local habitats versus a foreign transplant site. They

437 found that in over 70% of the studies the local plants consistently did better in their habitats of

438 origin, further supporting the argument for conservation of karst areas (Leimu et al. 2008).

439 Additionally, the unique karst habitat is difficult to replicate, and due to the economic

440 importance and biodiversity that karsts hold, *in situ* conservation is especially important as it

441 conserves an entire ecosystem.

442

443 **Conclusions**

444   The threats to limestone karst ecoystems in Southeast Asia are immense, and we still have

445 much to learn about them (Clements et al. 2006, Hughes et al. 2017). Laws to protect karst

446 ecosystems are severely lacking, and policies in place are often not enforced (Clements et al.

447 2006). Unfortunately, the lack of information about the ecosystems and the biodiversity they

448   house can make it more difficult to justify their conservation. For this reason, it is important to

449   conduct research in these ecosystems, and by combining available data across studies it can help

450   understand patterns and prioritize regions for protection. *Artocarpus annulatus* is just one of

451   many endemic species in an ecosystem that is as incredibly unique and diverse as it is fragile,

452   and points to the need for protection of the karst forests of Southeast Asia. The health of *A.*

453   *annulatus*, as a long-lived, fruit-bearing tree, is crucial for the complex web of plants and

454   animals that depend upon it. Finally, given that *A. annulatus* is the closest wild relative of two

455   important fruit tree species, it may be valuable for breeding programs to expand the growing

456   conditions of jackfruit and cempedak to limestone soils.

457

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463

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Figure 1

Artocarpus annulatus

a) Karst limestone habitat in Kuching Division, Sarawak, b) Roots growing on limestone outcrops, c) White exudate found throughout trees, d) Tree, e) Cauliflorous syncarp (multiple fruit structure), f) Close up of syncarp, and g) Male inflorescence with annulate rings. Photo credits: Nyree Zerega (a-c), Michael Lo (d and g) and Benedikt Kuhnhäuser (e and f)

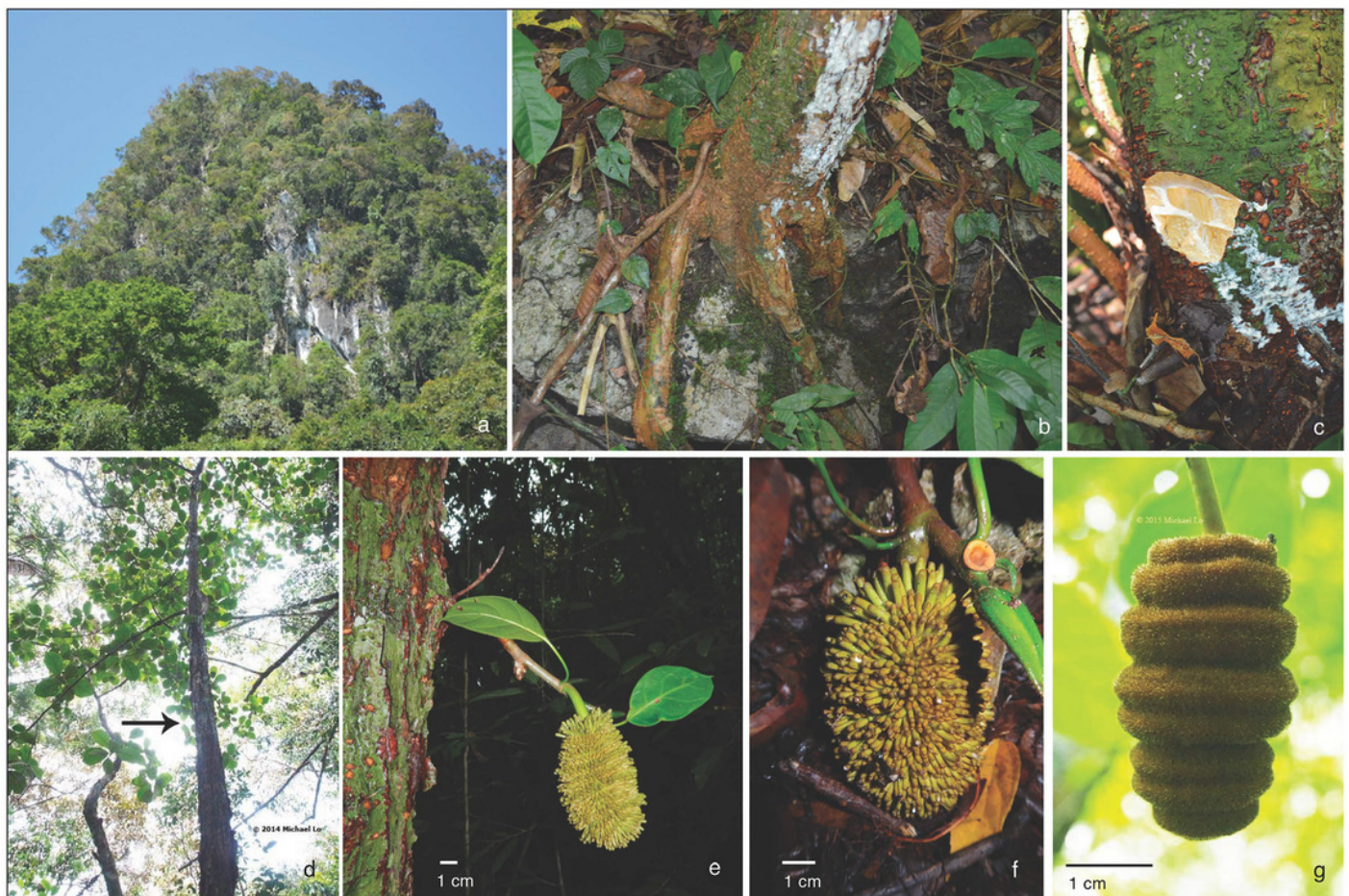



Figure 2

Artocarpus annulatus distribution

a) Malaysia is shown in dark gray; the area in Sarawak where *A. annulatus* populations are found is indicated by the white box, b) Close up of boxed area from above panel shows extant populations of *A. annulatus* (circles), locations of historical specimens (triangles), and locations that were searched in 2016 and no *A. annulatus* were found (squares), c) Close up of boxed area from above panel shows known extant *A. annulatus* populations that were sampled in this study. Map Credit: SimpleMappr (2019), CC 0.

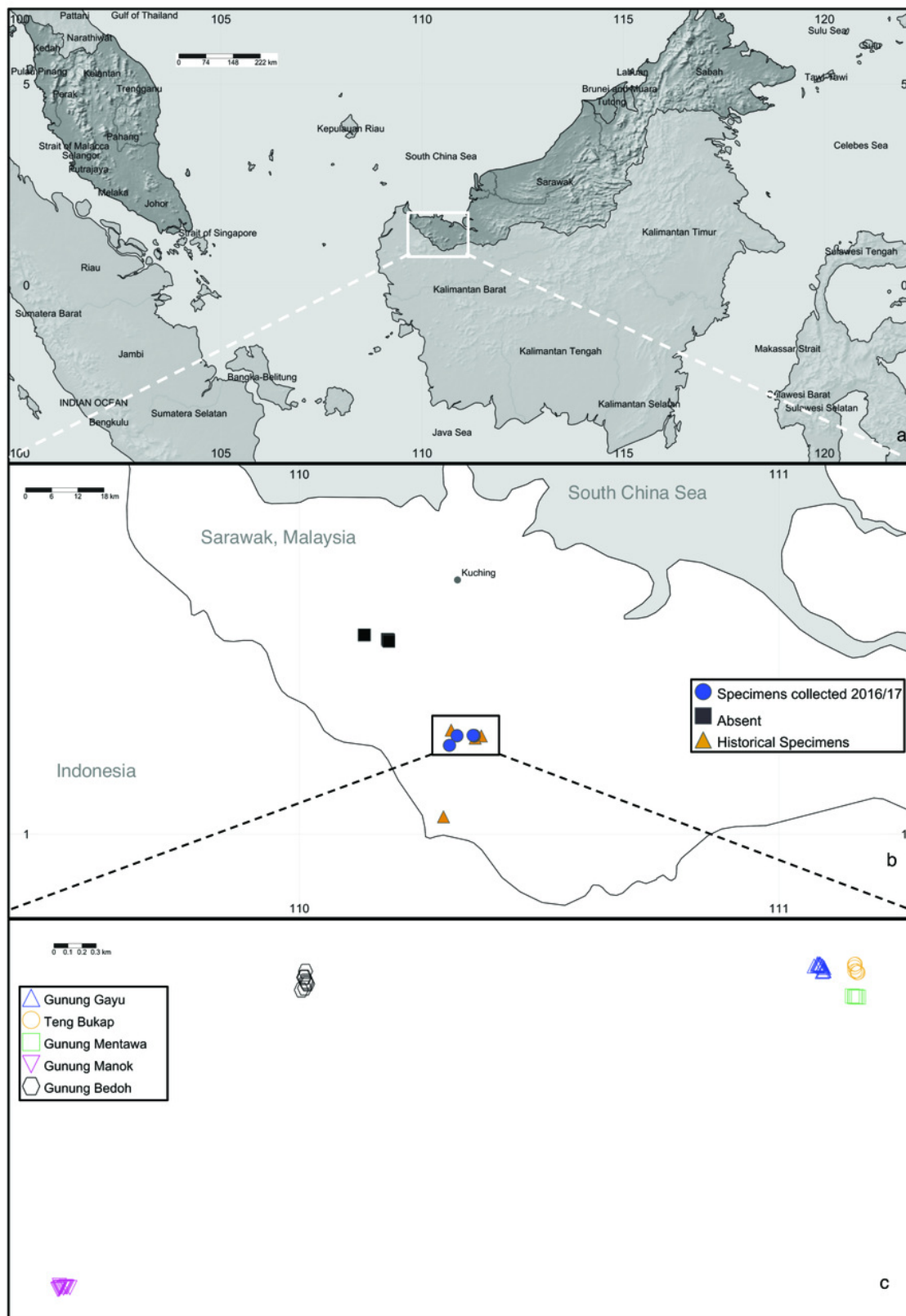
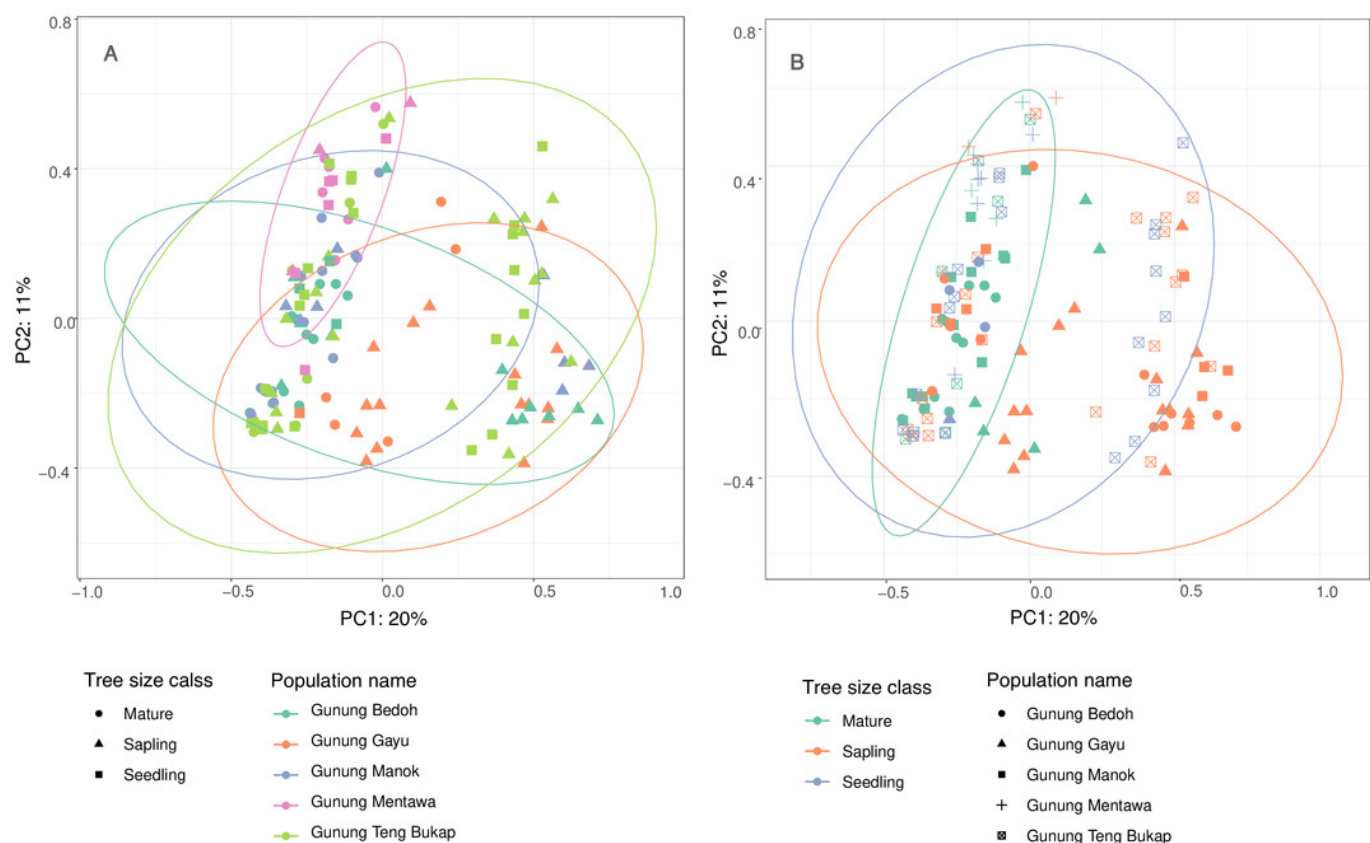



Figure 3

Principle Coordinate Analysis of five *A. annulatus* populations

a) Populations are displayed in different colors, tree size class is represented by different shapes, 95% confidence ellipses are drawn around populations; b) Tree size classes are displayed in different colors; populations are represented by different shapes; 95% confidence ellipses are drawn around tree size classes.

Genetic diversity measures of *A. annulatus* populations.

Number of individuals sampled in each population by size class. Numbers in parentheses after population names refer to number of individuals from each size class (mat = mature, sap = sapling, sdlg = seedling).

2

Population	N	N_a	N_e	A_R	H_o	H_e	F_{IS}	I	P_a
Gunung Gayu (5 mat, 16 sap, 1sdlg)	22	3.33	2.01	3.44	0.60	0.49	-0.23	0.85	5
Gunung Bedoh (11 mat, 12 sap, 3sdlg)	26	3.83	1.81	3.14	0.49	0.41	-0.20	0.72	6
Gunung Manok (13 mat, 18 sap)	21	2.00	1.50	2.53	0.42	0.29	-0.36	0.45	1
Gunung Mentawa (6 mat, 3 sap, 6sdlg)	15	2.17	1.56	2.96	0.31	0.29	0.05	0.48	0
Gunung Teng Bukap (6 mat, 20 sap, 21sdlg)	47	3.00	1.71	3.08	0.38	0.35	-0.06	0.63	3
Total (41 mat, 59, sap, 31 sdlg)	131								
P value	NA	0.07	0.31	0.18	0.46	0.40	0.40	0.26	0.20

4 N_a = no. of different alleles, N_e = no. of effective alleles, A_R = allelic richness, H_o = observed heterozygosity, H_e =
5 expected heterozygosity, F_{IS} = Fixation Index, P_a = number of private alleles, I = Shannon's Informational Index

Pairwise comparisons of F_{ST} values

Comparison of *A. annulatus* by population and tree size.
1

Pairwise F_{ST} Comparison by Populations

Gunung Gayu (Pop. 1)	Gunung Bedoh (Pop. 2)	Gunung Manok (Pop. 3)	Gunung Mentawa (Pop. 4)	Gunung Teng Bukap (Pop. 5)	
0.00	-	-	-	-	1
0.067	0.000	-	-	-	2
0.118	0.032	0.000	-	-	3
0.101	0.052	0.068	0.000	-	4
0.061	0.032	0.059	0.028	0.000	5

Pairwise F_{ST} Comparison by Tree Size

Sapling	Seedling	Mature	
0.00	-	-	Sapling
0.021	0.000	-	Seedlling
0.020	0.014	0.000	Mature

AMOVA

Results are shown among and within five *A. annulatus* populations, and among and within tree size classes across all five populations.

AMOVA across five populations					
Source	df	SS	MS	Est. Var.	%
Among populations	4	42.80	10.70	0.17	8
Within populations	257	534.27	2.08	2.08	92
Total	261	577.07	-	2.25	100
AMOVA across three tree size classes					
Among populations	2	28.15	14.08	0.14	6
Within populations	259	548.92	2.12	2.12	94
Total	261	577.07		2.26	100

3   df = degrees of freedom, SS = sum of squares, MS = mean square, Est. Var. = estimated variance
4

Genetic diversity measures by tree size class of *A. annulatus* across five populations.

Total values are listed for sample size (N) and private alleles (P_a). Mean values across all loci are listed for all other measures. If p-value are < 0.05, different superscript letters indicate significant difference between groups.

2

Size Class	N	N_a	N_e	A_R	H_o	H_e	F_{is}	I	P_a
Seedling	28	3.00	1.77	3.46	0.41	0.36	-0.16	0.65	0[a]
Sapling	46	4.33	1.80	4.02	0.45	0.42	-0.06	0.77	8[b]
Mature	37	4.00	1.73	3.30	0.43	0.39	-0.10	0.70	6[b]
P value	NA	0.26	0.97	0.12	0.95	0.89	0.79	0.84	**0.03**

4 N_a = no. of different alleles, N_e = no. of effective alleles, A_R = allelic richness, H_o = observed heterozygosity, H_e =
5 expected heterozygosity, F_{is} = Fixation Index, P_a = number of private alleles, I = Shannon's Informational Index
6

Comparison of genetic diversity (H_e).

Comparisons are shown between *A. annulatus* and its closest relatives, *A. heterophyllus* (jackfruit) and *A. integer* (cempedak) based on the same microsatellite loci. There is no significant difference between species for H_e, but there is for F_{IS} (indicated by superscript letters).

Locus	A. heterophyllus (N = 746)		A. integer var. integer (N=344)		A. integer var. silvestris (N=187)		A. annulatus (N = 131)	
	H_e	F_{IS}	H_e	F_{IS}	H_e	F_{IS}	H_e	F_{IS}
MAA26	0.774	0.106	0.348	0.341	0.480	0.154	0.427	-0.094
MAA156	0.373	-0.084	0.566	0.016	0.613	-0.088	0.287	-0.429
MAA182	0.554	0.223	0.178	0.360	0.594	0.182	0.087	-0.146
MAA178A	0.580	0.638	0.610	0.092	0.553	0.261	0.484	0.091
MAA178B	0.776	0.391	0.558	0.255	0.870	0.207	0.535	-0.105
MAA196	0.730	0.609	0.599	0.165	0.521	0.179	0.497	-0.439
Mean	**0.631**	**0.314[a]**	**0.477**	**0.205[a]**	**0.605**	**0.149[a]**	**0.386**	**-0.184[b]**