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Mating system variation in neotropical black mangrove, *Avicennia germinans*, at three spatial scales towards an expanding northern distributional limit

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Abstract

Climate-driven range expansion of ecosystem-defining foundation species can have wide-reaching ecological consequences. Expansion may also result in mating system changes in these foundation species because of the ecological characteristics of range margins, such as greater conspecific isolation and reduced pollinator availability. It is important to understand how mating systems may change during expansion due to their direct influence on intraspecific genetic and demographic dynamics. Here, we used 12 microsatellite loci to genotype progeny arrays of the neotropical black mangrove (*Avicennia germinans*) at six collection sites (n = 23 maternal trees; 1,612 genotyped propagules) along a latitudinal gradient towards a northern distributional limit on the Atlantic coast of Florida, USA (27.56 – 30.01°N), where mangroves have expanded into salt marsh over the past several decades. We assessed mating system variation at three spatial scales. First, at the species-distribution level, published outcrossing rates for tropical conspecifics were more than two times higher than those for subtropical Florida *A.*

germinans, consistent with reductions in pollinator diversity and in mangrove abundance with latitude. Second, at the population level, Florida outcrossing rates did not systematically decline towards the northern range limit, but instead, a more open pollen-dispersal neighbourhood at the transition from mangrove to salt marsh dominance may elevate outcrossing until conspecific abundances become too low towards the range limit. Third, at the individual level, outcrossing increased as conspecific cover increased at the Florida range margin, consistent with density-dependent plastic shifts in mating system. These findings suggest that ecological structure influences the *A. germinans* mating system at varying spatial scales. Further research needs to evaluate the effect of *A. germinans* mating system variation on the survival and fitness of offspring and on the extent of population-level local adaptation at expanding distributional limits.

Keywords: density-dependent; foundation species; outcrossing; pollinator diversity; range expansion; self-fertilisation

Introduction

Climate-driven redistributions of species are now commonplace and can lead to important changes in ecological communities, ecosystem function, and human well-being (Pecl et al., 2017). For instance, range expansion of ecosystem-defining foundation species (e.g., long-lived tree and shrub species) can result in entire biome shifts with wide-reaching ecological consequences (Beck et al., 2011; Peñuelas and Boada, 2003; Saintilan et al., 2014).

Colonisation of new habitat has long been associated with greater self-fertilisation in plants (i.e., Baker’s Law; Baker, 1955) as characteristics of expanding range margins, such as greater isolation among conspecifics (Eckert et al., 2010; Ghazoul, 2005) and reductions in pollinator availability (Kalisz et al., 2004; Moeller et al., 2012; Yin et al., 2016), are known to select for increased self-fertilisation (Hargreaves and Eckert, 2014). However, adaptive shifts in mating system are not thought to be general attributes of the expansion of long-lived trees and shrubs because these species generally maintain outcrossing independent of their environment (Barrett and Harder, 2017). Instead, density-dependent plastic shifts towards greater self-fertilisation may occur during initial colonisation (Morgan et al., 2005; Peterson and Kay, 2015), with subsequent changes post-colonisation as increased conspecific density favours shifts back towards greater outcrossing (Pannell, 2015). Understanding mating system variation is important because of its direct influence on genetic and demographic dynamics within a species (Barrett and Harder, 2017 and cites within).

Mangroves are an assortment of intertidal tree and shrub species that are ecologically-important coastal foundation species (Tomlinson, 1986). Mangroves originate in the tropics and decline in abundance towards latitudinal range limits that correspond to ecological thresholds in temperature and/or precipitation (Osland et al., 2017). The pantropical genus *Avicennia* consists of eight species, of which three are found in the Neotropics (Duke, 1992). Of these three neotropical *Avicennia* species, *Avicennia germinans* (neotropical black mangrove) is the most widespread, with a distribution across the tropics and into the subtropics (Lonard et al., 2017). On the Atlantic coast of Florida (USA), mangroves are the dominant coastal foundation species at lower latitudes where three mangrove species (*A. germinans*, plus *Rhizophora mangle* and *Laguncularia racemosa*) form dense forests, but the higher-latitude mangrove range margin consists of discrete patches of isolated individuals (almost exclusively *A. germinans*) in a salt marsh-dominated landscape (Kangas and Lugo, 1990). Lack of extreme winter freezes over the past several decades have been linked to proliferation and expansion of *A. germinans* at this northern distributional limit (Cavanaugh et al., 2019, 2014; Rodriguez et al., 2016) and further expansion is forecast with climate change (Cavanaugh et al., 2019, 2015). This ongoing shift from salt marsh to mangrove dominance at this expanding range margin will presumably lead to significant changes in ecosystem structure and services essential to human well-being, including nutrient storage, storm protection, and habitat availability for certain fauna (Doughty et al., 2017, 2016; Kelleway et al., 2017; Osland et al., 2018; Simpson et al., 2019).

Avicennia germinans is hermaphroditic with clusters of white flowers (each with one stigma and four stamens) on axillary or terminal inflorescences (Lonard et al., 2017), and is recognised as predominantly outcrossed via insect pollination (Tomlinson, 1986). However, higher-latitude mangroves may encounter far less diverse sets of pollinators than their tropical conspecifics (Hermansen et al., 2014b). Few pollination studies exist for *A. germinans*, but those that do demonstrate greater pollinator diversity at lower latitudes (Sánchez-Núñez and Mancera-Pineda, 2012) compared to higher latitudes (Landry, 2013). Consistent with this latitudinal decline in pollinator diversity, indirect genetic evidence (via inbreeding coefficients, F_{IS}) supports predominant outcrossing in *A. germinans* populations closer to the range centre of this species (Cerón-Souza et al., 2012; Mori et al., 2015; Nettel et al., 2008; Ochoa□Zavala et al., 2019; but, see Salas-Leiva et al., 2009), with observations of elevated inbreeding towards range limits (Kennedy et al., 2020a; Mori et al., 2015; Ochoa□Zavala et al., 2019). Direct evidence via progeny arrays with *A. germinans* in Mexico also supports predominant outcrossing closer to the range centre (Nettel-Hernanz et al., 2013), but we still lack equivalent direct estimates of mating system towards *A. germinans* distributional limits.

Here, we used progeny arrays to estimate *A. germinans* outcrossing rates at six collection sites along a latitudinal gradient towards the expanding northern distributional limit of this species on the Atlantic coast of Florida. We then assessed variation in mating system (i.e., outcrossing versus self-fertilisation rates; Neal and Anderson, 2005) at three spatial scales. First, at the species-distribution level, we compared published outcrossing rates based on progeny arrays from tropical conspecifics to rates observed here for subtropical *A. germinans*. Second, at the population level, we assessed whether outcrossing rates decreased along the Florida latitudinal gradient that reaches the northern range limit of this species. Third, at the individual level, we assessed whether conspecific cover influences outcrossing rates of individual trees at the expanding range margin. We tested the following predictions: (1) outcrossing rates will decline from the tropics to subtropics; (2) population-level outcrossing rates will decline along the latitudinal gradient in Florida; (3) tree-level outcrossing rates will increase as conspecific cover increases at the Florida range margin.

Materials and methods

2.1 Collection sites and sampling methods

On 7-8 October 2017, we collected ~100 propagules from and recorded GPS coordinates for each of 30 maternal trees at six collection sites (n = 5 trees per site) along a latitudinal gradient (27.56 – 30.01^{o}N) on the Atlantic coast of Florida. We systematically collected propagules from around the entire canopy of each maternal tree. A leaf was also collected from each maternal tree and dehydrated in silica gel to obtain maternal genotypes. Atlantic Florida mangroves are replaced by salt marsh as the dominant coastal foundation species at approximately 29 ^{o}N (Spalding et al., 2010). Our three most southern collection sites (27.56 – 28.37^{o}N) are areas within the *A. germinans* continuous range core where mangroves are the dominant coastal foundation species; whereas, our three most northern collection sites (29.41 – 30.01°N) are areas at the *A. germinans* range margin where salt marsh species are dominant and *A. germinans* exists as discrete patches of isolated individuals (Fig. 1). Population genetic data for each of these collection sites was previously collected in 2015 (Table 1; Kennedy et al., 2020a). All propagules from an individual tree were kept together in one plastic bag during field collections. Propagules that were eventually genotyped had their pericarps removed and were stored at -20°C until DNA extraction.

Our study design reflects two principal factors: (1) Atlantic Florida *A. germinans* exhibits a considerable reduction in genetic variation towards the northern distributional limit (Kennedy et al., 2020a) and (2) limited polymorphism can restrict our ability to detect differences using molecular markers (Arnaud-Haond et al., 2005). We were concerned that reduced genetic variation may inhibit our ability to quantify reliable outcrossing rates and, as such, we needed to focus more sampling effort on the number of offspring per tree. This concern proved valid, as we determined that a relatively large number of offspring were needed to obtain reliable tree-level outcrossing estimates (see *2.3 Descriptive analyses*). Hence, for practical reasons, we were limited in the number of sampled maternal trees that could be included in this study. We included 23 maternal trees. From south to north, we genotyped progeny arrays from n = 5, 2, and 2 maternal trees at each of the three range-core sites, and n = 4, 5, and 5 maternal trees at each of the three range-margin sites (Table 1). We focussed more effort on the range-margin sites to better address our third prediction (i.e., tree-level outcrossing rates will increase as conspecific cover increases at the range margin). Although only two maternal trees were analysed at two of the range-core sites, we attempted to capture variation across as large of an area as possible.

These two forest patches (code: MH, U; Table 1) extend for approximately 2.3 and 3.9 km, respectively, along the adjacent river channel, and we selected trees that were separated by 1.3 and 1.2 km, respectively (i.e., 56% and 31% of the linear extent of these collection sites).

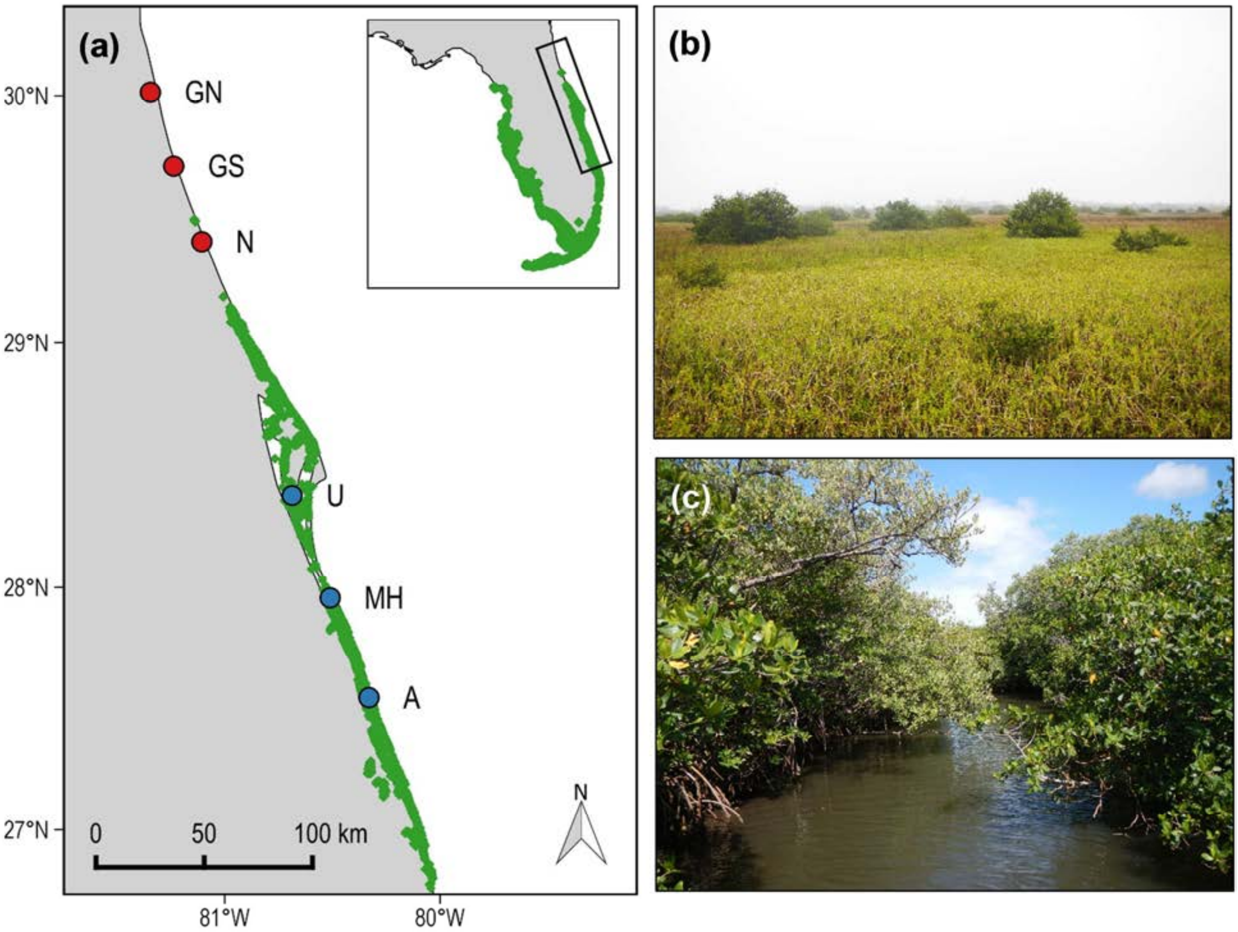

Fig. 1. Six collection sites along a latitudinal gradient (27.56 – 30.01°N) on the Atlantic coast of Florida (USA) for *Avicennia germinans* progeny arrays. (a) The three most southern sites (blue circles) are areas within the continuous range core of this species, where mangroves are the dominant coastal foundation species. The three most northern collection sites (red circles) are

areas at the range margin of this species, where salt marsh species are dominant and *A. germinans* exists in discrete patches. Mangrove distribution is shown in green (Giri et al., 2011). (b, c) Representative picture of range-margin and range-core community structure, respectively.

2.2 DNA isolation and microsatellite genotyping

For leaves from the 23 maternal trees, genomic DNA was isolated from 20 mg of dry tissue with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the standard protocol, with an extended incubation of 45 minutes. Trees were genotyped at 12 previously-developed nuclear microsatellite loci (Cerón-Souza et al., 2012, 2006; Mori et al., 2010; Nettel et al., 2005) following the protocol outlined in Kennedy et al. (2020a). We performed PCR on a Prime thermal cycler (Techne, Straffordshire, UK), analysed fragments on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA) with LIZ 500 size standard, and scored alleles in the R-package Fragman (Covarrubias-Pazaran et al., 2016). We amplified and genotyped DNA from each maternal tree twice to ensure we had the correct multi-locus genotype.

For each propagule, we removed the cotyledons and extracted DNA from portions of the hypocotyl and radicle, the eventual stem and root of the germinating seedling. Genomic DNA from propagules was isolated from 50 mg of frozen hypocotyl/radicle tissue with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany) following the standard protocol. Subsequent genotyping steps were identical to those for leaves, but we modified the PCR volumes outlined in Kennedy et al. (2020a). Each of the two multiplex PCR contained a total volume of 6 μL with 2.5 μL Multiplex PCR Master Mix, 0.5 μL primer mix, and 3 μL of genomic DNA. We also randomly re-amplified and re-genotyped 5% of our propagule DNA samples to estimate a study error rate (Bonin et al., 2004).

2.3 Descriptive analyses: maternal genotypes and sample sizes

We performed a discriminant analysis of principal components (DAPC) (Jombart et al., 2010) in the R-package adegenet 2.1.1 (Jombart and Ahmed, 2011) as a visual assessment of genetic differences among the 23 maternal trees. We retained nine principal components, the minimum number that explained ~90% of the total variance, identified two clusters, and retained three discriminant functions. We extracted each individual's coordinates on the two principal axes of the DAPC (i.e., ind.coord) and plotted them in ggplot2 (Wickham, 2011).

We performed an initial analysis to estimate how many genotyped propagules would be needed to provide reliable tree-level outcrossing estimates. For each of two trees (code: MH1, MH3), we genotyped as close to 100 propagules as possible (n = 87, 82, respectively) and estimated tree-level detectable outcrossing rates with the direct approach outlined below (see *2.4 Outcrossing calculations*). We then reduced the number of propagules in the data set by increments of five (i.e., n = 80, 75, 70, 65, 60, 55, 50, 45) and re-calculated outcrossing rates for each of these new subsets. This approach enabled us to visualise how outcrossing estimates changed as sample sizes decreased. Estimates of these subsets remained relatively consistent with the initial estimates (MH1: 0.6 – 6.8% change; MH3: 2.5 – 9.3% change) until $n \leq 55$ when values became more variable (MH1: 7.7 – 13% change; MH3: 16.1 – 43.1% change) (Fig. 2). As such, we determined that $n \geq 60$ propagules per tree should be sufficient to estimate reliable tree-level outcrossing rates.

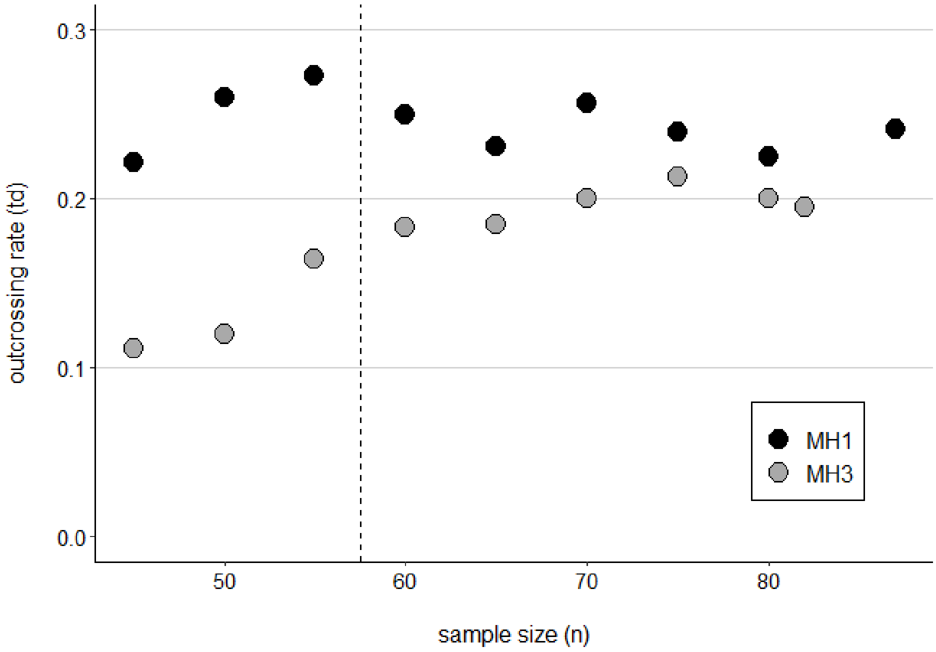

Fig. 2. Initial analysis to estimate an appropriate sample size of propagules to calculate tree-level outcrossing rates. Systematic reductions in sample size for two trees (MH1, MH3) continued to provide estimates of detectable outcrossing rate (t_d) relatively consistent with initial estimates until $n \leq 55$ when values became more variable (MH1: 7.7 – 13% change; MH3: 16.1 – 43.1% change). This threshold is shown with a vertical dashed line. We determined that $n \geq 60$ propagules should provide reliable estimates of tree-level outcrossing rates.

2.4 Outcrossing calculations: population-level and tree-level

To assess population-level mating system variation towards the Florida *A. germinans* range limit, we calculated multi-locus outcrossing rates (t_m) for each of the six collection sites with the maximum likelihood-based MLTR (Ritland, 2002). We also calculated levels of biparental inbreeding ($t_m – t_s$) and we used the proportion of offspring with the same father [$r_p(m)$] to calculate the number of effective pollen donors [1 / $r_p(m)$] for each collection site. We used default parameters, 500 bootstraps to calculate standard errors, and resampled among individuals within families.

To assess mating system variation among individual trees, we used a direct approach based on allelic differences between maternal genotypes and their sampled offspring to calculate apparent outcrossing rates (Cruzan et al., 1994). First, detectable outcrossing rates (t_d) were calculated as the number of propagules that possessed an allele not present in the maternal genotype (i.e., detectable outcross event) divided by the number of propagules genotyped. However, there may also be a percentage of offspring that appear to be the product of self-fertilisation, but are instead the result of outcrossing to individuals with similar genotypes to the maternal tree (i.e., undetectable outcross event). Hence, maternal genotypes with high-frequency alleles in a population will result in greater undetected outcrossing. Using allele frequency data from each of these six collection sites in 2015 (Kennedy et al., 2020b), we calculated the probability of an undetectable outcross event [$P(u)_j$] for each of the 23 maternal trees as the product across loci of the frequencies of each maternal allele within the corresponding collection site (Cruzan et al., 1994). For each maternal tree, we multiplied its specific $P(u)_j$ by the number of propagules that were not identified as outcrossed to obtain an estimate of the number of potential undetected outcross events. We then calculated apparent outcrossing rates (t_a) as the number of detectable outcross events plus the number of potential undetected outcross events divided by the number of propagules genotyped. We also estimated these tree-level outcrossing rates with the maximum likelihood-based MLTR, as described above for population-level estimates.

2.5 Estimates of conspecific cover at the range margin

It remains difficult to remotely assess mangrove cover at fragmented range margins (Bunting et al., 2018). However, mangrove patches within salt marsh can generally be manually identified with relative ease in satellite images, while also aided by knowledge of the collection site. For each of the three range-margin collection sites, we manually measured approximate estimates of area covered with *A. germinans* within a 10 m radius circle around each maternal tree (n = 14 GPS-referenced trees) with the polygon function in Google Earth Pro 7.3.2.5776. We chose this size because 10 m was the longest distance that permitted reliable estimates around maternal trees adjacent to terrestrial hammock forest patches.

2.6 Statistical analyses

We performed all statistical analyses in R v3.4.2 (R Core Team, 2013). To test prediction 1 (decline in outcrossing from tropics to subtropics), we compared mean multi-locus outcrossing rates (t_m) for these Florida collection sites (n = 6 sites) to published values for conspecifics in Mexico (n = 3 sites; Nettel-Hernanz et al., 2013) with a two-sample t-test. To test prediction 2 (decline in outcrossing along the Florida latitudinal gradient), we evaluated the relationship between t_m and latitude with a Spearman's rank correlation (n = 6 sites). To test prediction 3 (increase in outcrossing with increased conspecific cover at the range margin), we evaluated the effect of *A. germinans* cover around each range-margin maternal tree on tree-level apparent outcrossing rates (t_a) with a linear regression (n = 14 trees). We natural log-transformed *A. germinans* cover to meet the statistical assumption of normality.

Results

3.1 Maternal genotypes, sample sizes and study error rate

Each of the 23 maternal trees produced consistent multi-locus genotypes after being amplified and genotyped a second time. All 23 maternal genotypes were unique, with a range from 1 of 12 to 10 of 12 matching loci, and exhibited a clear separation between range core and margin genotypes (Fig. 3). We genotyped a total of 1,612 propagules, with a mean sample size per tree of 70.1 ± 5.3 (SD; range: 64 – 87). We re-amplified and re-genotyped DNA from 87 propagules (5.4% of all samples) and found an error rate of 0.01% (one error out of 1,044 locus comparisons). This locus-specific error was removed from the data set.

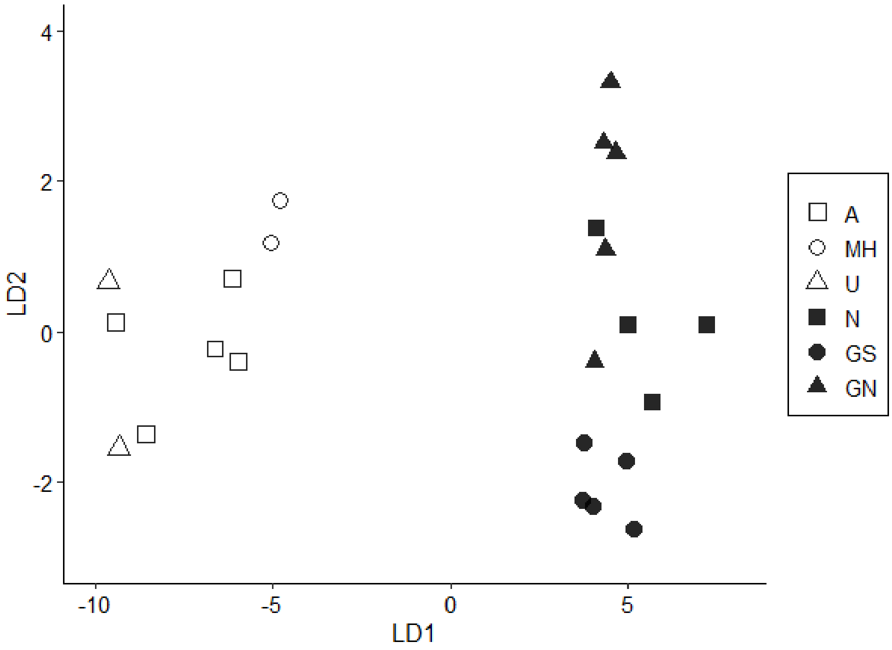

Fig. 3. Discriminant analysis of principal components (DAPC) of maternal tree multi-locus genotypes. All 23 maternal trees possessed unique multi-locus genotypes and exhibited a clear separation between range core and margin. Open shapes indicate range-core sites (A, Avalon;

MH, Maritime Hammock; U, Ulumay); filled shapes indicate range-margin sites (N, North Peninsula; GS, GTM South; GN, GTM North).

3.2 Population-level mating system variation

Population-level multi-locus outcrossing rates (t_m) in Florida ranged from 0.14 ± 0.02 (SE) to 0.49 ± 0.03 (Table 1). Florida t_m were, on average, more than two times lower than published values for tropical conspecifics in Mexico (mean t_m Florida: 0.32, Mexico: 0.71; $t_{4.8} = -4.8$, $p = 0.006$) (Fig. 4).

Population-level outcrossing (t_m) did not systematically decline along the Florida latitudinal gradient (Spearman's correlation, $r_s = -0.20$, $p = 0.70$). Instead, highest and lowest values were observed in range-margin sites (Fig. 4). All collection sites exhibited low levels of biparental inbreeding ($t_m - t_s = -0.01 – 0.06$) (Table 1). MLTR could not calculate the proportion of offspring with the same father [$r_p(m)$] for one range-margin site (code: GS), presumably because this site exhibited the lowest outcrossing rates (Table 1, 2). Estimates of $r_p(m)$ were calculated for all other collection sites and the number of effective pollen donors [$1 / r_p(m)$] varied from 2.5 to 20.4, with the lowest number of pollen donors at the northern range limit (code: GN) and the highest number at the most southern range-margin site (code: N) (Table 1).

Table 1 Population-level genetic diversity and mating system parameters of *Avicennia germinans* from six collection sites on the Atlantic coast of Florida (USA). AR, allelic richness; H_S, unbiased gene diversity; arrays, number of progeny arrays sampled; n, total number of propagules genotyped; t_m, multi-locus outcrossing rate; $t_m – t_s$, biparental inbreeding; $r_p(m)$, proportion of offspring with the same father; $1 / r_p(m)$, number of effective pollen donors. Standard error in parentheses. Note: estimates of $r_p(m)$ for collection site GS could not be calculated. [†]Data from Kennedy et al. (2020a) based on n = 30 adult trees per collection site genotyped at the same 12 nuclear microsatellite loci used here.

			genetic diversity†				progeny arrays								
Site	Code	Latitude	AR		H_S		arrays	n	t_m		$t_m - t_s$		$r_p(m)$		$1/r_p(m)$
Avalon	A	27.5468	3.40	(0.64)	0.42	(0.05)	5	336	0.37	(0.03)	0.03	(0.01)	0.16	(0.04)	6.2
Maritime Hammock	MH	27.9566	3.14	(0.48)	0.42	(0.05)	2	169	0.22	(0.04)	0.03	(0.01)	0.17	(0.07)	5.8
Ulumay	U	28.3756	2.33	(0.24)	0.31	(0.05)	2	135	0.39	(0.05)	-0.01	(0.02)	0.08	(0.12)	13.2
North Peninsula	N	29.4096	2.83	(0.61)	0.35	(0.06)	4	277	0.49	(0.03)	0.00	(0.02)	0.05	(0.02)	20.4
GTM South	GS	29.7159	2.85	(0.42)	0.40	(0.06)	5	342	0.14	(0.02)	0.02	(0.01)	-	-	-
GTM North	GN	30.0144	2.14	(0.29)	0.21	(0.05)	5	353	0.31	(0.02)	0.06	(0.01)	0.41	(0.07)	2.5

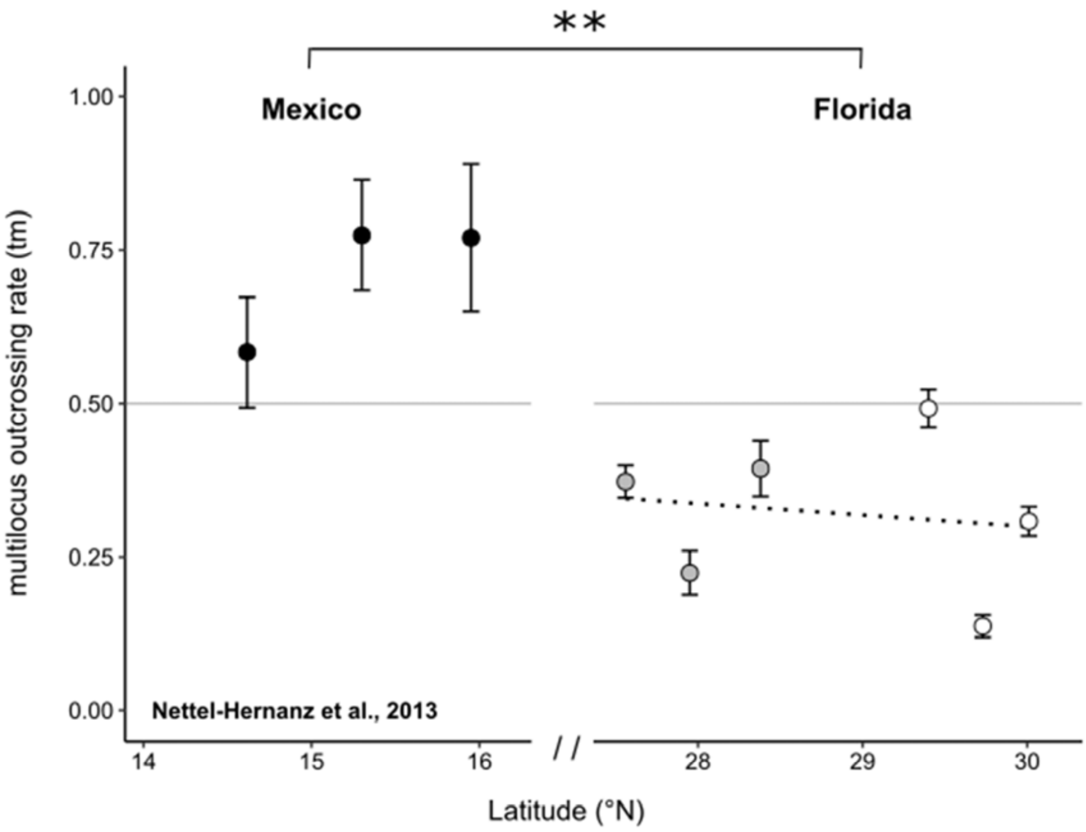

Fig. 4. Population-level multi-locus outcrossing rates (t_m) for tropical *Avicennia germinans* in Mexico (Nettel-Hernanz et al., 2013) and subtropical *A. germinans* in Florida (this study). Florida *A. germinans* mean t_m was more than two times smaller than their tropical conspecifics ($t_{4.8} = -4.8$, $p = 0.006$). Outcrossing (t_m) did not systematically decline along the Florida latitudinal gradient ($r_s = -0.20$, $p = 0.70$). Non-significant correlation is depicted with a dotted line. *A. germinans* in Mexico is shown in black, in Florida range core is shown in grey, and in Florida range margin is shown in white. Population error bars are standard error. **, $p < 0.01$.

3.3 Tree-level mating system variation

Tree-level detectable outcrossing rates (t_d) ranged from 0.05 to 0.58 (Table 2). Probabilities of an undetectable outcross event were generally low (0 – 0.09) and, as such, the number of potential undetected outcross events was also generally low (0 – 4.7) (Table 2). However, there were two notable exceptions with probabilities of 0.17 and 0.22, and a total of 9 and 12 potential undetected outcross events (tree: GN1, GN4; Table 2). These two exceptions were trees at the northern range limit, the least genetically-diverse collection site (Table 1). After incorporating potential undetected outcross events into our calculations, tree-level apparent outcrossing rates (t_a) still ranged from 0.05 to 0.58 (Table 2) and were highly correlated with t_d (Pearson's correlation, $r = 0.97$, $p < 0.0001$). Tree-level estimates calculated with MLTR were also highly correlated with both t_d ($r = 0.99$, $p < 0.0001$) and t_a ($r = 0.97$, $p < 0.0001$).

Conspecific cover at the range margin, measured as the area covered by *A. germinans* within a 10 m radius circle around each range-margin maternal tree, varied considerably from 15.5 to 177.4 m^2 (7 – 57% cover), with highest values at the most southern range-margin site (tree: N2-5; Table 2). Apparent outcrossing rates (t_a) increased as conspecific cover increased ($t_a = -0.46 + 0.20*\ln(\text{cover})$, $F_{1,12} = 21.0$, $p = 0.0006$, $r^2_{adj} = 0.61$), with a 2% increase in outcrossing with every 10% increase in *A. germinans* cover (m^2) (Fig. 5).

Table 2 Tree-level outcrossing rates and conspecific cover around range-margin trees. n, number of propagules genotyped; t_d, detectable outcrossing rate; $P(u)_j$, probability of an undetectable outcross event; undetected, number of potential undetected outcross events; t_a, apparent outcrossing rate; cover, *A. germinans* cover within a 10 m radius circle around each maternal tree at range-margin sites. Note: cover was not calculated for range-core collection sites.

Tree	Latitude	Longitude	n	t_d	$P(u)_j$	undetected	t_a	cover
A1	27.560644	-80.328730	72	0.33	0.000	0.0	0.33	-
A2	27.559835	-80.329391	67	0.49	0.050	1.7	0.52	-
A3	27.559641	-80.329857	66	0.42	0.002	0.1	0.43	-
A4	27.559870	-80.330614	64	0.17	0.003	0.2	0.17	-
A5	27.557966	-80.329410	67	0.42	0.014	0.6	0.43	-
MH1	27.958072	-80.515093	87	0.24	0.004	0.3	0.24	-
MH3	27.951081	-80.509048	82	0.20	0.001	0.1	0.20	-
U3	28.372275	-80.684404	70	0.23	0.088	4.7	0.30	-
U5	28.380151	-80.685456	65	0.52	0.003	0.1	0.52	-
N2	29.407971	-81.099778	65	0.40	0.006	0.2	0.40	177.4
N3	29.407679	-81.098927	70	0.40	0.035	1.5	0.42	44.5
N4	29.407872	-81.099167	70	0.53	0.026	0.9	0.54	111.1
N5	29.407941	-81.099617	72	0.58	0.002	0.1	0.58	173.2
GS1	29.729168	-81.240662	65	0.05	0.005	0.3	0.05	28.2
GS2	29.730158	-81.240638	66	0.06	0.038	2.3	0.10	21.3
GS3	29.730077	-81.241591	70	0.10	0.016	1.0	0.11	32.9
GS4	29.730190	-81.241972	71	0.14	0.013	0.8	0.15	54.1
GS5	29.729601	-81.242441	70	0.31	0.079	3.8	0.37	87.1
GN1	30.016524	-81.345922	70	0.23	0.222	12.0	0.40	45.5
GN2	30.016660	-81.345954	72	0.43	0.014	0.6	0.44	39.7
GN3	30.014781	-81.344683	72	0.49	0.081	3.0	0.53	93.2
GN4	30.014178	-81.344754	69	0.23	0.171	9.0	0.36	91.2
GN5	30.013645	-81.345022	70	0.06	0.026	1.7	0.08	15.5

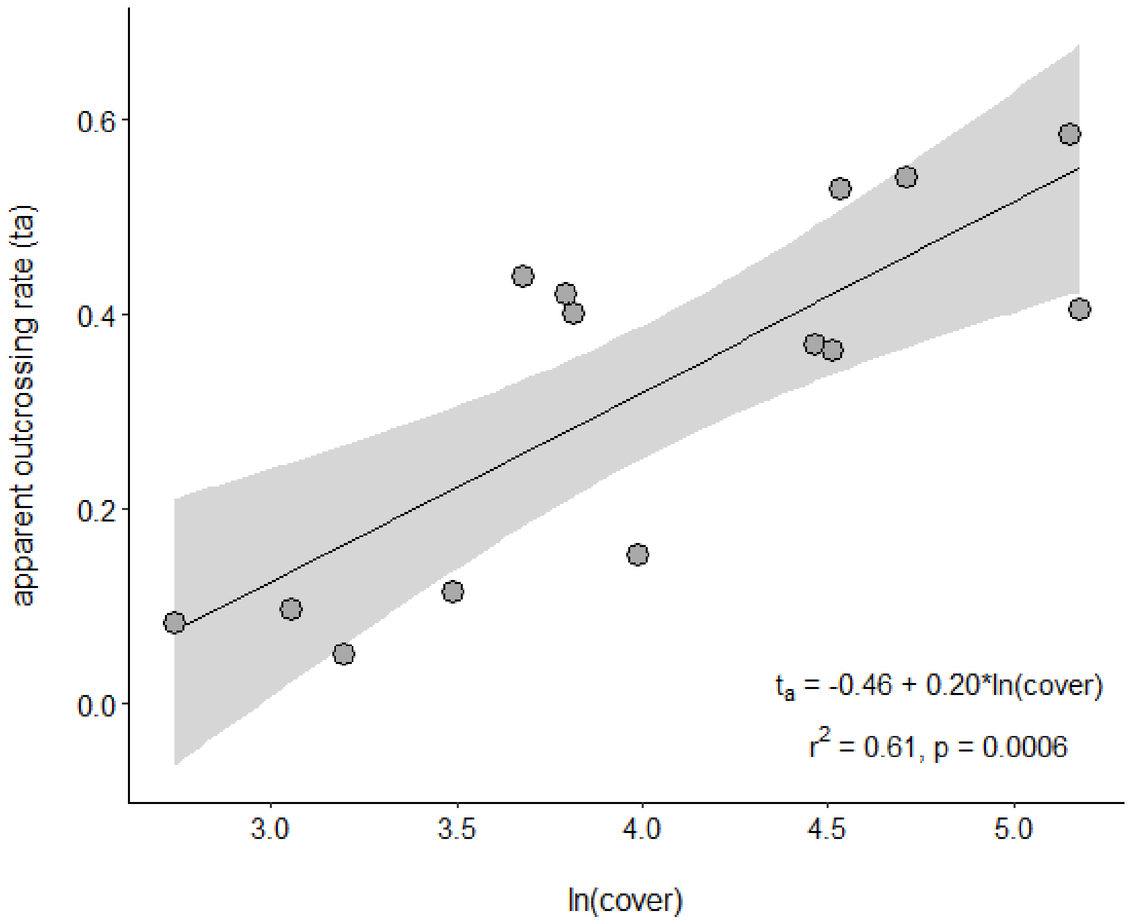

Fig. 5. Tree-level apparent outcrossing rates (t_a) increased as conspecific cover [ln(cover)] increased at the *A. germinans* range margin on the Atlantic coast of Florida (USA). Conspecific cover was measured as the area covered by *A. germinans* within a 10 m radius circle around each maternal tree at the three range-margin collection sites.

Discussion

We used progeny arrays to characterise mating system in *Avicennia germinans* at six collection sites along a latitudinal gradient towards the northern distributional limit of this species on the Atlantic coast of Florida (27.56 – 30.01°N). We assessed variation in mating system at three spatial scales: (1) at the species-distribution level, (2) at the population level along the Florida latitudinal gradient, and (3) at the individual level among conspecifics at the expanding Florida range margin. First, published outcrossing rates for tropical conspecifics in Mexico were, on average, more than two times higher than those for subtropical Florida *A. germinans*. Second, population-level outcrossing rates did not systematically decline with latitude towards the northern range limit in Florida. Third, tree-level outcrossing rates increased as conspecific cover increased at the Florida range margin.

4.1 Reduced outcrossing from tropics to subtropics

In contrast to the prevailing idea that *A. germinans* is predominantly outcrossed, we found that all Florida collection sites were predominantly self-fertilised. Tropical conspecifics in Mexico (14.61 – 15.95°N) exhibit predominant outcrossing (Nettel-Hernanz et al., 2013), with outcrossing rates more than double our observations for subtropical Florida *A. germinans*. Predominant outcrossing has also been documented for a co-occurring neotropical congener, *A. schaueriana*, at lower latitude (0.82°S) (Mori et al., 2015). Reduced outcrossing towards *A. germinans* distributional limits is consistent with a global decline in plant outcrossing rates towards higher latitude (Moeller et al., 2017). However, Moeller et al. (2017) found that this global pattern is mostly shaped by latitudinal changes in life history and growth form (i.e., lower latitude: greater frequency of perennial and tree species; higher latitude: greater frequency of annual and herbaceous species), and propose that evaluations of mating system variation in species with broad latitudinal distributions will help better understand the potential impact of plant-pollinator interactions in shaping latitudinal patterns in mating systems.

Reduced outcrossing in subtropical *A. germinans* is consistent with reductions in pollinator diversity across this species' broad distribution range. At lower latitude, four highly-effective pollinators were identified on the small, Caribbean island of San Andrés (12.54°N) (Sánchez-Núñez and Mancera-Pineda, 2012), and a diverse set of pollinators (including wasps, flies, and bees) was also identified for the neotropical congener, *A. schaueriana* (7.68°S) (Nadia et al., 2013). At higher latitude, only one principal pollinator, the exotic European honey bee (*Apis mellifera*), accounted for 87% of flower visits at a location in Southeast Florida (26.03°N) (Landry, 2013). However, we cannot disregard additional factors that could also impact the *A. germinans* mating system in Florida, such as reduced mangrove abundances towards distributional limits (Osland et al., 2017) and the highly-fragmented nature of Atlantic Florida mangroves due to anthropogenic modifications of these coastal ecosystems (Brockmeyer et al., 1996). Outcrossing often increases with conspecific density and pollinator abundance, but these two factors are not independent for animal-pollinated plants because dense groupings generally attract more pollinators (Ghazoul, 2005). Research near the southern latitudinal limits (33.94 – 34.63°S) of a widespread Indo-West Pacific congener, *A. marina*, highlights this point. Exotic European honey bees were also identified as the only significant pollinator for this species (Hermansen et al., 2014b) and smaller, more fragmented stands attracted fewer pollinators with altered foraging behaviour that may facilitate greater self-fertilisation (i.e., bees spent more time foraging on individual trees) (Hermansen et al., 2014a). As a result, these smaller *A. marina* stands exhibited reduced outcrossing compared to larger stands (Hermansen et al., 2015). Our observation of reduced outcrossing in subtropical Florida *A. germinans* may be the product of the interactive effects of reduced pollinator diversity and more fragmented mangrove area, that could also reduce pollinator abundances, compared to tropical mangrove forests. Further research needs to directly link assessments of mating system with plant-pollinator surveys across the broad, latitudinal distribution of *A. germinans*, as well as other *Avicennia* species, to provide definitive answers. In addition, research also needs to consider further environmental (e.g., seasonality; Chybicki and Dzialuk, 2014; Yin et al., 2016) and biological factors (e.g., fecundity, phenology; Ghazoul, 2005; Kameyama and Kudo, 2015) that can shape mating system variation.

Reductions in outcrossing are the product of a combination of biparental inbreeding and self-fertilisation. We found low biparental inbreeding at all collection sites, consistent with *A. germinans* progeny arrays in Mexico ($t_m - t_s = 0.01 - 0.06$; Nettel-Hernanz et al., 2013). However, progeny arrays with the neotropical congener, *A. schaueriana*, exhibited a greater effect of biparental inbreeding ($t_m - t_s = 0.15$; Mori et al., 2015), and even higher rates were found at the southern latitudinal limits of the Indo-West Pacific congener, *A. marina* ($t_m - t_s = 0.29 - 0.53$; Hermansen et al., 2015). Differences among these studies may be explained by interspecific variation in reproductive biology, variation in kinship structure among collection sites (Hasan et al., 2018), or simply methodological differences (e.g., number of progeny arrays, of propagules sampled, and of loci genotyped) that can impact these parameter estimates (Ritland, 2002). Our findings suggest that reduced outcrossing in subtropical Florida *A. germinans* is predominantly via self-fertilisation, either autonomous (within the same flower) or geitonogamous (among flowers on the same plant). Although we lack evidence for *A. germinans*, multiple *Avicennia* species are self-compatible, including the neotropical congener, *A. schaueriana* (Nadia et al., 2013), and three Indo-West Pacific congeners (Aluri, 1990; Raju et al., 2012), with a much higher fruit set via geitonogamous compared to autonomous self-fertilisation for all species. Geitonogamous self-fertilisation is common in bee-pollinated plants (Harder and Barrett, 1995; Mitchell et al., 2004), is thought to aid mangrove colonisation (Primack et al., 1981), and seems to be a likely mechanism in *A. germinans* as inflorescences have multiple flowers open simultaneously.

4.2 Density-dependent mating system variation towards range limits

We did not observe a systematic decline in outcrossing rates towards the expanding *A. germinans* northern range limit in Florida, consistent with the prevailing view that adaptive shifts in mating system are not common during range expansion of long-lived trees and shrubs (Barrett and Harder, 2017). Instead, density-dependent plastic shifts in mating system may occur, with transitions between increased self-fertilisation at low density and mixed mating at higher density (Morgan et al., 2005; Peterson and Kay, 2015). We found lowest outcrossing at the range margin, but we also observed highest outcrossing at the most southern range-margin site (Fig. 4). While low density and greater spatial isolation among conspecifics at range margins can reduce outcrossing (Ghazoul, 2005), the same can also be true in areas with higher species diversity that may increase competition for pollinators (Vamosi et al., 2006). Higher-density forests can also restrict routes of pollen vectors and lead to declines in the diversity of tree-level pollen donors (González-Varo et al., 2009). Lower-latitude Florida mangroves consist of dense forests with *A. germinans* and two other mangrove species (*Rhizophora mangle*, *Laguncularia racemosa*), with evidence of competition for pollinators between *A. germinans* and *L. racemosa* (Landry, 2013). In contrast, higher-latitude range-margin Florida mangroves are patches of almost exclusively *A. germinans* individuals surrounded by lower-stature salt marsh species (Kangas and Lugo, 1990). A more open pollen-dispersal neighbourhood and limited interspecific competition at the higher-latitude range margin could facilitate inter-tree pollination among *A. germinans*, consistent with our observation of highest outcrossing and largest number of effective pollen donors at the most southern range-margin site. However, this greater potential for inter-tree pollination would be overshadowed farther north where limited numbers of conspecifics would provide very few potential pollen sources, as evidenced by the lowest number of effective pollen donors at the northern range limit.

At the range margin, tree-level outcrossing increased as conspecific cover increased. Lowest outcrossing ($t_a = 0.05$) at an *A. germinans* cover of 28.2 m^2 (7% of surrounding area covered by *A. germinans*) was more than ten times smaller than highest outcrossing ($t_a = 0.58$) at a cover of 173.2 m^2 (57% covered by *A. germinans*). We presume that continued proliferation of *A. germinans* at this range margin, forecast with climate change (Cavanaugh et al., 2019, 2015), will drive further mating system changes as increased conspecific density shifts mating systems towards greater outcrossing (Pannell, 2015). As *A. germinans* becomes more abundant, inter-individual distances will decline, presumably these denser patches will attract more pollinators, and tree-level outcrossing will increase. Hence, documented loss of genetic diversity towards *A. germinans* range limits (Kennedy et al., 2020a; Mori et al., 2015; Ochoa□Zavala et al., 2020, 2019; Sandoval-Castro et al., 2014), and for other *Avicennia* species (Arnaud-Haond et al., 2006; Binks et al., 2019; De Ryck et al., 2016; Maguire et al., 2000), may be the product of founder effects combined with density-dependent plastic shifts towards greater self-fertilisation. Areas that experience subsequent proliferation and immigration will then transition towards greater outcrossing and increased offspring genetic diversity, but will exhibit unique genetic signatures because of the disproportionate contribution of initial colonisers to the gene pool (e.g., Goldberg and Heine, 2017).

Plastic shifts towards greater self-fertilisation at range margins can be advantageous as a form of reproductive assurance (Hargreaves and Eckert, 2014) and tend to elevate seed production when pollinators or mates are unreliable (Morgan et al., 2005). However, these advantages may be offset by the genetic costs associated with inbreeding depression (e.g., reduced offspring survival and fertility) (Charlesworth and Willis, 2009). These costs are documented near the southern latitudinal limits of the Indo-West Pacific congener, *A. marina*, where smaller, less-outcrossed stands exhibited reduced reproductive success, reduced propagule size, and reduced seedling recruitment compared to larger stands (Hermansen et al., 2017). However, our finding of predominant self-fertilisation in *A. germinans* runs contrary to mangrove performance at the Atlantic Florida range margin. These mangroves have undergone proliferation and expansion for several decades (Cavanaugh et al., 2019, 2014; Rodriguez et al., 2016), with evidence from a co-occurring mangrove species, *Rhizophora mangle*, of precocious reproduction and increased propagule size (Dangremond and Feller, 2016) and greater reproductive success (Goldberg and Heine, 2017) compared to Florida conspecifics farther south. Characterisation of *A. germinans* mating system coupled with assessments of offspring performance at this range margin is needed to garner insights into the potential influence of mating system on these expanding populations.

4.3 Considerations

Mating system assessments are a balance between the number of progeny arrays and the number of offspring genotyped per progeny array. Here, we focussed our efforts more on genotyping larger numbers of offspring per progeny array. Our sampling design was shaped by our concern that reduced genetic variation towards this northern distributional limit could inhibit our ability to quantify outcrossing rates. This concern was valid as we found that a substantial tree-level effort ($n \geq 60$ propagules per tree) was likely needed to obtain reliable estimates. In addition, although the probability of an undetectable outcross event was low across most maternal trees, two trees at the northern range limit exhibited relatively high probabilities, with 9 and 12 potential undetected outcross events. Estimates based on smaller sample sizes per tree, comparable to research in the tropics (14-18 propagules on average per tree; Nettel-Hernanz et al., 2013), could have been overwhelmed by undetectable outcross events and potentially underestimated outcrossing at this northern range limit.

However, a greater investment of resources at the tree-level inevitably limited our ability to genotype progeny arrays from a larger number of maternal trees (n = 2-5 trees per collection site). Our sampling design may have provided robust tree-level estimates, but our estimates scaling up to the population level should be interpreted with caution. Sampling few maternal trees can bias these estimates towards tree-specific outcrossing rates that may not be representative of the entire collection site. Although our estimates (based on n = 23 maternal trees) are consistent with a substantial reduction in outcrossing compared to estimates from tropical conspecifics (based on n = 22 maternal trees; Nettel-Hernanz et al., 2013), and are not consistent with a systematic adaptive shift in mating system towards the northern range limit, further supported by evidence of density-dependent variation at the range margin, more intensive sampling of progeny arrays at each collection site is needed to obtain more definitive estimates of population-level mating system variation. Further research into mating systems at range margins, or areas with reduced genetic variation, should consider an investment in both more progeny arrays and large numbers of offspring per progeny array to generate reliable population-level outcrossing estimates.

4.4 Conclusions

This research suggests that ecological structure influences the mating system of the neotropical black mangrove, *A. germinans*, at varying spatial scales towards its expanding northern distributional limit on the Atlantic coast of Florida. First, subtropical Florida *A. germinans* exhibited significant reductions in outcrossing compared to tropical conspecifics, consistent with reductions in pollinator diversity and mangrove abundance with latitude. Second, the transition from mangrove to salt marsh dominance along Atlantic Florida may create a more open pollen-dispersal neighbourhood that is conducive to elevated *A. germinans* outcrossing, until conspecific abundances become too low towards the range limit. Third, greater inter-individual isolation at the range margin resulted in drastic reductions in tree-level outcrossing, consistent with density-dependent plastic shifts in mating system that we presume will continue to shift towards greater outcrossing as these mangroves continue to proliferate with forecast climate trends. Further research needs to evaluate the effect of *A. germinans* mating system variation on the survival and fitness of offspring and on the extent of population-level local adaptation at expanding distributional limits.

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Highlights

- Range expansion of foundation species may lead to shifts in their mating systems.
- Subtropical Florida *Avicennia germinans* exhibited predominant self-fertilization.
- Outcrossing did not systematically decline towards the Florida range limit.
- Outcrossing increased with conspecific cover at the Florida range margin.
- Ecological structure may impact mangrove mating systems at varying spatial scales.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: