Whole-genome sequencing reveals the extent of heterozygosity in a preferentially self-fertilizing hermaphroditic vertebrate

Luana S.F. Lins, Shawn Trojahn, Alexandra Sockell, Muh-Ching Yee, Andrey Tatarenkov, Carlos D. Bustamante, Ryan L. Earley, and Joanna L. Kelley

Abstract: The mangrove rivulus, Kryptolebias marmoratus, is one of only two self-fertilizing hermaphroditic fish species and inhabits mangrove forests. While selfing can be advantageous, it reduces heterozygosity and decreases genetic diversity. Studies using microsatellites found that there are variable levels of selfing among populations of K. marmoratus, but overall, there is a low rate of outcrossing and, therefore, low heterozygosity. In this study, we sequenced whole-genome data to assess the levels of heterozygosity in different lineages of the mangrove rivulus and infer the phylogenetic relationships among these lineages. We sequenced whole genomes from 15 lineages that were completely homozygous at microsatellite loci and used single nucleotide polymorphisms (SNPs) to determine heterozygosity levels. More variation was uncovered than in studies using microsatellite data because of the resolution of full genome sequencing data. Moreover, missense polymorphisms were found most often in genes associated with immune function and reproduction. Inferred phylogenetic relationships suggest that lineages largely group by their geographic distribution. The use of whole-genome data provided further insight into genetic diversity in this unique species. Although this study was limited by the number of lineages that were available, these data suggest that there is previously undescribed variation within lineages of K. marmoratus that could have functional consequences and inform us about the limits to selfing (e.g., genetic load, accumulation of deleterious mutations) and selection that might favor the maintenance of heterozygosity. These results highlight the need to sequence additional individuals within and among lineages.

Key words: Kryptolebias marmoratus, heterozygosity, self-fertilizing.
Introduction

Self-fertilization is a mode of reproduction employed by many plants and invertebrates, and comes with inherent advantages and disadvantages (reviewed in Shimizu and Tsuchimatsu 2015). Selfing assures reproduction when few mating partners are available. While selfing can result in coadapted suites of alleles that confer high fitness in a given environment (Allard 1975), it can also drive populations quickly toward extinction and dampen responses to selection (Noel et al. 2017). Many selfing species also can outcross (i.e., a mixed mating system), which introduces genetic diversity at the individual and population levels. Recent studies have shed light on the genomic and evolutionary consequences of selfing (Burgarella et al. 2015; Noel et al. 2017), but the extent to which genetic diversity is maintained in mixed-mating species remains an open question.

Mangrove rivulus fish (Kryptolebias marmoratus) are an excellent model in which to address such a question because individuals can exist as hermaphrodite or male (Mackiewicz et al. 2006a). The most common mode of hermaphrodite reproduction for K. marmoratus is self-fertilization (Harrington 1961), which can generate isogenic lineages characterized by complete homozygosity (at 32 neutral markers; Mackiewicz et al. 2006a). Predominant self-fertilization in K. marmoratus has both disadvantages and advantages. Selfing can create barriers to gene flow between adjacent populations, resulting in varying levels of differentiation among populations. For example, a study utilizing microsatellite data found that the genetic differentiation among populations only 112 km apart was high, with differentiation values (measured by Fs) approaching 0.26 between some populations (Tatarenkov et al. 2012). Rare outcrossing events occur between males and hermaphrodites and result in a burst of heterozygosity, which is then reduced through subsequent generations of selfing (Mackiewicz et al. 2006b). Additionally, previous studies have highlighted the impact self-fertilization has on the immune system and body size of these fish, with a reduction in heterozygosity (measured via microsatellites) leading to an increased parasite load (Ellison et al. 2011) and smaller adult male size (Molloy et al. 2011). Alternatively, selfing can maintain locally well-adapted genotypes (Avise and Tatarenkov 2012). Selfing can also potentially have a positive fitness effect in individuals by saving energy otherwise invested in courtship.

This species has a broad geographic distribution ranging from Florida, the Bahamas, the Caribbean, to Central America (Davis et al. 1990). Preferentially inhabiting mangrove forests, mangrove rivulus are particularly well-adapted for this constantly changing habitat (Davis et al. 1990). Individuals are able to survive a wide range of environmental conditions, including high hydrogen sulfide levels, low oxygen levels, and a large range of salt concentrations (Taylor 2012). Individuals can also leave the water (emersion) for extended periods of time to avoid these extreme conditions (Abel et al. 1987). The ability to survive such extreme environmental fluctuations coupled with their preference for self-fertilization allows for genetic variation to be maintained within a location as subpopulations with distinct genotypes.

There is substantial evidence that outcrossing occurs in most mangrove rivulus populations (Lubinski et al. 1995; Mackiewicz et al. 2006a), which is likely driven by male–hermaphrodite matings. Crossing between hermaphrodites has not been observed either in the laboratory or in the wild; therefore, it is likely that males must be present for an outcrossing event to occur (Turner et al. 2006; Furness et al. 2015). Males can arise in a population of K. marmoratus in two ways: either via temperature-dependent sex determination during embryogenesis or via temperature-dependent sex change (Harrington 1961, 1967, 1968; Turner et al. 2006; Ellison et al. 2015). Both mechanisms can lead to the spontaneous occurrence of males in a population. The percentage of males varies among wild populations, with males making up between 2% (Florida) and 25% (Twin Cayes, Belize) of the population (Davis et al. 1990). This difference in sex ratios may lead to varying outcrossing rates among populations (Turner et al. 2006; Tatarenkov et al. 2015). While outcrossing events appear to be rare in most populations, the amount of outcrossing varies drastically by site, with some sites having a selfing rate of 80%–90% (Florida populations) and others roughly 40% (Twin Cayes population), which further contributes to the varying levels of differentiation among populations (Mackiewicz et al. 2006a). A high level of homozygosity within populations of K. marmoratus but high levels of differentiation among lineages, as shown by previous studies utilizing DNA fingerprinting (Turner et al. 1990) and microsatellite data (Tatarenkov et al. 2010). To date, no study has combined both mitochondrial and full genome sequencing data to study genomic variation in this species. These types of data are essential to determine whether diversity seen in only a few markers (e.g., microsatellites) adequately describes genetic diversity and, ultimately, to explore whether and through which evolutionary mechanisms heterozygosity produced through male–hermaphrodite outcrossing is maintained in the genome.

This study utilizes high-throughput sequencing data to infer genetic relationships among 14 laboratory-reared lineages of K. marmoratus and one laboratory-reared lineage of the sister species Kryptolebias hermaphroditus, the only other preferentially self-fertilizing vertebrate. Additionally, this study aims to determine the levels of intra-individual heterozygosity using single nucleotide polymorphisms (SNPs) in lineages that have been maintained in the laboratory for as short as 1 and as many as 11 generations.

Material and methods

Sample collection

One sample from each of 14 isogenic lineages of K. marmoratus and one sample from the sister species K. hermaphroditus (GTMO) were included in the study (Table S1'). It should be noted that recent genetic and taxonomic analyses showed that earlier names—K. bonairensis and K. heyi—are available for Caribbean populations of species designated here as K. hermaphroditus (Tatarenkov et al. 2017a). As a result, the GTMO sample will ultimately bear one of these names. However, because taxonomy of this species is still in a state of flux, here we chose to use the recognized name K. hermaphroditus. Data from the K. marmoratus lineage RHL is the same as that used by Kelley et al. (2016) to construct the reference genome used in this study. All samples were obtained from laboratory stocks in the Earley Lab. The lineages were sampled from throughout the species range (Fig. 1; Table S1'). Representative samples from each of the isogenic lineages were genotyped for 32 microsatellites (Fig. S1'); note that samples genotyped were not the same individuals as those used in this study. Individuals were euthanized with a lethal dose of sodium bicarbonate-buffered Finquel® (MS-222, tricaine methanesulfonate) and muscle tissue was dissected and flash frozen at −80 °C.

Library preparation and sequencing

DNA was extracted from ~50 mg flash frozen tissue using the Qagen Genta Puregene Tissue kit with the following modifications: samples were placed in Covaris TT1 bags, immersed in liquid nitrogen, and then pulverized using a Covaris CryoPrep system. The pulverized tissue was then incubated at 56 °C for 3 h in 300 μl cell lysis buffer with 15 μl Proteinase K to complete cell lysis. All steps following cell lysis were performed per the Genta Puregene protocol. Genomic DNA was checked for high molecular weight content before sequencing by agarose gel electrophoresis.

Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2017-0188.
by running on a 2% agarose Invitrogen E-gel, and concentration was
determined using a Thermo Fisher Qubit Fluorometer.

Sequencing libraries were fragmented using either sonication
or Illumina Nextera tagmentation technology (Table S2). For the
libraries prepared using sonication, 500 ng of genomic DNA was
sheared to an average size of ~500 base pairs (bp) using a Covaris
sonicator. The KAPA BioSystems Library Preparation Kit for Illu-
mina was used for end repair, adapter ligation, and amplification.
Libraries were amplified with eight cycles of PCR using KAPA’s
recommended standard cycling conditions. Size selection was per-
formed using a 0.6X Agenecourt AMPure bead cleanup to select for
an average fragment size of 400 bp. For the libraries prepared using
the Illumina Nextera tagmentation technology, the standard
protocol was followed except that the tagmentation was followed
by size selection on a PerkinElmer Labchip XT 750. The quality
of all libraries was assessed using an Agilent Bioanalyzer,
and concentration was determined by Qubit. Libraries were then
pooled to achieve an equimolar concentration of each library
prior to sequencing at the Stanford Genome Sequencing Service
Center on a HiSeq 2000 with the paired read 101 bp option.

Nuclear genome analysis—data processing and analysis

Prior to mapping, raw reads were inspected using FastQC
(Arends 2010). The adapters were trimmed with a minimum
overlap of 5 bp, and the reads were trimmed based on the quality
with a minimum value of 28 PHRED score, additionally depending
on base composition the reads were trimmed at the 5’ end of both
reads using TrimGalore! (Table S2) (Krueger 2015). Coverage was
estimated based on the mapped reads using Picard Tools (http://
broadinstitute.github.io/picard) (Table S2). Reads from each sample
were mapped to the reference genome (GCA_001663955.1, (Kelley
et al. 2016) including the mitochondrial genome (Tatarenkov
et al. 2017b) using the Burrows–Wheeler aligner algorithm BWA-MEM
(Li 2013). The resulting SAM files were converted to BAM format
using SAMTOOLS 1.2 (Li et al. 2009), and read group information
was added using Picard Tools. Variants were called on each sample
using the Genome Analysis Toolkit (GATK version 3.7) (Li 2013)
HaplotypeCaller module. The files were combined using the com-
bineGVCFs module, and then joint genotyping was performed
across samples using GATK module GenotypeGVCFs. Sites for
which a genotype could be determined (callable loci) across the
genome were identified using the GATK module CallableLoci,
with minimum coverage of 4 and maximum coverage of 250. SNPs
were extracted using the GATK module SelectVariants. The SNPs
were flagged using VariantFiltration in GATK using the following
criteria: QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < –12.5,
ReadPosRankSum < –8.0. Sites that passed the filter criteria were
kept using VCFtools (v0.1.15) (Danecek et al. 2011). Sites missing
more than 15% of genotypes were excluded from the analysis,
which is equivalent to excluding sites where at least two individu-
als are missing genotypes.

Summary statistics were calculated using VCF-stats (Danecek
et al. 2011). To calculate the ratio of heterozygous to homozygous
sites, the number of heterozygous sites were divided by the num-
We also performed a principal component analysis (PCA) using PLINK (1.07) (Purcell et al. 2007) with data that were thinned to exclude SNPs that were within 5 kb of each other using VCFTools (Danecek et al. 2011) to minimize the effect of linkage disequilibrium. A final set of 140 081 SNPs was included in the PCA analysis. Identity by decent relatedness was calculated using VCFTools (Manichaikul et al. 2010; Danecek et al. 2011).

Inference of population splits from the nuclear genome

The VCF file containing filtered SNPs was converted to plink format using VCFTools (Danecek et al. 2011). PLINK was used to determine allele frequencies for each lineage at each site (Chang et al. 2015). The allele frequencies were used to create a TreeMix-formatted file utilizing the plink2treemix python script included in the TreeMix package (v1.13) (Pickrell and Pritchard 2012). TreeMix was run using SNPs grouped in windows of 500, sample size correction was turned off, and GTMO was specified as the root. A bootstrap analysis with 1000 replicates was performed; bootstrap support (bs) through the manuscript is presented as percentage. The TreeMix analysis was also performed with samples grouped by location.

Mitochondrial phylogenetic analysis

Mitochondrial genomes were assembled for 14 lineages of K. marmoratus and 1 lineage of K. hermaphroditus with ARC (Hunter et al. 2015), subsampling the raw genomic sequence reads to achieve approximately 30x coverage as per the ARC user manual (Table S22). Sequences for the 13 protein-coding genes were identified using MitoAnnotator (Iwasaki et al. 2013). Mitochondrial phylogenetic analyses were performed using the 13 protein-coding genes. The 15 samples from this study were combined with 4 samples obtained from GenBank (Kim et al. (2016) (accession number: NC_032387.1), Lee et al. (2001) (accession number: AF283503), Rhee et al. (2017) (accession number: PRJNA37650), and Tatarenkov et al. (2017b) (accession number: KT893707)). Nucleotide sequences for each gene were aligned using the default options in MUSCLE (Edgar 2004). Alignment files were concatenated using FASCONcat (Kück and Meusemann 2018). Two K. hermaphroditus samples were used as an outgroup: a new sequence from this study (GTMO) and one sequence from GenBank (accession number: NC_032387.1) (Kim et al. 2016). We assigned an independent model of nucleotide substitution to each gene, chosen using PartitionFinder 2.1.1 (Lanfear et al. 2016). Model K81+I (for position 1 in all genes), Model HKY+I (for position 2 in all genes), and Model TRN+C (for position 3 in all genes). We performed both maximum likelihood and Bayesian analyses on the mitochondrial genome dataset. Maximum likelihood analysis was performed using RAxML 8.2.9 (Stamatakis 2014). Node support was estimated using 1000 rapid bootstrap replicates. Bayesian analysis was conducted in MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003) using default priors. The Markov chain Monte Carlo was run for 10 million generations sampling every 1000 generations, with two parallel runs each with four chains (three hot and one cold). Convergence was considered reached on the basis of the standard deviation of split frequencies (<0.01). The first 10% of trees were discarded as burn-in.

Data deposition

Sequence data have been deposited under NCBI BioProject PRJNA385014.

Results

There were 2 106 131 SNPs in the entire whole-genome resequencing dataset, 1 168 538 of which are variable in K. marmoratus. Using these SNPs, we determined the level of heterozygosity present in K. marmoratus lineages that were identified as being completely homozygous using 32 microsatellite markers. The percent of heterozygous sites per individual ranged from 0.0305% (RHL) to 0.0554% (LION2) per_callable region of the genome (Table S32). For K. marmoratus, the count of private alleles (alleles that are only found in one lineage) varied between 7279 (UNK) and 46 557 (R2) and for K. hermaphroditus (GTMO) there were 914 466 private alleles (Table S33). The heterozygous to homozygous ratio ranged from 0.83 (R2) to 1.54 (SLCE8) (Fig. S22). As the data for RHL were used to assemble the reference genome (Kelley et al. 2016), there are very few homozygous non-reference sites and RHL was excluded from the analysis of heterozygous to homozygous ratios.

The correlation between the percent of heterozygous sites and the number of generations in the laboratory was not significant (Fig. S32; R^2 = 0.14, p = 0.23). There were 1 468 846 predicted effects of the variants determined by SnpEff from the 1 168 538 SNPs specific to K. marmoratus. While 9.8% of the genome is coding, only 3.5% of the predicted effects were found within coding regions. Additionally, 54.4% of the SNPs found within coding regions were missense and 44.7% were silent. The number of heterozygous sites in each individual ranged from 126 649 (VOL) to 251 480 (UNK) and the percent of heterozygous sites found within coding regions ranged from 4.417% (RHL) to 3.970% (Vol). Finally, the Ts/Tv ratio was calculated to be 1.76, which is slightly lower than the expected 2.0. There were 13 overrepresented GO terms associated with genes that had greater than one SNP resulting in a missense mutation present (Table S42). The lineage with highest proportion of long ROH in the genome shows a positive correlation with the number of generations in the laboratory (Fig. S52; R^2 = 0.67, p = 0.025).

To investigate the genetic structure of the lineages, we performed PCA using the nuclear SNPs and phylogenetic analyses with both nuclear SNPs and mitochondrial sequences. PCA separated the sister species K. hermaphroditus (GTMO) from K. marmoratus on PC1 (Fig. S62; percentage of variance explained by PC1 = 17.21%, PC2 = 2.47%). In the PCA performed with only populations of K. marmoratus, the populations clustered by geographic location except for DAN2K (Fig. 2; PC1 = 2.57%, PC2 = 1.6%). Additionally, this study found one misplaced lineage (UNK), a problem which has been previously noted (Tatarenkov et al. 2010); based on the PCA, the lineage clusters closely with the Belizean lineage DAN2K. Lineages from Florida clustered closely together in the PCA space; therefore, we estimated relatedness among the Floridian lineages. The identity by descent probability relatedness (r) among only Floridian K. marmoratus lineages

![Fig. 2. Principal component analysis (PCA) of genetic variation among samples of Kryptolebias marmoratus. PCA performed using PLINK (Purcell et al. 2007). To minimize the effects of linkage disequilibrium, the data were thinned to exclude SNPs that were within 5 kb of each other using VCFTools (Danecek et al. 2011). A total of 140 081 SNPs were included in the analysis. PC1 explains 2.57% of the variation and PC2 explains 1.60% of the variation.](image-url)
shows that BBSC, FDS1, LION2, and SLC8E are closely related (Fig. S7*).

The maximum likelihood topology for the genomic SNP data given by TreeMix (Fig. S8*) groups the Floridian lineages with the Bahamian lineage (bs = 100). The lineage labeled UNK grouped with the lineage from Belize DAN2K (bs = 100). The Honduran lineages did not group together; HON grouped with the lineages from Belize (DAN2K, FW2, BWN3) and the lineage labelled UNK (bs = 100). R2 appeared as an early branching lineage; however, there was no support for this position. Additionally, when grouping individuals by sampling location, Belizean and Honduran lineages clustered together (bs = 95) and the lineage from the Bahamas is the first branch to appear (bs = 100) (Fig. S9*).

The topologies of the maximum likelihood and the Bayesian analyses of the mitochondrial genomes were identical based on 13 protein-coding genes with 11 436 sites; of those, 579 are variable and 487 are parsimony informative (Fig. 3; Fig. S10*). There were several samples that were identical to each other (FW2 and BWN3; UNK and DAN2K; FDS1 and FDS08). All seven lineages from Florida (including FDS08 from Tatarenkov et al. (2017b)) and one from the Bahamas (RHL) grouped together in a single clade (bs = 57, posterior probability (pp) = 0.78). Samples from the studies by Lee et al. (2001) and Khee et al. (2017) grouped together (bs = 100, pp = 1). The lineage labeled UNK grouped with the lineage from Belize (DAN2K; bs = 100, pp = 1). The sister lineage of all above-mentioned lineages is the clade with the Honduran lineage HON9 and the two Belizean lineages FW2 and BWN3. R2 did not group with the other Honduran lineage (HON9), and instead it was an early branching lineage among K. marmoratus lineages.

**Discussion**

*Kryptolebias marmoratus* has always been regarded as having high rates of selfing in the wild meaning that outcrossing has been considered as a minor component of the mating strategy, with the exception of lineages in Twin Cayes, Belize (Lubinski et al. 1995). These conclusions have been drawn by the fact that males exist at very low frequencies (Vrijenhoek 1985) and that microsatellite markers are often highly homozygous in wild populations (Turner et al. 1990). Given the homozygous nature of the micro-satellites for lineages used in this study, the heterozygosity results show that there is previously undescribed variation in individuals of *K. marmoratus*. This variation is rare, as most SNPs were found as singletons and were found mainly in intergenic regions of the genome, with only 3.5% of the SNPs found in coding regions even though ~9.8% of the genome is made up of coding sequences. Looking closer at the SNPs within the coding region, specifically focusing on genes that have greater than one missense mutation, we found that SNPs resulting in a missense mutation are more likely to fall within genes associated with the immune system. This is the first genomic evidence in *K. marmoratus* of the importance of genetic variation in genes associated with the immune system and supports the findings of Ellison et al. (2011), which showed that outcrossed fish had a lower parasitic load than selfing lineages. Other studies showed that there appears to be considerable heterozygosity in major histocompatibility complex (MHC) genes and maintenance of diverse MHC supertypes, despite persistent homozygosity at neutral markers and non-MHC loci (Sato et al. 2002; Ellison et al. 2012). Collectively, these data suggest the potential for parasite and/or pathogen-mediated selection on the maintenance of genetic diversity within some regions of the genome. Many of the remaining genes that had more than one SNP resulting in a missense mutation are associated with reproduction and merit further study to determine the role they play in this self-fertilizing species.

The ratio of heterozygous to homozygous sites for some lineages indicates that outcrossing may be frequent among individuals of *K. marmoratus* and that it may play a role in maintaining variation within and among lineages (Fig. S2*). Although the ratios in *K. marmoratus* were lower than in a randomly mating population (the expected heterozygous to homozygous ratio in randomly mating populations is 2 (Jun et al. 2012)), the values for several of these lineages were greater than 1.25. A possible interpretation is that rare outcrossing events occur between distant lineages, which is supported by the findings of Lomax et al. (2017) showing that in some seasons egg laying increased as a function of increased genetic dissimilarity in *K. marmoratus*. Additionally, Ellison et al. (2013) showed that males prefer genetically dissimilar hermaphrodites, which should promote more outcrossing between genetically distinct versus genetically similar lineages. The extensive number of variable sites provided by whole-genome
data allowed us to uncover this heterozygosity that was not identified previously by studies using microsatellites.

Similarly, we found that inbreeding, when estimated by the percentage of the genome covered by long ROH, was lower than expected. Long ROH are the result of processes that reduce effective population size and increase homozygosity (Szpiech et al. 2013; Curik et al. 2014), such as selfing. Because K. marmoratus has high levels of selfing, we expected to find a large portion of the genome covered by long ROH. However, only a maximum of 9.5% of the genome that is contiguous enough to determine long ROH was covered by long ROH, which is lower than the values found in some human populations (range between 1% and 19% of the genome, Szpiech et al. 2013). Our reference genome is fragmented, with an N50 scaffold length of 111 539 bp (Kelley et al. 2016), which inherently decreases our ability to identify ROH in this species, especially long ROH.

This study shows that Florida was colonized in one event, and indicates that the lineage present in the Bahamas is closely related to the populations in Florida. The lineages from the geographically close locations, Belize and Honduras, did not form a monophyletic clade in either mitochondrial or individual nuclear analysis, suggesting a complex colonization of the region. Although the swimming abilities of adult K. marmoratus are limited and they typically live their entire life within a short distance from where they hatched (Davis et al. 1990; Taylor 2012), adults have been found within log hollows that presumably can float to distant areas during storms (Mackiewicz et al. 2006a; Tatarenkov et al. 2007). Additionally, eggs are resistant to desiccation making them capable of long distance dispersal on floating material, which could explain how K. marmoratus can colonize new areas and explains how movement between Florida and the Bahamas can occur.

The phylogenetic distribution of K. marmoratus lineages derived from protein-coding mitochondrial sequences is quite consistent with lineages having dispersed with the Caribbean and Florida currents (see Fig. 1). Indeed, several studies have identified migration events that are likely explained by prevailing ocean currents (Tatarenkov et al. 2017b). Given the currents in the Gulf of Honduras, it is thus not surprising to find complex phylogenetic relationships among the Belizean and Honduran lineages. The strong Florida current might explain why lineages from the Florida Keys (LION1, LION2, LK1), the Bahamas (RHL), and the east coast of Florida (SLC8E, VOL) are closely related: it seems quite reasonable that eggs and fish could disperse on flotsam from southern Florida to the eastern peninsular coast and the Bahamas. Lineages from western Florida (BBSC, FDS1) are most derived and most closely related to lineages from the Florida Keys, which might be explained by the relatively weak surface currents that move northward from the Keys to places like Fort Myers (BBSC) and Tampa (FDS1).

Inferences of population splits from the nuclear data provide additional insights into the complexity of the phylogeographic relationships. The population split inference when grouping samples from the same location, showed Belize and Honduras in the same clade. Moreover, even with the individual nuclear analysis, relationships among Floridian lineages do not follow the prevailing currents as clearly. Discrepancies between the mitochondrial and nuclear data could suggest biased migration, incomplete lineage sorting, or a combination of the two. The overall relationship among individuals sampled in Florida, the Bahamas, Honduras, and Belize are concordant with topologies estimated in other studies such as Tatarenkov et al. (2017b) that used different lineages to the ones in this study.

Earlier studies suggested that the low levels of heterozygosity were due to the fact that there are few naturally occurring males; however, there was evidence for multiple isogenic lineages at a specific sampling site and the identity of those varied from year to year. As additional data have been collected, there is clear evidence for outcrossing in the wild. Although our study has only examined one specimen per lineage, we were able to uncover variation at a finer scale than has been achieved by microsatellites. This result and the discrepancies in our phylogenetic analyses are strong motivation for further collection of whole-genome data from more specimens throughout the K. marmoratus range. This will enable genome-wide comparisons within and among populations, which will elucidate whether outcrossing is as rare as previously thought (Vrijenhoek 1985) and (or) whether outcrossing preferentially occurs between genetically dissimilar lineages as suggested by Ellison et al. (2013).

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