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Genome-Wide SNP Analysis Reveals Multiple Paternity in Burmese Pythons Invasive to the Greater Florida Everglades

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ABSTRACT.—**Reproductive strategies are an essential component of invasion ecology that influence invasion success and rates of population growth. Burmese Pythons (*Python bivittatus*) are large constrictor snakes that were introduced to the Greater Everglades Ecosystem of southern Florida, USA, from Asia. Since their introduction, these giant constrictors have spread throughout wetlands of southern Florida while increasing in abundance and causing declines in the native species upon which they prey. Multiple paternity in reproduction could facilitate invasion success by increasing the genetic diversity produced within each reproductive event. We used Diversity Arrays Technology genome-wide genotyping to assess multiple paternity in the progeny of wild Burmese Pythons in Florida. We analyzed $>$4,000 single nucleotide polymorphisms from 153 neonates belonging to 4 clutches collected in southwestern Florida. Complementary hierarchical and *K*-means clustering analyses of the genetic distances within clutches revealed that three clutches were each fertilized by two sires, with a fourth fertilized by a single sire. The proportions of offspring attributable to each sire within multiple paternity clutches ranged from nearly even to highly skewed. Analysis of multivariate dispersion showed significantly increased genetic variability in the multiple paternity clutches. These results improve our understanding of the reproductive strategy and invasion potential of a giant constrictor with significant ecological impacts.**

Upon introduction into a novel environment, invasive species face challenges associated with low genetic variability, low propagule pressure, and uneven sex ratios. Studies on the reproductive characteristics of invasive species have uncovered many different strategies for establishing in, expanding into, and colonizing novel areas. Asexual reproduction can reduce the negative impacts of inbreeding in founding populations (Roman and Darling, 2007), but for sexually reproducing species, there must be other adaptations that mitigate inbreeding and outbreeding depression that can occur during a founding event and subsequent invasion (Peer and Taborsky, 2005). Often, colonization success by a nonnative species is improved with increased genetic variation through the promotion of adaptive potential and the reduction of negative effects from inbreeding depression (Allendorf and Lundquist, 2003; Roman and Darling, 2007).

Multiple introductions of a species can allow for additional, sometimes even novel, combinations of alleles to be added to the population to facilitate successful establishment (Roman and Darling, 2007). In some sexual animals, such as the Burmese Python (*Python bivittatus*), sporadic parthenogenesis in females is employed as a reproductive strategy and can increase the numbers of females and the general population size (Groot et al., 2003). However, this strategy does not increase the genetic diversity found in the invasive population.

Polyandry, which is defined as females that mate with multiple males, is a widespread reproductive strategy that functionally increases adaptability and reduces vulnerability to stochastic events for recently introduced species. Fertilization by multiple males can increase diversity among sibling offspring potentially allowing for the use of more divergent resources and habitat to reduce competition (Ridley, 1993), and increase the chances of successful genotypes in unpredictable or novel environments (Loman et al., 1988; Zeh and Zeh, 1996). When females are unable to recognize close relatives among potential mates, polyandry decreases the chance of inbred offspring (Stockley et al., 1993). Alternatively, some females may be able to discriminate among sperm from closely related males and preferentially store and use sperm from more distantly related males (Bretman et al., 2009). At the population level, polyandry is expected to increase effective population sizes relative to monogamy for species where females produce multiple offspring during each reproductive event (i.e., clutches, broods, litters, etc.; [see Sugg and Chesser, 1994; Pearse and Anderson, 2009]). Thus, a propensity for mating with multiple males could facilitate the colonization process for nonnative species by mitigating the negative impacts of low genetic diversity in the invading population.

Burmese Pythons (*P. bivittatus* [previously *P. molurus bivittatus*]) are large constrictors (up to 6 m and 90 kg) native to Southeast Asia. They were common in the pet trade and are now recognized as an established species in Everglades National Park in southern Florida, USA (Meshaka et al., 2000). Burmese Pythons have caused severe population declines in several species upon which they prey, which include reptiles, mammals, and birds (Snow et al., 2007; Dove et al., 2011; Holbrook and Chesnes, 2011; Dorcas et al., 2012). Since their introduction, Pythons have spread throughout southern Florida, covering thousands of square kilometers. The current invaded range includes southwestern Florida and the Florida Keys, and is expected to continue expanding northward beyond Loxahatchee National Wildlife Refuge (Hunter et al., 2015, 2019; Hanslowe et al., 2018). The invasion success of Burmese Pythons could be facilitated by a polyandrous reproductive strategy that mitigates issues of a small founding population during population establishment. Polyandry is known to be common among reptiles, particularly snakes (Uller and Olsson, 2008), and is likely to be found in Burmese Pythons because they often form breeding aggregations of up to seven males per female during the breeding season (Smith et al., 2016). Additionally,

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initial evidence from microsatellite analysis of eight neonates from a single clutch of Burmese Pythons indicated the potential for half sibling relationships indicative of multiple paternal contributions (Hunter et al., 2018). In this study, we examine genome-wide genetic markers among the progeny of free-living Burmese Pythons in southern Florida to determine whether individual clutches were sired by multiple males.

METHODS

We collected 153 total neonate snakes from clutches deposited in the wild by 4 dams in southwestern Florida. Three of the four dams were radiotelemetered as part of a long-term tracking study ("Valentina," "Orra," and "Sweet Pea"; Bartoszek et al., 2021). One opportunistic egg clutch was found ("Little AG") through excavation by heavy equipment. The neonates were collected between May and July 2016 and in June of 2017. We extracted genomic DNA from muscle tissue of neonate tails utilizing Qiagen DNeasy Kit protocol (Qiagen, Valencia, California) following manufacturer guidelines. We quantified DNA concentrations using an Epoch microplate spectrophotometer (BioTek) and 2-μL samples with concentrations >40 ng/μL were run on a 0.8% agarose gel to confirm high molecular weight DNA. If there was no visible indication of fragmentation, we incubated 1 μL of each sample in a restriction enzyme buffer (CutSmart® Buffer, New England BioLabs, Ipswitch, Massachusetts) at 37°C for 2 h and ran the DNA on a 0.8% agarose gel as a final quality-control check for high molecular weight DNA.

Sequencing and genotyping were conducted by Diverse Arrays Technology (DArT Pty Ltd.) following standard protocols. DArTseq genotyping by sequencing (GBS) uses next generation sequencing to identify variable single nucleotide polymorphisms (SNPs) for genotyping of the sample population (Baloch et al., 2017). Briefly, genomic DNA was digested by the restriction enzymes *PstI* and *SphI*. Illumina adapters and indices were ligated to the resulting fragments, which were then sequenced on an Illumina HiSeq 2500 sequencer (Wenzl et al., 2004). Quality filtering and identification of SNPs were conducted by DArT following methods of Akbari et al. (2006) and Grewe et al. (2015), respectively. We conducted all further data analyses in Program R version 3.6.0 (R Core Team, 2019). A single-row format biallelic SNP matrix was converted to a GenLight object for analysis using the Dartr package (Gruber et al., 2018). To reduce nonindependence due to linkage among adjacent SNPs, which could skew the results, we randomly selected a single SNP within each DNA sequence fragment and discarded the rest. The total SNP matrix was then split into separate matrices for each clutch (four in total).Within each matrix, alleles that were not variable (monomorphic alleles) were removed. Each SNP matrix was then used to calculate a Jaccard dissimilarity matrix using the vegdist() function in the Vegan package (Oksanen et al., 2020), yielding all pairwise distances for each snake within each clutch. SNP data for the parents of the neonate clutches were not available, so we used de novo clustering analyses based on genetic distance to assess multiple paternity, rather than pedigree analysis. We used two clustering methods to determine the number of sires (male parents) that had contributed to each clutch.

We also calculated pairwise kinship coefficients for all snakes in the dataset (popkin package; Ochoa and Storey, 2016). For each clutch, we assessed presence of multiple paternity by visualizing the distribution of within-clutch kinship coefficients as a density plot. Kinship coefficients for single paternity clutches were expected to be unimodal, indicating all full sibling relations within the clutch. Multiple paternity clutches were expected to have a bimodal distribution of kinship coefficients, representing both full sibling and half sibling relationships within the clutch.

We estimated the number of paternities for each clutch and specific relationships among intraclutch individuals using *K*-means clustering and visualized results via nonmetric multidimensional scaling (NMDS). The number of distinct clusters in each clutch (*K*) was determined to assess clustering error according to two methods, at all values of *K* ($K = 1$–10). *K*-means clustering was conducted according to the method of Hartigan and Wong (1979) implemented in R using 100 random starts (package '*stats*'; R Development Core Team, 2010). Scree plots were produced by plotting each *K* against the total within-cluster sums of squares, and silhouette scores calculated in R (package '*cluster*'; Maechler et al., 2021). The best supported hypothesis of *K* was considered by examining the scree plots using the "elbow" method for the sums of squares and maximizing the silhouette value. The elbow method is a standard heuristic method for determining the appropriate value of *K* by examining the scree plot to find the value of *K* at which increasing *K* no longer results in a substantial decrease of within-group sums of squares. In each of the four clutches examined, both the scree plot and silhouette methods agreed on the best supported *K*. Ordination by NMDS of the Jaccard distance matrix was used to visualize genetic clusters in each clutch using the metaMDS() function of the vegan package (Oksanen et al., 2020).

We then applied an additional and fundamentally different clustering method, hierarchical clustering, to verify the results of *K*-means clustering. For each clutch, a Jaccard distance matrix was calculated using the vegdist() function of the vegan package and clustering was implemented by the hclust() function in the stats package using the "ward.D2" method. Based on the proportion of clutches for which we found polyandry, we used the Wilson method (Wilson, 1927) to calculate a 95% confidence interval (CI) for the proportion of polyandrous clutches in the population. We compared the amount of genetic variability within each clutch using a distance-based test for homogeneity of multivariate dispersions (Anderson, 2006), implemented by the betadisper() function in the vegan package. In this test, genetic variability is measured as the average distance of all individuals from the centroid of each group (i.e., clutch).

RESULTS

DArT sequencing identified 4,414 SNPs from 153 neonate snakes (see Supplementary Online Material for SNP data). This was reduced to 4,167 SNPs after keeping only one SNP per fragment. We identified four individual clutches by their dam as "Little AG," "Orra," "Sweet Pea," and "Valentina." After splitting the data by clutch and filtering for monomorphic SNPs, we retained 2,606 SNPs from 55 neonates in clutch "Orra," 2,545 SNPs from 21 neonates in "Little AG," 1,671 SNPs from 23 neonates in "Sweet Pea," and 2,743 SNPs from 54 neonates in "Valentina."

Analysis of SNPs indicated that three out of four clutches were sired by two males, whereas the fourth clutch ("Sweet Pea") was sired by a single male. The density plot of kinship coefficients in "Sweet Pea" was unimodal, indicating all full

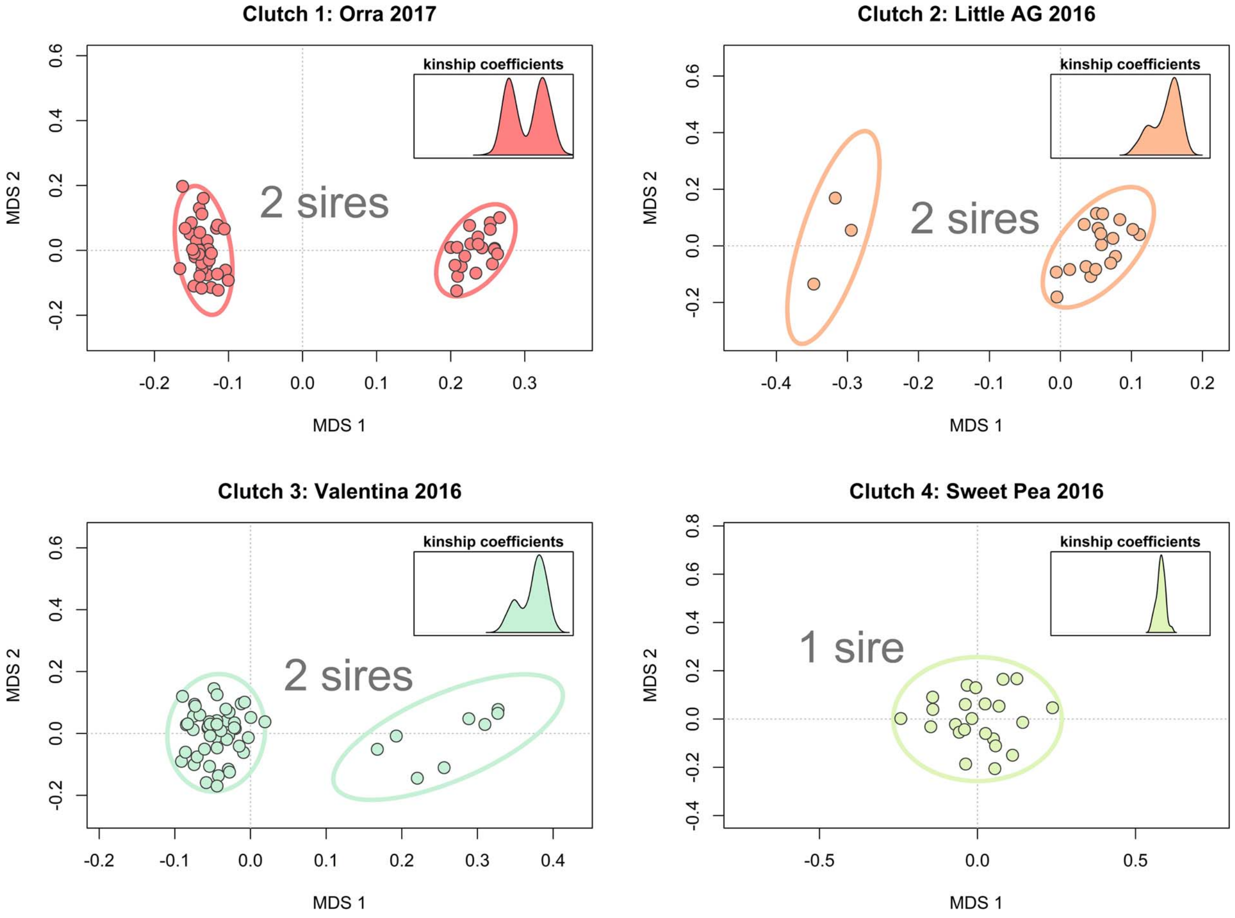

FIG. 1. Multiple paternity in three of four Burmese Python egg clutches. Panels show nonmetric multidimensional scaling (NMDS) ordination of genetic distances among snakes within each egg clutch. The number of sires was inferred from the number of distinct genetic clusters determined by *K*-means clustering analysis. Insets show distributions of kinship coefficients, which is unimodal in the single paternity clutch (“Sweet Pea”) and bimodal in the multiple paternity clutches, reflecting full sibling (right peak) and half sibling (left peak) relationships.

sibling relationships, whereas the density plots for the other three clutches were all bimodal, indicating both full sibling and half sibling relations (Fig. 1 insets). For each clutch with multiple paternity (“Orra,” “Valentina,” and “Little AG”), two distinct clusters of genotypes were found by *K*-means clustering, indicating two paternal contributions (Fig. 1). In each case, both methods for selecting the best number of clusters (i.e., elbow and silhouette methods) yielded the same results. The results of hierarchical clustering perfectly agreed with results of *K*-means clustering in terms of number of inferred sires in each clutch, and the memberships of each neonate to full sibling groupings within each clutch (Fig. 2).

Table 1 provides the number of neonates in each paternal cluster, the mean or median kinship coefficients, and the minimum range of kinship coefficients separating the clusters. We estimated a 95% CI for the proportion of polyandrous clutches to be 30–98% (range is 95% CI). Results of the betadisper analysis revealed significantly different amounts of genetic variability among clutches for “Sweet Pea,” whose clutch had less variability than the three other clutches ($P <$ 0.001; Fig. 3).

DISCUSSION

Using ∼4,000 SNPs, we were able to conclusively identify polyandry in Burmese Pythons in three out of four clutches, which were fertilized by a minimum of two sires. The findings were consistent across multiple analytical approaches. This is supported by previous findings using fewer multilocus markers (Hunter et al., 2018). By sampling numerous loci throughout the genome, we were able to identify genetic patterns of multiple paternity despite the challenges presented by low genetic variability and high levels of inbreeding in this invasive population.

As these clutches were opportunistically collected from the wild population, polyandry may be commonplace among Burmese Pythons within their invaded range in southern Florida. During the 2016 breeding season, “Valentina” was observed with two individual male pythons on separate dates prior to laying a successful clutch of eggs. We estimated that 30–98% of python clutches are polyandrous in the invaded population; however, this estimate is based on a small sample of clutches ($n = 4$) and more extensive sampling is needed for a robust estimate of the frequency of polyandry among these snakes. The indicated level of polyandry is consistent with the frequency found in other reptiles, which is often >50% of clutches, particularly in snakes (Uller and Olsson, 2008). Further it is supported by the finding of female Burmese Pythons to be accompanied by several male snakes during breeding events (Smith et al., 2016; Bartoszek et al., 2021). Polyandry may increase the number of male snakes that can be found and removed through the snake ‘scout’ program, which radiotracks male pythons in order to find and then remove females (Smith

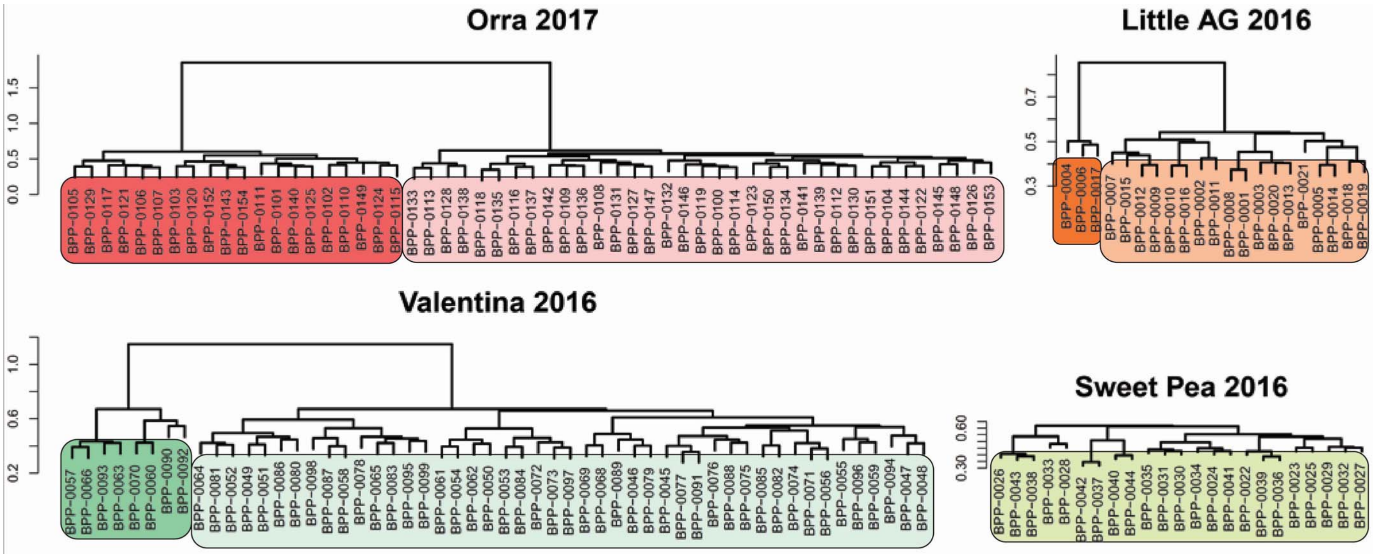

FIG. 2. Dendrograms of hierarchical clustering of each clutch identified multiple paternity in three of four clutches. Hierarchical clustering was conducted on Jaccard distance matrices. To aid comparison of the results of the two clustering methods, color codes within each dendrogram correspond to clusters identified by *K*-means clustering analysis.

et al., 2016). Polyandry in Burmese Pythons may be helping with their continuing success and population expansions (Hunter et al., 2019). Polyandry and increased diversity has been shown to make genetic-based population control measures more difficult in other invasive species (Manser et al., 2019).

Two of the primary challenges faced by invading species are reduced genetic diversity following founder events and adaptation to novel environments (Allendorf and Lundquist, 2003; Roman and Darling, 2007). Polyandry could help Burmese Pythons address both challenges. As the result of a recent bottleneck, likely in the pet trade and/or during population establishment, the introduced population of Burmese Pythons in Florida has low genetic diversity, and consequently, a low effective population size (Hunter et al., 2018). Through multiple paternity leading to increased diversity, the likelihood of inbred offspring is lower (Stockley et al., 1993) and the effective population size maybe be increased (Sugg and Chesser, 1994; Pearse and Anderson, 2009). Further, suitable genotypes may be generated to the novel environment and natural selection and adaptation can occur more rapidly (Loman et al., 1988; Zeh and Zeh, 1996). Indeed, rapid evolution was identified across 10 yr of samples from the invasive Burmese Python population, likely through strong ecological and climatic selection pressures causing adaptation to cold events (Card et al., 2018).

TABLE 1. Kinship characteristics of the four invasive Burmese Python clutches assessed for multiple paternity. The number of neonates identified in each paternal cluster are given, followed by the mean kinship coefficient for each of those density peaks, in addition to the minimum range of kinship coefficients separating the two clusters. "Sweet Pea" neonates formed a single paternal cluster with a unimodal distribution and a median value of kinship (*).

Clutch dam	Small cluster	Kinship	Large cluster	Kinship	Minimum range	Distribution
Little AG	3	0.173	18	0.303	0.196–0.248	Biomodal
Valentina	8	0.180	46	0.291	0.200–0.240	Biomodal
Orra	20	0.283	35	0.160	0.204–0.249	Biomodal
Sweet Pea	0	-	23	*0.367	-	Normal

Previously detected low levels of hybridization between Burmese Pythons and the closely related Indian Python (*P. molurus*) could increase genetic diversity and confer adaptive traits for expansion of the invaded range, because Indian Pythons are better adapted to cooler and dryer habitats (Hunter et al., 2018). By mating with multiple males, female Burmese Pythons could increase the likelihood of hybrid progeny that could confer adaptive benefits in the newly invaded environment. This is an interesting possibility, but it is currently unknown whether hybridization between these species occurs within the invaded range, are remnants of species crosses in the

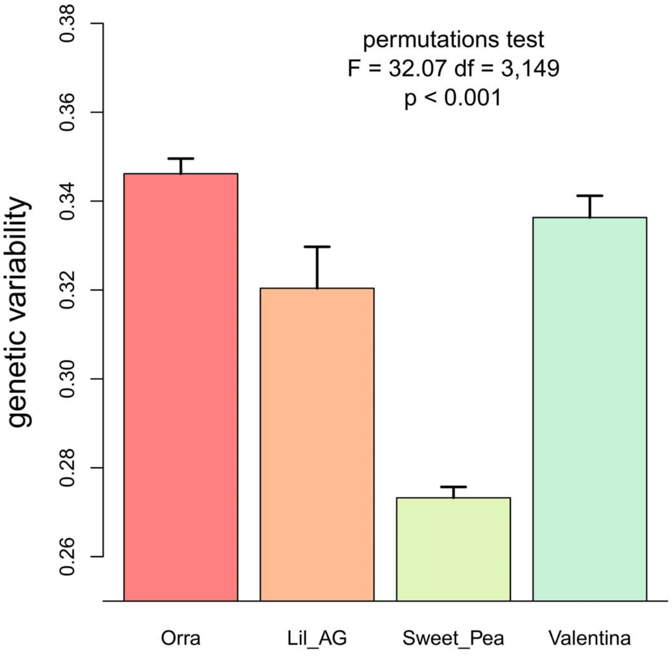

FIG. 3. Test of multivariate homogeneity of variance showed that the only single paternity clutch "Sweet Pea" had significantly lower genetic variability among offspring than all other clutches. Genetic variability within each clutch represented by colored bars. Standard error is indicated by black lines on each bar.

native range prior to captivity, or occurred in captivity prior to release (Hunter et al., 2018).

Our results support that clutches of Burmese Pythons in southwestern Florida are frequently fertilized by more than one sire. The analyses do not rule out the possibility of a small number of neonates being fertilized by more than two sires, but the large separation of the clusters and the lack of outliers indicates that a third sire is unlikely in this sample set. The minimum kinship levels separating the two paternal clusters were <25%, suggesting that the two sires of each clutch were not likely to be closely related (Table 1). Two potential modes of polyandry known in reptiles are sperm storage by dams across reproductive cycles and copulation with multiple sires during a single reproductive cycle (Uller and Olsson, 2008). It remains unknown which fertilization strategy the identified polyandry arises from in Burmese Pythons, or whether it could include both strategies. Future work is needed to determine the mechanism of polyandry in Burmese Pythons, the percentage and number of sires of polyandrous clutches, and whether polyandry confers adaptive benefits to improve the success of the invasive population.

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Supplementary Data

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