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Sex, males, and hermaphrodites in the scale insect *Icerya purchasi**

Andrew J. Mongue,[1,2] Sozos Michaelides,[3,4] Oliver Coombe,[3] Alejandro Tena,[5] Dong-Soon Kim,[6] Benjamin B. Normark,[7] Andy Gardner,[3,8] Mark S. Hoddle,[9] and Laura Ross[1,3,7,10]

[1]*Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JT, United Kingdom*

[2]*E-mail: ajmongue@gmail.com*

[3]*Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom*

[4]*Department of Biology, Concordia University, Montreal, Quebec H4B 1R6, Canada*

[5]*Institut Valencià d'Investigacions Agràries (IVIA), Centre de Protecció Vegetal i Biotecnologia, Unitat d'Entomologia, Moncada 46113, Spain*

[6]*Majors in Plant Resource Sciences and Environment, College of Applied Life Science, SARI, Jeju National University, Jeju 63243, Republic of Korea*

[7]*Department of Biology and Graduate Program in Organismic and Evolutionary Biology, University of Massachusetts, Amherst, Massachusetts 01003*

[8]*School of Biology, University of St Andrews, Fife KY16 9TH, United Kingdom*

[9]*Department of Entomology, University of California, Riverside, California 92521*

[10]*E-mail: laura.ross@ed.ac.uk*

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Androdioecy (the coexistence of males and hermaphrodites) is a rare mating system for which the evolutionary dynamics are poorly understood. Here, we investigate the cottony cushion scale, *Icerya purchasi*, one of only three reported cases of androdioecy in insects. In this species, female-like hermaphrodites have been shown to produce sperm and self-fertilize. However, males are ocassionally observed as well. In a large genetic analysis, we show for the first time that, although self-fertilization appears to be the primary mode of reproduction, rare outbreeding events do occur in natural populations, supporting the hypothesis that hermaphrodites mate with males and hence androdioecy is the mating system of *I. purchasi*. Thus, this globally invasive pest insect appears to enjoy the colonization advantages of a selfing organism while also benefitting from periodic reintroduction of genetic variation through outbreeding with males.

KEY WORDS: **Androdioecy, haplodiploidy, mating systems, microsatellite markers, population genetics, scale insects.**

Reproductive systems are remarkably variable across life, yet much of this variation is poorly understood in evolutionary terms. The forces that favor different modes of reproduction, as well as those that shape their taxonomic and phylogenetic distributions remain elusive. The evolutionary and zoological literature has predominantly focused on the distinction between sexual and asexual reproduction (Innes et al. 2000; Silvertown 2008; Gibson et al. 2017). However, much of the variation in reproductive systems is found among those that reproduce sexually (White 1973; Charnov et al. 1976; Normark 2003; Bachtrog et al. 2014). For example, although most animals are gonochoristic (i.e., have separate sexes) with genetic sex determination, more than 20% of species deviate from this familiar system (Tree of Sex Consortium 2014).

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One such system is androdioecy, which is characterized by the coexistence of males and hermaphrodites in the absence of females. Androdioecy is a rare mating system. To date, it has been found in only 115 species of animals (Weeks 2012), including just three species of vertebrate, all of which are fish (Smith 1965; Taylor et al. 2001). Outside of the animal kingdom, it has also been found in several plant species (Lloyd 1975; Liston et al. 1990; Pannell 1997; Saumitou-Laprade et al. 2010). Thus, androdioecy appears to have independently evolved several times in eukaryotes and is often thought of as an advantageous strategy for colonizing new locations: enjoying the benefit of reproductive assurance (i.e., allowing even a single hermaphrodite to found a new population through self-fertilization) without incurring the inbreeding cost of pure selfing as the production of males permits outcrossing (Weeks 2012). Yet this logical hypothesis has not been formally tested and it remains unclear how organisms anatomically and cytologically acquire the capacity to produce both gamete types in one sex morph but not the other.

In plants, androdioecy is considered an intermediate condition in the evolutionary transition from hermaphroditism to separate sexes (Charlesworth and Charlesworth 1978). In animals, however, most transitions appear to go in the opposite direction (from separate sexes to hermaphroditism then androdioecy, e.g., barnacles; Høeg 1995). Another difference between plant and animal androdioecy is that, in animals, hermaphrodites can either self-fertilize or mate with males but are typically unable to mate with each other (Weeks et al. 2006). This suggests either that the forces shaping the evolution of androdioecy are different in the two kingdoms or that the evolution of outbreeding hermaphrodites is constrained differently by the anatomy of plants and animals. Indeed, it has been argued that the evolution of cross-fertile hermaphroditic animals poses a greater evolutionary challenge as it would require not only changes to gamete production, but also novel rearrangement of the reproductive tract to allow for bidirectional copulation (Pannell 2002; Weeks et al. 2006, 2009). Moreover, plants often possess multiple, independently formed reproductive structures (e.g., flowers), in contrast to the single-origin reproductive structures of animals. It stands to reason that, with the evolution of novel reproductive strategies, plants may rescue some fitness from subfunctional reproductive organs, whereas in animals, sterility/fertility tends to be a binary trait. As such, androdioecy in animals may be the more tractable evolutionary route to outcrossing. The presence and frequency of males in such systems has been interpreted in terms of selection for outbreeding opportunity and of conflict between males and hermaphrodites over mating. Males in androdiecious species can only pass genes on through mating with hermaphrodites, but if the cost of selfing is lower than 50%, hermaphrodites pass on their genes at a higher rate via selfing (Chasnov 2010).

To date, the best-studied androdiecious animal systems are the nematode worm *Caenorhabditis elegans* and the shrimp *Eulimnadia texana* (Anderson et al. 2010; Chasnov 2010). In both cases, hermaphrodites appear to have evolved mechanisms to limit mating with males. In *C. elegans*, hermaphrodites are physiologically differentiated from females of closely related dioecious species (Chaudhuri et al. 2015). In *E. texana*, hermaphrodites are behaviorally differentiated from females, spending less time with males when given the choice (Ford and Weeks 2018). Both of these lines of evidence are suggestive of evolutionary responses to conflict, but, with effectively only two observations, it is difficult to say how crucial such changes are to the evolution and maintenance of androdioecy. To understand the generalizability of traits involved in the evolution of this rare mating system requires the identification of more, independently evolved, androdiecious taxa.

Insects are the most species-rich clade of animals and display exceptionally variable reproductive systems, including chromosomal sex determination, haplodiploidy, and—very rarely—hermaphroditism (de la Filia et al. 2015; Blackmon et al. 2017). The only known cases of hermaphroditism are found in a tribe of scale insects (plant-feeding insects in the order Hemiptera), the Iceryini. Hermaphroditism has been recorded in at least three species within this tribe, with likely multiple evolutionarily independent origins (Hughes-Schrader 1925, 1963; Hughes-Schrader and Monahan 1966; Unruh and Gullan 2008a; Tree of Sex Consortium 2014). Hermaphroditic individuals display a female phenotype (scale insects exhibit strong sexual dimorphism with relatively large wingless females and very small winged males, see Fig. 1a and b) and are almost certainly incapable of mating with each other. Specifically, hermaphrodites possess a vaginal opening leading to an internal ovitestis (a modified gonad structure that produces both sperm and eggs) and sperm storage organs (spermathecae), but lack an intromittent organ with which to transfer sperm to one another (Johnston 1912; Hughes-Schrader 1925). This suite of sexual traits matches the expectation for an obligate selfing hermaphroditic animal, but there is some evidence that these species are actually androdiecious.

First of all, true males are observed in natural populations (the male in Fig. 1b is from one of these hermaphroditic species, *Icerya purchasi*), although they are rare (Royer 1975; Hamon and Fasulo 2005; Kim et al. 2011), with reported frequencies differing substantially between populations (from 0.01% to 10%). Second, these rare males can physically mate with the hermaphrodites (Fig. 1c). However, if males do not contribute genetically to the next generation, their presence is irrelevant from a reproductive perspective and the system is, genetically at least, purely hermaphroditic.

If confirmed, the presence of androdioecy in Iceryini would be remarkable as it would not simply be the only known

Figure 1. ***Icerya purchasi*** **hermaphrodites (with white egg case and juveniles) are most commonly observed (A) but rare males have been reported (B) and we have observed matings between the two sexes under laboratory conditions, with the hermaphrodite enabling mating by raising their body (C). It is has previously been unclear whether or not these matings lead to the fertilization of eggs.**

instance of this mating system in insects, but also the first record of androdioecy having evolved from haplodiploidy (in which females develop from fertilized eggs and males from unfertilized eggs; Schrader and Hughes-Schrader 1926). Cytogenetic studies have shown that, while hermaphrodites are diploid ($2n = 4$), both males and the sperm-producing section of the ovitestis within hermaphrodites are haploid ($n = 2$, Hughes-Schrader 1925; Kokilamani et al. 2014). It has been suggested that the evolution of androdioecy in the Iceryini could result from conflict between males and females over the proportion of eggs that are fertilized, a conflict that is characteristic of haplodiploid species (Normark 2009; Gardner and Ross 2011). Thus, this system would be a valuable addition to the understanding of male-hermaphrodite conflict in androdiecious species.

Setting aside the presence of males for a moment, the physiology of hermaphrodites themselves deserves more scrutiny. At the cellular level, the presence of an unusual haploid tissue within the ovitestis of hermaphrodites is poorly understood and unique to Iceryini. The first study that described this phenomenon proposed that the haploid tissue arises due to the random loss of one haploid copy of the genome (Hughes-Schrader 1925) from germ cells destined to produce sperm, like more familiar mechanisms of spermatogenesis (Fig. 2a). However, a later study suggested instead that the haploid tissue originates from supernumerary sperm cells transmitted by males during mating (Royer 1975): Cytogenetic observations show that oocytes are penetrated by multiple sperm cells, one of which fertilizes the eggs and gives rise to the diploid hermaphrodite soma, whereas the other sperm cells persist and divide to apparently give rise to a haploid male germline (Fig. 2b). By "infecting" females with sperm cells that form male gametes inside the offspring, males effectively mate not only with the female, but also with her daughters, ensuring reproductive fitness for generations to come. Under this scenario, offspring-producing individuals might better be considered chimeras of a genetically female soma and ovary with male gonadal tissue incorporated, rather than "true" hermaphrodites. For simplicity's sake, we will refer to these individuals as hermaphrodites throughout, as modern genomic inference has yet to verify the presence of persistent sperm cell lines. This unique mechanism for generating self-fertilizing individuals does merit further consideration though, as a mathematical model of this infectious sperm lineage hypothesis has suggested that this could lead to the evolution of androdioecy (Gardner and Ross 2011). These two possible mechanisms of haploid male germ-tissue formation in hermaphrodites should be distinguishable from each other in a purely selfing system (see expected offspring genotypes in Fig. 2a and b). However, if males genetically contribute to the next generation, the inference is more complicated, so this potential needs to be assessed first (Fig. 2c).

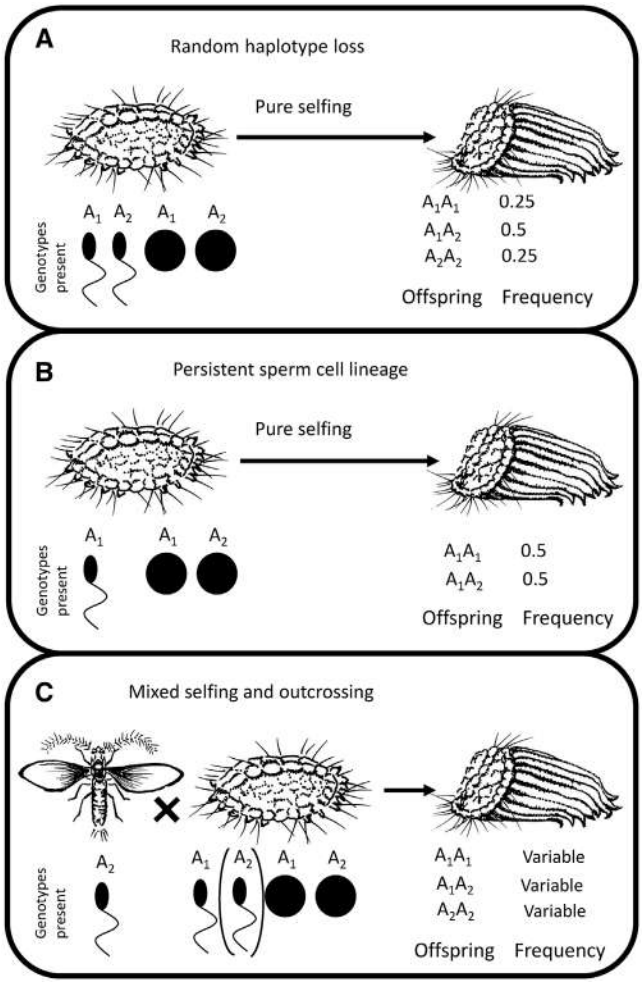

Figure 2. Hypothesized mating systems for *Icerya purchasi*. (A) Sperm is formed within the diploid hermaphrodite by traditional meiotic processes resulting in all possible genotypes at Mendelian ratios. (B) A historic outbreeding event with a haploid male results in the fertilization of an egg nucleus plus the establishment of a sperm cell lineage that persists over generations. Only two of three allelic combinations are possible under this scenario. (C) Rare males mate with hermaphrodites, fertilizing some proportion of their eggs, resulting in offspring of all possible genotypes with frequencies depending on the fertilization success rate of males. Note that in this scenario distinguishing between selfing mechanism (a or b) would be impossible based solely on genotype frequency information.

Here, we focus on one species of hermaphroditic scale insect in the Iceryini: the cottony cushion scale insect, *I. purchasi*. This species is a highly polyphagous pest, feeding on urban ornamental plants (e.g., *Magnolia*), agricultural crops such as *Ficus* and *Citrus* (Singh and Kaur 2017; Liu and Shi 2020), and plants of conservation importance threatened by *Icerya's* invasive potential (e.g., Galapagos endemics, Hoddle et al. 2013). *Icerya purchasi* spread from its native range in Australia over a century ago and it is now globally distributed; climate models predict that changing environmental conditions will permit further geographic expansion in the future (Liu and Shi 2020).

Here, we present an investigation into the reproductive biology of *I. purchasi* primarily to test for signatures of outcrossing in nature, assess the status of the species as purely hermaphroditic or androdiecious, and secondarily to investigate potential mechanisms of selfing within hermaphrodites. We developed a panel of polymorphic microsatellite markers for use in a genetic analysis to estimate outbreeding rates in natural populations of *I. purchasi*. Hermaphrodites are able to self-fertilize and therefore most of the population should consist of highly homozygous strains under strict selfing. However, if the mating system is androdiecious, we would also expect to find some heterozygous individuals resulting from recent outbreeding events. We therefore estimated outbreeding rates by examining the difference between allele and genotype frequencies in multiple populations collected globally. We found multiple lines of evidence consistent with androdioecy in wild populations. The inferred genetic contribution of males to the next generation obfuscated the dynamics of self-fertilization in hermaphrodites however, and we were unable to distinguish whether hermaphrodites were genetic chimeras or true hermaphrodites in the familiar sense.

Methods

SPECIMEN COLLECTION

We initially collected 343 adult and 536 egg samples from 27 locations in 10 countries on five continents (see Supporting Information Table S1). When possible, we collected hermaphrodites as well as their egg masses. These specimens were stored in individual tubes in 99% ethanol at −20°C. We were unable to obtain male specimens from field populations, either because they are rare, because the short lifespan of adult males makes them hard to detect, or both. For each hermaphrodite, we dissected the tip of the head (<1 mm) to use for the analysis. This avoided the inclusion of the ovitestis and allowed us to distinguish between a true heterozygous female or a homozygous female fertilized by and carrying embryos from a male with a different genotype. For each mature hermaphrodite that had an egg mass (white waxy ovisac, Fig. 1a), we also analyzed two embryos (or more if the parent was found to be heterozygous). However, due to the limited insight gained from these pedigrees we omit them here; see Supporting Information for details on parent–offspring analyses.

MICROSATELLITE MARKERS

For the purpose of this study, we designed a set of 12 polymorphic microsatellite markers. We identified the markers based on one lane of 454 (titanium) sequencing data from a pooled sample of hermaphrodites that came from a laboratory population in

California. This resulted in 94,553 reads that could be assembled to 1772 contigs with an average length of 909 base pairs. We used the software package msatfinder (Thurston and Field 2005) to detect microsatellite repeats in the sequence data. We selected only those primers that had a tri-nucleotide repeat and had more than 10 repeats. This resulted in 40 possible microsatellite loci. We tested each of these primers under standardized PCR conditions with an annealing temperature of 55°C. Those primers that amplified were tested for polymorphism on six individuals (three from a UK and three from a French population). Based on these tests, we selected 12 polymorphic primers (details of these primers can be found in Supporting Information Table S2). To speed up the analysis, we also designed a multiplex method so that the 12 primers could be analyzed in four PCR 10 μL reactions and three ABI runs (see Supporting Information Table S1).

MICROSATELLITE ANALYSIS

DNA was extracted by using the Qiagen DNAeasy kit (Hilden, Germany). Microsatellite loci were amplified with the primers described in Supporting Information Table S2 and repeat length was assessed via the microsatellite plugin in Geneious R8 (Kearse et al. 2012). We only counted alleles that were found in at least two separate individuals. The embryos we analyzed were very small and so it was not always possible to get enough DNA for a reliable analysis. To avoid calling erroneous alleles in low-quality samples, we removed samples in which fewer than two-thirds of the loci amplified. We repeat-genotyped for 18 samples for all loci and 154 for a subset of loci (48 at five loci and 106 at three loci) to estimate overall genotyping error and to confirm unexpected genotype calls (cases where embryos showed different genotypes than the parent).

DATA ANALYSIS

We carried out two sets of analyses based on microsatellite genotyping of *I. purchasi*. In the first, we considered only wild caught adult hermaphrodites from populations around the world and estimated allele frequencies, using the R package *polysat* (Clark and Jasieniuk 2011) to calculate F_{ST} and create an input for population structure analyses. For this objective, we considered only individuals with at least two-thirds of loci amplifying (i.e., 8 of 12, $n = 295$ across nine countries) and generated 10 independent runs of population structure inference from $k = 1$ to 26 populations (Pritchard et al. 2000). We aggregated runs and used the webtool CLUMPAK to infer the best k (Kopelman et al. 2015). With these F_{ST} estimates, we tested for a relationship between genetic and geographic distance. We used a Mantel test to compare matrices of genetic differentiation and physical distance (Diniz-Filho et al. 2013), as implemented in the R package "ade4" (Dray and Siberchicot 2020); the resulting p-value for isolation by distance was the result of 10,000 permutations of the distance matrices. We used shortest distances (i.e., straight lines, over water if necessary) when comparing two locations. For countries with multiple sampling locations, we averaged the latitudes and longitudes of sampling locations within the country.

To estimate per-population selfing rate and assess evidence for androdioecy, we used two approaches. For the first, we used the R package *adegenet* (Jombart 2008) to calculate the inbreeding rate (F_{IS}) on individuals with at least two-thirds of loci amplifying (the same used for F_{ST} analyses). All analyses were carried out in R version 3.6.0 (Team 2019). For individuals with especially low F-values (<0.4), we directly examined their called genotypes for evidence of outcrossing between common haplotypes. Separately, we estimated the selfing rate (s) with the software RMES (David et al. 2007).

Results

GLOBAL GENETIC VARIATION

After filtering, we analyzed 295 individuals from nine countries (details in Supporting Information Fig. S1). On average, we found 2.67 alleles per locus across 12 microsatellite markers, indicating low levels of variation. For instance, the samples from Korea did not show any genetic variation, all sharing a single haplotype. Globally, one haplotype dominated our samples with 46% of all specimens analyzed having the same homozygous diploid genotype across all 12 markers. The next most common haplotype, which differs at a single homozygous locus from the first, accounts for another 44% of the samples. Consequently, 90% of our sampled individuals with full genotypes showed no genetic variation across 11 of 12 microsatellite loci.

Population structure analyses indicated the existence of two distinct populations based on the biggest change in likelihood between sequential k values. This level of division separates heterozygous individuals and those carrying rare alleles from the two most common haplotypes (Fig. 3, top). At $k = 3$ divisions, the three clusters reflect the pattern described above, with one inferred population for each of the two dominant haplotypes and a third for the rarer haplotypes (Fig. 3, middle). Some individuals from the relatively more diverse populations of Australia and France appear as distinct clusters at $k = 4$ (Fig. 3, bottom), but beyond level of subdivision no new nonadmixed clusters are formed. Globally, genetic differentiation between countries is variable, and ranges from 0.02 to 0.92, driven mainly by which of the two most common haplotypes is fixed locally, with no evidence for increased isolation by distance ($P = 0.31$). The relationships between populations can be seen more easily in Supporting Information Table S4, in which we have calculated F_{ST} for each pair of countries.

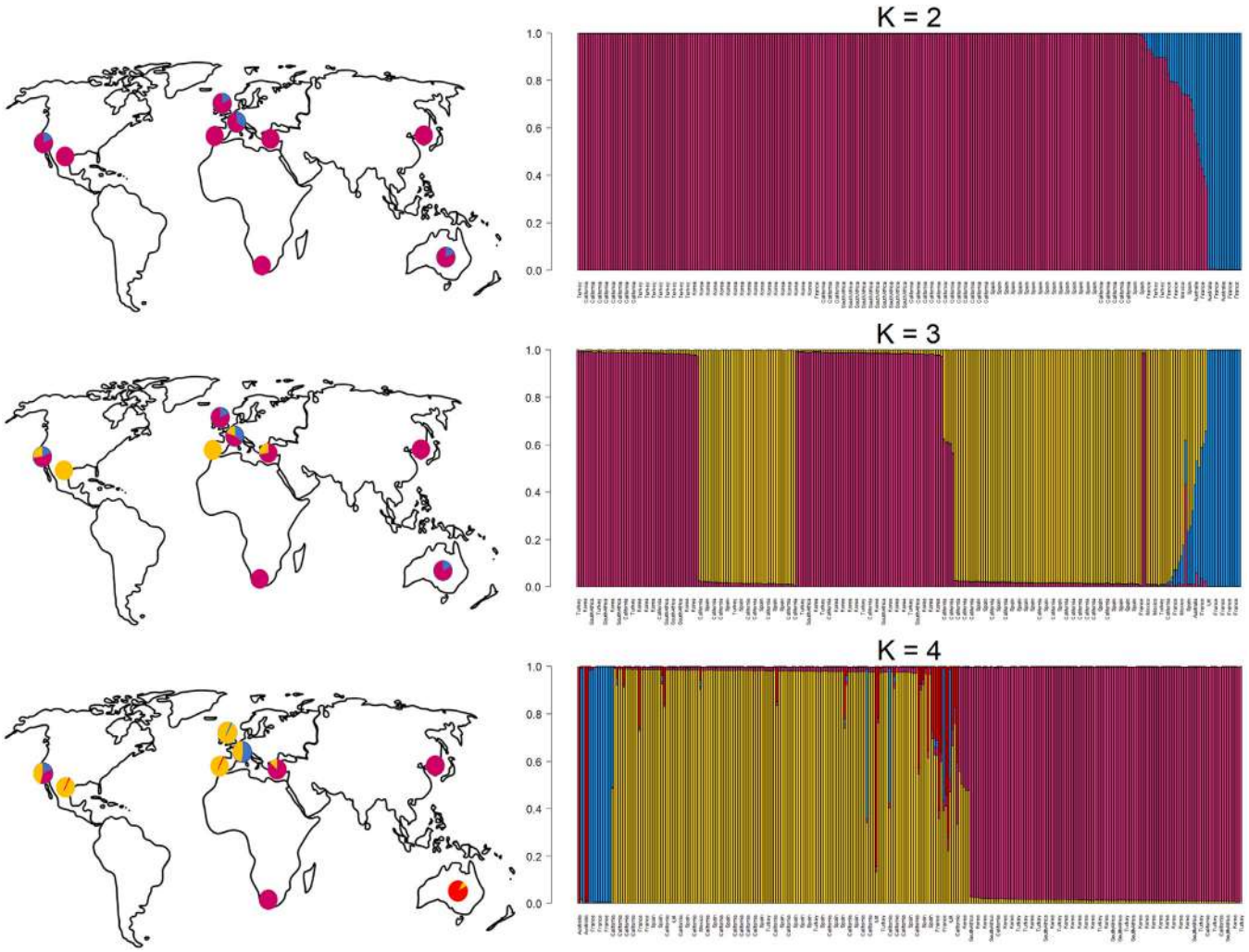

Figure 3. Map of sampled wild individuals and inferred population structure. Sample sizes can be found in Supporting Information Figure S1. Top: Structure plot for the most likely number of populations ($k = 2$). Purple individuals are homozygous for either of two common haplotypes (~90% of individuals). Blue individuals have some heterozygous loci and/or rare alleles. There is no geographic signal of differentiation. Middle: Plot for three inferred populations. Here, the two most common homozygous haplotypes (which differ only at one locus) are split into two clusters (purple and gold), with more heterozygous individuals in blue. Bottom: Plot for four populations, here again the dominant haplotypes are purple and gold, but two countries with greater diversity are separated into new clusters: Australia (red) and France (blue). Apparently admixed individuals (bars showing multiple colors in composition) are those with greater than average heterozygosity, as expected of recent outbreeding events. The most extreme of these are explored in Figure 4 and Table 1.

OUTBREEDING RATE

Evidence for outbreeding (i.e., androdioecy) was assessed in two ways. Fiirst, we estimated per-population inbreeding rates (Fig. 4) based on differences between observed and expected heterozygosity. These analyses suggest that selfing is pervasive, but not complete. The global mean F_{IS} is 0.70 and 90% of individuals have $F_{IS} > 0.5$. However, roughly 7% of individuals show quite low F_{IS} values (<0.2), as expected if rare males create occasional outbreeding events.

We further investigated these low F_{IS} individuals and found that they were more heterozygous than expected by chance. Because many DNA samples were depleted in initial genotyping, we could not repeat genotyping of these samples. Thus, rather than assuming genotypes were correct, we assessed heterozygous individuals as derived from binomial sampling probabilities of genotypes, to see if heterozygous loci occurred more frequently than expected under random genotyping error (see Supporting Information Fig. S2). These heterozygous individuals did indeed carry significantly more heterozygous loci than expected from genotyping error. Moreover, the alleles at these heterozygous loci were a combination of the two homozygous genotypes found in the nearby populations. In other words, putatively outbred individuals appeared to be the result of recent matings between locally common homozygotes. We have presented the

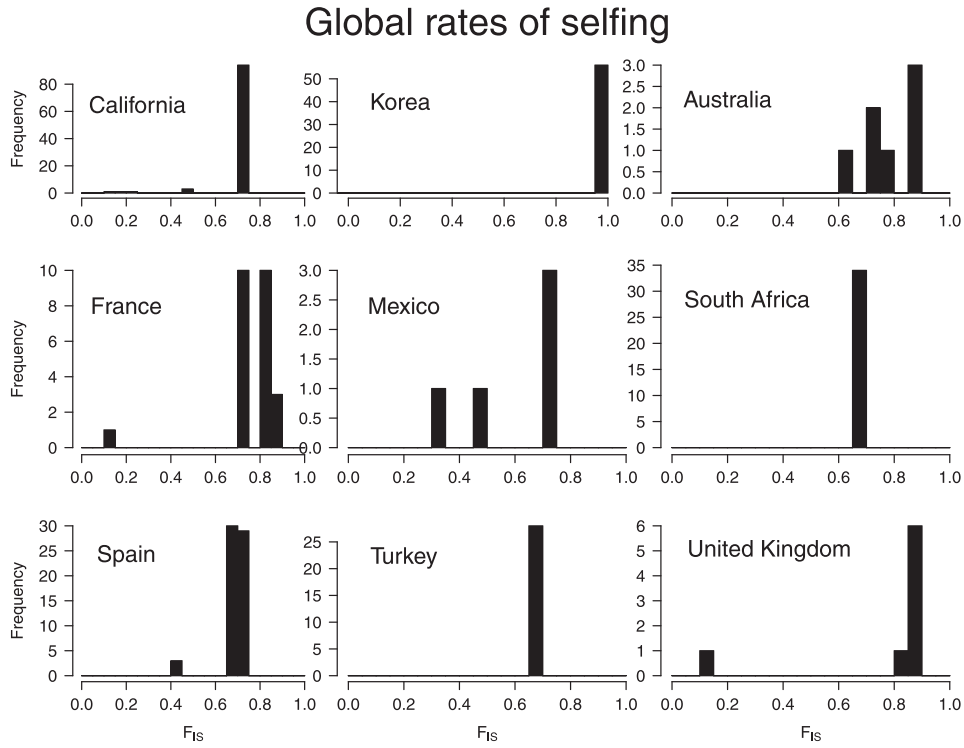

Figure 4. **Inferred inbreeding coefficient (F_{IS}) for individuals across nine populations. Here, we included only individuals for which at least two-thirds of loci (eight of 12) amplified. Each population shows a mean F_{IS} above 0.5 but many (e.g., the United Kingdom and France) also contain a minority of individuals showing relatively little evidence for inbreeding. Note that owing to a lack of clear population structuring, we pooled sampling locations within countries (namely, California in the United States, France, and Turkey).**

five individuals for which this evidence is strongest in Table 1.

This line of inference almost certainly underestimated the rate of outbreeding, as mating events between individuals with shared genotypes at our 12 loci will go undetected. To leverage our whole genotyping dataset, we also estimated the selfing rate (s) via the software RMES. Many populations were discarded due to lack of variation, precluding per-population estimates, but we obtained a global selfing rate of 0.85 (95% confidence interval: 0.84, 0.92). These rare inferred outbreeding events provide further indirect evidence that outcrossing, presumably involving males based on the reproductive anatomy of hermaphrodites, occurs in the wild.

Discussion

Three species of scale insects in the tribe Iceryini have been identified as hermaphroditic based on the cytology of their gonads: *Icerya purchasi* (Hughes-Schrader 1925; Royer 1975), *Gigantococcus bimaculatus* (previously known as *Icerya bimaculata*, Hughes-Schrader 1963), and *Crypticerya zeteki* (previously known as *Icerya zeteki*; Unruh and Gullan 2008b; Hughes-Schrader and Monahan 1966). Another two species, *Auloicerya acaciae* (Gullan 1986) and *Icerya aegyptiaca* (Gavrilov 2007 lists as parthenogenic; but see Kokilamani et al. 2014), have also been claimed to be hermaphroditic, though with limited or contradictory evidence. In each of these species, the precise reproductive system remains unclear. Under pure selfing, hermaphrodites can self-fertilize their eggs with their own sperm for an indefinite number of generations, rapidly exhausting genetic variation and leading to high levels of homozygosity and predictable genotype frequencies in offspring. However, the observations of occasional males raise the possibility that some portion of offspring is the result of outbreeding events between hermaphrodites and these males.

Here, we use a genetic analysis to examine the reproductive system of *I. purchasi* in detail. Based on genotypes at 12 microsatellite loci, we estimate both the selfing rate ($s = 0.82$) and the inbreeding coefficient (mean $F_{IS} = 0.70$) are substantial but not 100%, indicating some outcrossing occurs in nature. Moreover, there is considerable variation within populations in the inbreeding rate, as would be expected if some sampled individuals are derived from more recent outbreeding events than others.

In principle, this pattern could also arise from completely selfing populations if some other mechanism generates and

Table 1. Evidence for outbreeding in wild adult hermaphrodites.

Region	Population	Picp21		Picp39		Plcp45		Plcp71		Plcp58		Plcp62		Plcp101		Plcp37		Plcp93	
France	FA	186	186	165	165	179	179	108	108	137	137	173	173	98	107	206	206	115	115
France	FA	186	186	165	165	179	179	108	108	137	137	173	173	98	98	206	206	115	115
France	FR	186	186	165	165	179	179	108	108	137	137	173	173	98	95	206	206	115	115
France	FR	183	186	165	171	173	179	99	108	131	137	173	176	98	107	206	215	115	121
France	FP	183	183	171	171	173	173	99	99	131	131	176	176	128	128	215	215	121	121
France	FP	183	183	165	171	173	173	99	99	131	131	176	176	128	128	215	215	121	121
France	FP	183	183	171	171	173	173	99	99	131	131	176	176	128	128	215	215	121	121
UK	CH	186	186	165	165	179	179	108	108	137	137	173	173	98	98	NA	NA	NA	NA
UK	CH	183	186	165	171	173	179	99	108	131	137	173	176	98	107	206	215	115	121
UK	CH	183	183	171	171	173	173	99	99	NA	NA	NA	NA	107	107	215	215	121	121
UK	CH	183	183	171	171	173	173	99	99	131	131	176	176	NA	NA	NA	NA	NA	NA
California	CCS1	186	186	165	165	179	179	108	108	131	137	173	173	98	98	206	206	115	115
California	CCS1	186	186	165	171	173	179	108	108	131	137	173	176	98	107	206	215	115	121
California	CCS1	186	186	165	171	173	179	108	108	131	131	173	176	98	107	206	215	115	121
California	CCS1	183	183	165	171	173	179	99	99	131	131	173	173	98	107	206	215	115	115
California	CCS2	183	183	171	171	173	173	99	99	131	131	176	176	107	107	215	215	121	121
California	CCS2	183	183	171	171	173	173	99	99	131	131	176	176	107	107	215	215	121	121

Rows represent individuals with columns denoting individual alleles. For each putatively outbred individual ($F_{IS} < 0.2$ from Fig. 4, highlighted to the left), several individuals from the same country are shown above and below it. Alleles are colored by population in which they are most commonly found. Putatively outbred individuals are heterozygous for the locally common homozygous alleles, suggesting that they are the result of recent mating between the haplotypes.

maintains heterozygosity. First, it is worth considering that, without a better understanding of the genomic features of *I. purchasi*, it is possible that at least some of our markers are subject to selective rather than neutral forces. If some of our microsatellites are linked to recessive lethal variants, then we might expect variation to be maintained at low levels via selection against homozygotes at these loci (Kimura et al. 1963). However, we have no reason to think our markers should be effectively nonneutral, and moreover, given the substantial amount of selfing, homozygous genotypes should frequently arise and genetic load should be effectively purged by selection in a few generations (Fox et al. 2008), as has been seen with inbreeding in other insects (Haikola et al. 2001; Mongue et al. 2016). Second, consider that the observed heterozygosity may not be due to the maintenance of alleles, but de novo mutation. Microsatellite repeats are known to be more variable than point mutations, with mutation rate estimates as high as 10^{-3} per gamete per generation (Brinkmann et al. 1998; Schlötterer et al. 1998). But even this high rate of mutation would not be enough to generate the number of heterozygous loci we observe in a small sample of *I. purchasi*. Considering the pedigree data in the Supporting Information, roughly 13% of offspring loci were heterozygous at sites apparently homozygous in their hermaphroditic parent. Some of these are likely erroneous, but if even one in 13 is correct, and *not* a result of outbreeding, it would require a de novo mutation rate an order of magnitude higher than reported in other species. Moreover, there is some evidence that microsatellites generally evolve faster in species with monocentric chromosomes (Jonika et al. 2020), and mutation rate is positively related to genome-wide heterozygosity (Amos 2016). Given that *I. purchasi* is a holocentric species (Melters et al. 2012) with very low heterozygosity, the expectation is that microsatellites should evolve slower than average. Finally, the same patterns of heterozygosity could be observed without males if hermaphrodites occasionally mate with each other; however, hermaphrodites do not possess any sort of intromittent organ with which to transfer sperm (Johnston 1912) and appear to be largely sessile at maturity. Thus, although our observations are consistent with each of the above scenarios, they all strain credulity.

A much more plausible explanation is the reintroduction of heterozygosity into lineages via outcrossing between hermaphrodites and males, in other words, androdioecy. Our genotyping data point to evidence of these matings in the wild. First, individuals vary in inbreeding coefficient and, crucially, lower F_{IS} values appear to be crosses of two locally common haplotypes. And, while less compelling, one family from supplemental pedigree analyses showed a tri-allelic locus between parent and offspring, which could only result from outbreeding or genotyping error. Moreover, we observed males mating with hermaphrodites in our laboratory population. This combination of genetic and behavioral evidence makes a strong case for androdioecy.

Apart from obtaining evidence consistent with androdioecy, we also attempted to use our genotypic data to distinguish mechanisms of selfing. Unfortunately though, due to the low global diversity there were very few heterozygous parents, and even fewer for which sufficient eggs were available for genotyping. In the end, we were only able to consider a single family from which we obtained genotypes for the parent and 10 of their eggs. The presence of all three genotypic combinations in the offspring argues against a chimeric system of pure selfing with a persistent sperm lineage, which would generate only two of the three genotypes. Considering genotype frequencies at heterozygous loci, we would expect a Mendelian ratio of homozygotes and heterozygotes if sperm are created by the random meiotic elimination of one of the two alleles during spermatogenesis. On the contrary, all nine heterozygous loci deviate from this expected ratio. It is likely that some of the loci are in linkage disequilibrium with each other due to a high selfing rate and the low chromosome number in *I. purchasi*; in other words, much of the genome is physically linked and the decay of heterozygosity from selfing will create longer distance association between alleles. Therefore, we did not formally test for significant differences at each locus. Moreover, the possible genetic contribution by a male to the offspring makes distinguishing between mechanisms of selfing very difficult because the progeny may be a mix of outcrossed and self-fertilized embryos. Therefore, our results are inconclusive and controlled laboratory observation of these insects will be required to confirm how hermaphrodites self-fertilize.

POPULATION STRUCTURE OF *ICERYA PURCHASI*

As predicted for a species with a substantial selfing rate, *I. purchasi* populations are highly homozygous, with 90% of samples having an identical diploid genotype at 11 of 12 markers. This homogeneity corroborates the narrative of global expansion through repeated, (presumably) accidental introductions by humans into new areas. No significant relationship between genetic and geographic distance is detectable. For example, South Africa and South Korea have nearly identical genotypes ($F_{ST} = 0.01$), whereas South Korea and Spain ($F_{ST} = 0.87$) have the highest divergence of any pair of populations, being essentially fixed for two different haplotypes. And more generally, although genetic differentiation does not vary predictably with physical distance, the presence of identical haplotypes around the world might suggest that these populations share a common origin (i.e., derived populations all arose from the same ancestral population rather than secondary spread from introduced populations). But these inferences are based on a small number of markers; a much larger sample of loci (e.g., from whole-genome SNP data) could reveal population variability and differentiation not detected here.

Finally, *I. purchasi* is thought to be Australian in origin (Prasad 1989) and, consequently, one might expect genetic diversity to be highest there. Speaking to this, there are at least 10 private alleles not seen in any other population from this initial sample. Needless to say, it is difficult to accurately assess this pattern with a mere 12 loci from seven individuals. As above, a larger set of genetic markers and increased sampling across this native range will be needed to fully resolve the population variability and differentiation.

ANDRODIOECY IN ECOLOGICAL CONTEXT

Although the present genetic markers do not permit us to resolve the colonization history of this pest insect, it is clearly globally distributed and androdioecy likely contributed to its colonizing success following accidental introduction into new areas by humans (most likely through trade in live plants). Its ability to self-fertilize allows a single hermaphrodite to establish a new colony on its own, whereas occasional outbreeding can maintain variation on which selection can act, allowing it to adapt to new ecological conditions faster than clonal reproduction would allow.

This system, although physiologically distinct from that of (fellow hemipteran) aphids, bears striking ecological similarity to their parthenogenesis with occasional sexual reproduction (Blackman 1980). In each system, a single individual has the ability to found a new population: either through selfing in *I. purchasi* or through the parthenogenetic production of daughters in aphids. Likewise, both systems retain a capacity for sexual reproduction via mating with rare males. Scale insects and aphids share a similar phytophagous lifestyle, so it is worth considering that selective pressures to exploit the geographic and phylogenetic range of host plants have produced complimentary reproductive dynamics in these herbivores. Understanding these systems is thus important not only for evolutionary theory, but also for the development of management strategies for these crop pests.

Finally, it is worth considering our initial findings with *I. purchasi* in the context of other androdiecious species. The relatively high rate of selfing is surprising compared to those of androdiecious plant systems (e.g., 0.08–0.35; Fritsch and Rieseberg 1992) but perfectly in line with the two most studied androdiecious animals. In the clam shrimp, *E. texana*, outcrossing seems to be much less common than selfing (with an estimated selfing rate of 0.80–0.98; Hollenbeck et al. 2002). In *C. elegans*, selfing rates also range from roughly 0.78–0.99, depending on the population and methodology in question (reviewed in Anderson et al. 2010). Thus, three independently evolved systems have converged on similar outcrossing rates. These similarities could be the result of basic differences in animal and plant biology: hermaphroditic plants tend to be cross-fertile, whereas hermaphroditic animals are not (Weeks et al. 2006), but the high selfing rate may also be partly attributable to parallel adaptations in animal systems. In both *E. texana* and *C. elegans*, hermaphrodites have evolved traits to discourage outcrossing (Chaudhuri et al. 2015; Ford and Weeks 2018). It remains unclear if *I. purchasi* has behaviorally or physiologically changed to avoid outcrossing, but it is an obvious line of further research.

Conclusions

Here, we have presented evidence, both at the population and pedigree scale, that *I. purchasi* is most likely an androdiecious insect. Self-fertilization of hermaphrodites appears to be the most common mode of reproduction, leading to low levels of genetic variation at (putatively) neutral markers. Yet physical observation and genetic data reveal that males occasionally mate with hermaphrodites and wild hermaphrodites occasionally outcross, thus preventing a complete loss of genetic variation. This mixed system defies clear predictions for the presence and frequencies of genotypes in offspring, thus it remains an open question with what cytogenetic mechanism hermaphrodites self-fertilize. Nevertheless, this corroborating evidence of androdioecy lays the groundwork to investigate how males are produced and why they vary substantially in frequency between populations, how this rare and unusual system of reproduction compares to other androdiecious animals, and the implications for managing this invasive pest.

AUTHOR CONTRIBUTIONS

AJM conceived of and carried out analysis, wrote the manuscript, and coordinated input from all other authors. SM and OC carried out the genotyping. AT, DSK, and MSH collected most of the samples, enabling the worldwide scope of the analysis. AG and BBN offered expertise in both the design of the experiment and the write up of results. LR conceived of the project, designed primers, coordinated sample collection and genotyping, and provided in-depth feedback for the manuscript. All authors reviewed and edited the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

DATA ARCHIVING

All data and analysis scripts can be found at the following git repo: https://github.com/RossLab/Icerya_purchasi/. Information on primer sequences can be found in the Supporting Information Materials.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Additional collecting information for samples, denoting country, location, and population code used in data files.

Table S2. *Icerya purchasi* microsatellite primer information for the markers used in this study.

Table S3. Allelic richness of loci examined in this study. Note that in spite of high levels of homozygosity, all loci included in analyses showed some variation globally.

Table S4. Global pairwise F_{ST} for each population included in the population structure analysis (i.e., those with more than one individual with genotypes at eight or more of the 12 markers used here).

Table S5. Investigation of offspring genotypes from a wild-caught hermaphrodites.

Figure S1. Sample sizes for each location used in genotypic analyses. Locations match the maps accompanying the structure plots in Figure 3.

Figure S2. Evidence for outbreeding based on excessively heterozygous individuals.