

Mate availability contributes to maintain the mixed-mating system in a scolytid beetle

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Abstract

We investigated the mating system and population genetic structure of the beetle, *Coccotrypes dactyliperda*, with life history characteristics that suggest the presence of a stable mixed-mating system. We examined the genetic structure of seven populations in Israel and found significant departures from the Hardy–Weinberg equilibrium and an excess of homozygosity. Inbreeding coefficients were highly variable across populations, suggesting that low levels of outbreeding occur in nature. Experiments were conducted to determine whether the observed high inbreeding in these populations is the result of a reproductive assurance strategy. Females reared in the laboratory took longer to mate with males from the same population (inbreeding) than with males from a different population (outbreeding). These results suggest that females delayed inbreeding, and were more inclined to outbreed when possible. Thus inbreeding, which predominates in most populations, may be due to a shortage of mates for outbreeding rather than a preference for inbreeding. We conclude that *C. dactyliperda* has a mixed-mating system that may be maintained by a reproductive assurance strategy.

Introduction

Breeding systems in plants and animals vary and may occur at any point on a continuum between the two extremes of self-fertilization (an extreme case of inbreeding) and outbreeding (Jarne & Charlesworth, 1993; Barrett, 2003).

Selfing is promoted, for reasons of advantages in terms of increased gene transmission and avoiding the cost associated with searching for a mate. The advantages of outbreeding include the masking of deleterious alleles and an increase in genetic variability, which is favoured in changing environments (Keller and Waller, 2002). Early comparative data as well as mathematical models describing the evolution of breeding systems demon-

strated that plants and animals should evolve towards a stable state at either end of the breeding continuum (Lloyd, 1979; Lande & Schemske, 1985; Lande *et al.*, 1994). Intermediate rates of inbreeding (mixed-mating systems) were considered to be only a transitional state or an incidental by-product of other adaptive mechanisms (Schemske & Lande, 1985). Models (e.g. Lande & Schemske, 1985) predicted that the distribution of inbreeding rates (F_{IS} -estimates) will be completely bimodal (Schemske & Lande, 1985; Vogler & Kalisz, 2001): either high selfing rates ($F_{IS} = 0.7$) or high outcrossing rates ($F_{IS} = 0.1$) with low variation (Lande & Schemske, 1985; Jarne & Charlesworth, 1993; Goodwillie *et al.*, 2005). Species with a transient mixed-mating system are expected to have some populations with intermediate selfing rates and others with extreme F_{IS} values that characterize complete outbreeding or inbreeding populations (Jarne & Charlesworth, 1993).

Despite the predicted evolutionary dichotomy of the two mating systems, all recent extended surveys show a

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much lower representation of predominantly inbreeding or outbreeding taxa than expected (Goodwillie *et al.*, 2005). Species with intermediate levels of inbreeding are characterized by variation in selfing rate both within-populations (plants: Dudash *et al.*, 1997; Koelewijn, 1998; Mutikainen & Delph, 1998; animals: Jokela *et al.*, 2006; Viard *et al.*, 1997; Henry *et al.*, 2005; Wiehn *et al.*, 2002) and between populations (plant: Rajimann *et al.*, 1994; Routley *et al.*, 1999; animals: Charbonnel *et al.*, 2005; Jokela *et al.*, 2006). The variation within the range of intermediate rates of inbreeding is considered to be an integral characteristic of stable mixed-mating systems (Rauscher & Chang, 1998).

In small or recently colonizing populations and in island populations (Jarne & Charlesworth, 1993), which are characterized by large genetic differentiation between populations (Larson & Barrett, 1998), predominant inbreeding or outbreeding in a mixed-mating system is likely to occur. If there is one founder or related founders, inbreeding will predominate and if the founders are unrelated predominantly, outbreeding is expected. Recent theoretical (Uyenoyama, 1986; Cheptou & Mathias, 2001; Cheptou & Schoen, 2002; Tsitrone *et al.*, 2003a; Goodwillie *et al.*, 2005) and empirical studies (Tsitrone *et al.*, 2003a; Goodwillie *et al.*, 2005) suggest a variety of possible mechanisms in which mixed-mating systems can become evolutionarily stable. Mixed mating can be promoted by genetic factors, for example over-dominance (Campbell, 1986; Charlesworth & Charlesworth, 1990) or by behavioural mechanisms to ensure reproduction when outbreeding possibilities are limited (Lloyd, 1992; Kalisz & Vogler, 2003; Tsitrone *et al.*, 2003a).

Reproductive assurance mechanisms are suggested in ecological models in which the components of reproductive success may vary depending on ecological conditions. Delayed inbreeding is usually considered a fixed strategy in genetic models. However, flexibility in the timing of inbreeding may be of great importance in species that can perform selfing, but nonetheless prefer to outbreed. For example, when inbreeding depression is severe and exceeds the advantages associated with selfing (Keller and Waller, 2002), inbreeding species that have the potential to outbreed should prefer to outbreed (Tsitrone *et al.*, 2003a). Outbreeding, however, is not always possible when the availability of sexual partners is limited. The optimal reproductive strategy in this case might be to wait for a partner for a certain period of time, and then switch to inbreeding if no suitable mates are encountered (Tsitrone *et al.*, 2003a). When inbreeding depression is weak, selection for reproductive assurance will favour early inbreeding (Lloyd, 1992; Elle & Hare, 2002). This reproductive assurance strategy may lead to rapid changes in the average inbreeding coefficients of the populations (Charbonnel *et al.*, 2005).

There are several models based on reproductive assurance that predict stable mixed mating, some of which

predict variable selfing rates within and others between populations (reviewed in: Goodwillie *et al.*, 2005). The actual mechanisms that are considered to stabilize mixed mating differ. Several models consider selfing and mixed mating to evolve when outcrossed pollen is chronically limited (Lloyd, 1979, 1992; Vallejo-Marin & Uyenoyama, 2004) or variable in availability (Schoen & Brown, 1991; Sakai & Ishii, 1999; Morgan & Wilson, 2005). For example, Schoen & Brown (1991) predict that selfing will be induced only in the absence of cross-fertilization and will be selected whenever pollination is variable and the inbreeding depression is not extremely high. Some models show that mixed mating can be evolutionarily stable only if self-pollination reduces the export of pollen to flowers on other plants, i.e. pollen discounting (Johnston, 1998; Morgan & Wilson, 2005).

Models of reproductive assurance were developed primarily to explain breeding strategies in plants, and later expanded to self-fertilizing animals such as snails that preferentially outbreed (Jarne & Delay, 1990; Stadler *et al.*, 1995; Coutellec-Vreto *et al.*, 1997). In these models mechanisms that are considered to stabilize mixed mating in plants are suggested to play an essential role also in animals; for example, limited mate availability can act in the same manner as pollen limitation (Jarne & Auld, 2006). The relevance of the mate availability model has not yet been tested on species with other life history traits. In this study, we investigated population genetics and mating decisions of a haplo-diploid beetle species, testing whether populations maintain a mixed-mating system by means of a female reproductive assurance strategy.

The palm-seed borer *Coccotrypes dactyliperda* Fabricius (Coleoptera: Scolytidae) is a haplo-diploid organism with a mixed-mating system. This system is characterized by (i) mother-son mating, where an unfertilized mother produces haploid sons and mates with them, (ii) sib-mating and (iii) mating with an individual from the same or a different population. Mother-son mating and other inbreeding possibilities might be part of a reproductive assurance strategy when females experience low availability of less related males. We studied the possible role of the reproductive assurance strategy in the mating system of *C. dactyliperda*. We first assessed the genetic background of various populations to estimate their inbreeding rates and degree of genetic differentiation and then conducted behavioural experiments to test whether females will preferentially outbreed when given the opportunity to do so. By using this approach we tested the hypothesis that *C. dactyliperda* maintains a mixed-mating system that is consistent with a female strategy of reproductive assurance. If reproductive assurance determines the mating system in this species, we expect to find the following combination of traits: (i) large variability in inbreeding rates of populations, typical of a mixed-mating system, and (ii) low male availability, together with (3) a strong tendency to outbreed. All three traits together will suggest a reproductive assurance

strategy, i.e. a preference for outbreeding, but with limited outbreeding opportunities.

Materials and methods

Study organism

The palm-seed borer beetle, *C. dactyliperda* develops in seeds of the date palm, *Phoenix dactylifera*, and other palm species. Seed colonization usually takes place individually, but in high beetle densities several females may colonize a single seed. Colonization and oviposition occur only after the fruit drops to the ground. The eggs are laid in clusters in the distal part of a gallery excavated by the female. Mated females may disperse or stay in the natal seed whereas males usually do not disperse. Each colonizing female excavates a brood chamber with a single entrance hole (Herfs, 1950; Bar-Shalom & Mendel, 2003).

Coccotrypes dactyliperda populations are highly female-biased, with males ranging from 0% to 16% of the total population (D. Gottlieb, unpublished data). Preliminary studies showed that ovipositing females respond to the relatedness of other females colonizing the same date by adjusting their offspring sex ratio as predicted by Hamilton (1967) under local mate competition (D. Gottlieb, unpublished data). These observations suggest that *C. dactyliperda* can distinguish kin from nonkin and may select a preferred mating partner accordingly.

Populations studied and sampling design

Seven date plantations were sampled in June 2005 over a wide geographic range, from the western Negev in the south of Israel to the Jordan Valley and the Sea of Galilee in the north (SG = Sha'ar Hagolan, BZ = Beit Zera, PC = Pardes Chana, BD = Beit Dagan, Ch = Chatzor, Be = Ein Habsor and Sa = Sa'ad, Fig. 1). Between 50 and 100, seeds were collected haphazardly under five trees located in the centre of each date plantation. At least 10 beetles were collected from each site and the beetles were extracted from 10 seeds, immediately after collecting the seeds from the field, preserved in 95% ethanol and stored in the laboratory at 4 °C until DNA extraction.

DNA analysis

Microsatellite primers were selected from previously developed primer pairs (Berg *et al.*, 2003). Six loci were chosen for their polymorphism and unambiguous amplification patterns (Holzman *et al.*, 2009). Fluorescent dye labelled versions of chosen primers (FAM-6, VIC, PET, NED) were obtained from Applied BioSystems (Foster City, CA, USA). Temperature conditions for genomic amplification of microsatellite loci followed Berg *et al.* (2003). PCR amplifications were performed in a total volume of 25 μ L, containing 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 0.5 unit of Taq, 50 μ M of dNTPs,



Fig. 1 Map of study area indicating the approximate location of *Coccotrypes dactyliperda* populations: On = Haon, SG = Sha'ar Hagolan, BZ = Beit Zera, Ash = Ashdot Yakov, PC = Pardes Chana, BD = Beit Dagan, Ch = Chatzor, Be = Ein Habsor, Sa = Sa'ad and Zi = Zikim.

40–60 ng μ L⁻¹ of DNA template and 5 μ M of fluorescent primer. For multiplexing, 3 μ L of diluted multiplexed PCR product was mixed with 8 μ L hi-di formamide, and 0.4 μ L of GENESCAN-500 LIZ-labeled molecular weight standard (Applied BioSystems). The samples were then denatured at 95 °C for 1 min before loading onto an ABI 3100 automated sequencer. ABI default genotyping run conditions for Pop-4 polymer were used (Applied BioSystems) and the default run time was extended to 1700 s from 1500 s. GENEMAPPER 3.7 software was used to score alleles (Applied BioSystems).

Within-population genetic diversity

Genetic diversity at a single locus was described using standard parameters: the frequency of alleles per locus and allelic diversity (i.e. average number of alleles per locus). The mean observed heterozygosity and expected heterozygosity (i.e. gene diversity; Nei, 1987) were calculated per population. The unbiased estimator of Wright's inbreeding coefficient F_{IS} was calculated according to Weir & Cockerham (1984). Genotypic disequilibria between pairs of loci and departures from Hardy-Weinberg equilibrium were evaluated in each population

using Fisher's method (Fisher, 1925). All calculations and tests above were performed using GENEPOP 3.1C (Raymond & Rousset, 1995).

Among-populations genetic structure

The level of genetic differentiation between populations was calculated by F_{ST} estimates following Weir & Cockerham (1984) using the computer program GENEPOP 3.1C (Raymond & Rousset, 1995). To test for the impact of stepwise mutations on genetic structuring, R -statistics (Goldstein & Pollock, 1997) were compared with the corresponding F -statistics using the computer program SPAGED1 1.2 (Hardy & Vekemans, 2002). This comparison indicates the relative effect of mutation (R_{ST} estimates) vs. genetic drift (F_{ST} estimates) on the level of the estimated genetic differentiation between populations (Balloux & Lugon-Moulin, 2002).

Pairwise estimates of F_{ST} were regressed on spatial distance (shortest distance, see below). This allowed us to estimate the different patterns of migration between populations. Isolation by distance was tested using the method of Rousset (1997). In isolation by distance, a correlation is expected between the logarithm of the geographical distance (the shortest distance between two points in a two-dimensional habitat) and $F_{ST}/(1-F_{ST})$. This correlation was tested using a Mantel test and a rank correlation coefficient (Rousset, 1997). The effective number of migrants (N_m) for the populations sampled in this study was estimated according to Slatkin (1985) using the computer program GENEPOP 3.1C (Raymond & Rousset, 1995).

Behavioural experiment: inbreeding vs. outbreeding

As natural populations are highly female-biased there is only a small chance of a female encountering two or more males simultaneously. Therefore, we tested a female's inclination to mate with a single male provided to her. We compared the time until mating with males from the same population (inbreeding) and males from a distant population (outbreeding). We used individuals from seven well-separated populations (Fig. 1): Sha'ar Hagolan (SG), Beit Zera (BZ), Haon (On), Beit Dagan (BD), Ashdot (Ash), Saad (Sa) and Zikim (Zi). Individuals from these populations were collected as described above and brought to the laboratory where they were kept in 25 ± 0.5 C, and 14 : 10 L : D. Offspring pupae were kept individually in vials to produce virgin males and females. Males and females 1- to 2-days-old were used in the experiment. The females were placed in a small Petri dish (6×1 cm), subjected to one of the two following treatments: (i) inbreeding (female presented with a male from the same population); (ii) outbreeding (female presented with a male from a different population). Time was measured from the introduction of the male into the female's dish until mating occurred.

Table 1 Origin of individuals in mating trials. Females were presented with males from the same or a different population.

Female origin	Inbreeding		Outbreeding	
	Male origin	<i>N</i>	Male origin	<i>N</i>
SG (I)	SG (I)	32	BD	36
SG (II)	SG (II)	20	On	16
On	On	13	SG	19
Ash	Ash	7	BZ	10
Sa	Sa	10	Zi	10
Zi	Zi	10	Sa	10

N = number of pairs.

We used this delay in time before mating as an indication of mating inclination: a shorter delay indicates that a female is more inclined to mate with the presented male (Tsitrone *et al.*, 2003a). Observations were stopped when mating occurred and lasted no more than 8 h after male beetles were presented to the females. Preliminary observations indicated that females that do not mate during 8 h are unlikely to mate at all, and this behaviour occurred equally in inbreeding and outbreeding treatments. Therefore, pairs that did not mate during 8 h of observation were excluded from the analysis. We report here on six inbreeding and outbreeding experiments, including two reciprocal crosses (see Table 1). High mortality rate of pupae in two populations reduced the number of crosses analyzed. Differences between time until mating in inbreeding and outbreeding females were analyzed by ANCOVA, with the time until mating as the dependent factor, male relatedness to the female (inbreeding or outbreeding) as independent fixed factor and the different origins of females (different populations) as covariates. Interaction between female origin and treatment allowed us to understand if there is a different effect of treatment on females from different populations. We analyzed the data with SPSS 14.0 (Bühl & Zöfel, 2002). For all results means and standard error of means are presented.

Results

Within-population genetic diversity

All six microsatellite loci that were scored for all *C. dactyliperda* populations examined were found to be polymorphic. Of these six microsatellites, three (Dact10, Dact13 and Dact6) showed fairly low levels of variation and were monomorphic in some populations. The other three polymorphic loci were variable in all populations and showed intermediate levels of diversity, with the average number of alleles per locus in a population (*A*) ranging from 2.17 (Be population) to 3.88 (BD population) and the percentage of polymorphic loci within a population (*P*) ranging from 66% to 100%. Mean gene

Table 2 Microsatellite variation averaged over all loci (Goodwillie *et al.*).

Population	N	A	P (%)	H_O	H_S	F_{IS}	Selfing rate
All loci							
Be	14	2.166	83	0.201	0.301	0.331***	0.497
BZ	14	3.000	100	0.246	0.404	0.391***	0.562
Ch	25	2.666	100	0.233	0.4233	0.448***	0.619
SG	11	2.666	83	0.328	0.432	0.241**	0.388
PC	15	3.000	83	0.344	0.415	0.170***	0.291
Sa	25	2.333	66	0.139	0.379	0.634***	0.776
BD	17	3.833	83	0.358	0.626	0.427***	0.598
Mean		2.809	85	0.264	0.426	0.377	0.548
Polymorphic loci							
Be		2.666					
BZ		3.333					
Ch		3.333					
SG		3.000					
PC		3.333					
Sa		3.333					
BD		3.667					
Mean		3.238					

N , sample size; A , mean number of alleles per locus; P , percentage of polymorphic loci; H_O , observed heterozygosity; H_S , expected heterozygosity (calculated using Weir & Cockerham (1984)) and F_{IS} (Nei, 1987).

Hardy–Weinberg test when H_I = heterozygote deficiency; * P < 0.01; ** P < 0.001; *** P < 0.0001.

diversities (observed heterozygosity, expected heterozygosity and F_{IS}) were highly variable (Table 2). Observed heterozygosity ranged from 0.14 in Sa population to 0.36 in BD population and F_{IS} ranged from 0.17 in PC population to 0.63 in Sa population (Table 2).

All samples analyzed displayed a significant departure from the Hardy–Weinberg equilibrium (global test, P < 0.001, Table 2) with an excess of homozygotes. To facilitate a better comparison of microsatellite variation with other studies that often use only the most polymorphic loci in their analysis (e.g. Awadalla & Ritland, 1997), here too we calculated microsatellite variation for polymorphic loci only, and it ranged between 2.66 and 3.66.

Given the high F_{IS} levels of some *C. dactyliperda* populations, some level of linkage disequilibria was expected. Indeed, tests for genotypic disequilibrium across all populations detected significant deviations

from the null hypothesis of independent assortment in two of the thirteen pairs of loci (Dact1 & Dact5: $\chi^2 = 27.842$, d.f. = 16, $P = 0.033$, and Dact1 & Dact6: $\chi^2 = 26.164$, d.f. = 10, $P = 0.004$). All other pairs of loci (13 pairs) had $P > 0.05$.

Among-population differentiation

Large differences in genetic variation were observed between populations. Nearly half of the alleles (16 of 39) were private alleles, i.e. alleles specific only for one population, and occurred at low frequencies (ranging between 0.023 and 0.368). In general, there were large differences among populations in the allele frequency distributions of most abundant loci [ranging between 0.023 (Be) and 0.833 (PC)]. Accordingly, most of the differentiation between the populations was significant, with large values of F_{ST} and R_{ST} (Table 3). Both, F_{ST} and R_{ST} indicate that there is little gene flow between the *C. dactyliperda* populations examined. F_{ST} values were regarded as more robust than R_{ST} values as (i) there was no significant contribution of the Stepwise Mutation model (R_{ST}) to genetic differentiation ($P = 0.8761$), and (ii) four alleles among the dinucleotide loci appeared to be separated by only one nucleotide which leads to inconsistencies and mis-scoring of R_{ST} (Chauvet *et al.*, 2004).

The pairwise F_{ST} values estimated over all loci averaged 0.135 (Table 3). The lowest pairwise F_{ST} value (Table 4) was found to be between Sa and BZ ($F_{ST} = 0.0148$) and the highest value was between populations from BD and Be ($F_{ST} = 0.3186$). Migration rate calculations using the private allele method and including all populations showed an estimated low number of migrants, $N_m = 0.869$ per generation (GENEPOP, option 4). Furthermore, Mantel tests using F_{ST} as a measure of genetic distance resulted in no significant correlations at various spatial scales ($r^2 = 0.0363$, $P = 0.626$).

Behavioural experiments

Females mated with related males (inbreeding: $N = 92$, 167 ± 12 min) took significantly more time to mate compared with mating with none related males (outbreeding: $N = 101$, 106 ± 9 min), (Table 5, Fig. 2). There was no significant interaction between the effect of male relatedness and female origin (Table 5, Fig. 2).

Table 3 Measures of population differentiation for five different parameters: Microsatellite (6 loci).

	A	P (%)	H_T	F_{ST}	R_{ST}	$F_{ST}/(1-F_{ST})$
All populations	2.80	85	0.336–0.732 (0503)	0.0148–0.3186 (0.135)	–0.0461–0.420 (0.141)	0.01–0.467 (0.226)
Holzman <i>et al.</i> (2009)	1.70	58.7	0.036–0.580 (0.283)	–0.051–0.455 (0.088)	–0.045–0.477 (0.093)	–0.04–0.834 (0.096)

F_{ST} and R_{ST} and average Nei's (1987) genetic distance ($F_{ST}/(1-F_{ST})$). F_{ST} and R_{ST} are the proportion of the total genetic variation that can be attributed to variation among populations.

A , Mean number of alleles per locus; P , percentage polymorphic loci; H_T , total gene diversity.

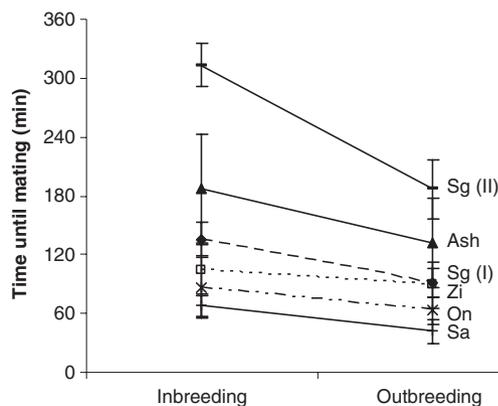
Table 4 Genetic differentiation between populations* (F_{ST}) values.

	Be	BZ	Ch	SG	PC	Sa
BZ	0.081**					
Ch	0.060***	0.101***				
SG	0.241**	0.297**	0.167***			
PC	0.437***	0.376***	0.278***	0.188***		
Sa	0.034	0.015*	0.060***	0.192**	0.332**	
BD	0.4677***	0.462***	0.289***	0.183***	0.084*	0.388***

Significance after Bonferroni correction, * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

Table 5 ANCOVA on the effects of treatment (inbreeding and outbreeding) and female origin on time until mating.

Source	N	F	Sig.
Treatment (inbreeding, outbreeding)	193	8.255	0.005
Female origin	193	9.081	0.002
Treatment × Females origin	193	1.193	0.160

**Fig. 2** Time until mating with males from the same population (inbreeding) and from a distant population (outbreeding). Figure shows mean \pm 1 SE.

Discussion

Most haplo-diploid animal species studied so far show high levels of microsatellite variation: the percentage of polymorphic loci (P) can range from 75% to 100%, mean number of alleles per locus (A) ranges from 2.8 to 7.7, and the mean gene diversity varies from 0.40 to 0.75 (Van Der Strate *et al.*, 2002). For the haplo-diploid beetle *C. dactyliperda*, we observed levels of microsatellite variation (Tables 3 and 4) similar to the lower values reported in typical haplo-diploid species (Van Der Strate *et al.*, 2002). Similar low levels of variation in microsatellite loci were observed in Californian populations of *C. dactyliperda* (Holzman *et al.*, 2009) (Table 3). These low

levels of variability are complemented by inbreeding values (F_{IS}) that average around 0.33 across all sampling sites. Moreover, the chosen microsatellites have been found in previous works to be in linkage equilibrium (i.e. genotypes on one locus are independent of genotypes on the other locus; Holzman *et al.*, 2009). Our tests for genotypic disequilibrium detected significant deviations from the null hypothesis of independent assortment for two pairs of loci. The low levels of heterozygosity and linkage between the two pairs of loci may result, in part, from the fact that allele frequencies differ between collecting sites, but the data are also consistent with mating between siblings (Herfs, 1950; Hamilton, 1993). Thus, high levels of variation in microsatellite loci, low levels of heterozygosity and linkage disequilibrium between loci confirm an expected large tendency towards inbreeding. Nevertheless, inbreeding coefficients varied considerably across populations, ranging from 0.17 to 0.63 and *C. dactyliperda* did not show extremely high F_{IS} values, i.e. above 0.7 (Chauvet *et al.*, 2004), as is often the case when selfing is the predominant form of mating. Taken together, these results suggest that individuals may move and outbreed with mates of a different genotype.

Genetic differentiation among populations

With microsatellite data, genetic structure can be deduced either on the basis of allelic identity (F_{ST}) or from differences in the allele size (R_{ST}) (Balloux & Lugon-Moulin, 2002). We found no substantial differences between global values of F_{ST} and R_{ST} (Table 3). This result suggests that stepwise-like mutations did not contribute to the differentiation among populations (Balloux & Lugon-Moulin, 2002), and implies that genetic drift with low gene flow among populations is sufficient to counteract the effects of mutation. Moreover, high pairwise F_{ST} values between most populations also indicate low levels of gene flow between most pairs of the examined populations.

We found no correlation between the level of genetic differentiation and geographic distance. A similar absence of significant correlation between genetic differentiation and geographic distance was observed in the Californian populations (Holzman *et al.*, 2009). This lack of correlation and the large genetic differentiation among *C. dactyliperda* populations examined suggest that the populations have not reached demographic equilibrium, as is reasonable if colonization is a recent evolutionary event (Slatkin, 1993). Recent colonizations can be attributed to the effects of anthropogenic habitat fragmentation, or to active transportation and introduction of palm trees to new locations. This idea is further supported by the lack of a significant difference between R_{ST} and F_{ST} . Different populations may have been separated for an insufficient time for mutations to accumulate (Hardy *et al.*, 2003).

Behavioural experiment

By experimentally testing individuals from various populations (regardless of geographical distances), we created extreme situations of choice of inbreeding vs. outbreeding that do not commonly occur in nature. In all cases females were quicker to mate with males from a different population than with males from their own population. This result is consistent with the fact that all populations that were tested for inbreeding rates had intermediate to high inbreeding rates yet did not show the extreme inbreeding rates found in predominantly selfing species (Chauvet *et al.*, 2004). Thus, although the estimated migration rate was low, migrating males may be favoured by local females that prefer to outbreed over mating within the population, and females that stay in their original population will delay mating to wait for outbreeding opportunities before inbreeding. These results suggest that by delaying inbreeding, females may increase the chance of encountering a male of a different genotype. Yet, genetic data of the beetle populations suggest that inbreeding occurs commonly and thus may constitute a reproductive assurance strategy (Tsitrone *et al.*, 2003a,b), resulting ultimately in a mixed-mating system.

Mixed mating as an evolutionarily stable system

The behavioural and population-genetic results of this study suggest that mate availability contributes to the maintenance of the mixed-mating system in *C. dactyliperda*, a species characterized as predominantly inbreeding but with occasional outbreeding. Thus, the mating pattern of *C. dactyliperda* supports the reproductive assurance theory, i.e. delayed inbreeding is expected to occur depending on the availability of outbreeding opportunities. In contrast to these results, previous studies in plants have suggested that early inbreeding is expected when the probability of finding a male for outbreeding is low (Lloyd & Schoen, 1992; Elle & Hare, 2002). The delayed inbreeding behaviour observed in *C. dactyliperda* may have two explanations: (i) In *C. dactyliperda* there is evidence for a high cost to inbreeding, expressed as a reduction in the number and size of offspring (D. Gottlieb, unpublished data). This is different from plants, where early inbreeding was observed in most cases when inbreeding depression was low (Lloyd & Schoen, 1992; Elle & Hare, 2002). (ii) When the supply of eggs is limited, early inbreeding may reduce the number of eggs available later, when outbreeding opportunities arise.

Results of the behavioural experiments suggest that female *C. dactyliperda* are more inclined to outbreed, whilst the genetic data indicate that inbreeding occurs commonly in all populations tested. Combining the results of the behavioural experiments and the population genetics suggests that mixed mating is stable, rather

than an ephemeral condition leading to fixation of pure selfing or outcrossing. This is further supported by (Holzman *et al.*, 2009), who suggest that variation in the level of inbreeding in *C. dactyliperda* is due to behavioural alteration of the reproductive strategy, rather than a side effect of population size or other demographic changes that could alter the amount of intra-population variation.

Similarly, Ciszek (2000) demonstrated through behavioural experiments that the prevalence of inbreeding in the naked mole rat is due to low mate availability rather than a preference for inbreeding over outbreeding (Reeve *et al.*, 1990; Faulkes *et al.*, 1997). Both our study and that of Ciszek (2000) show that in addition to population-genetic information it is necessary to conduct behavioural tests to understand the selective forces acting to maintain the mixed-mating system.

Our results contradict earlier mathematical models (Lloyd, 1979) that suggest that plants and animals should evolve towards a stable state only at either one of the two ends of the breeding axis (Lande & Schemske, 1985; Lande *et al.*, 1994). In contrast, we suggest that *C. dactyliperda* maintains a mixed-mating system through a reproductive assurance strategy: (i) females are inclined to outbreed in spite of the fact that there is at least one brother to mate with in most offspring galleries (Herfs, 1950), (ii) males hardly disperse, indicating that there are few outbreeding opportunities (Herfs, 1950; Bar-Shalom & Mendel, 2003) and (iii) there is high variability in inbreeding rates between populations, and these include only intermediate rates, whereas extreme selfing or outbreeding was not observed.

In plants, mixed-mating systems occur most commonly in hermaphroditic plants, where reproduction is achieved by both self-fertilization (selfing) and mating with other individuals (outcrossing). Mixed-mating systems in the animal kingdom are an extremely heterogeneous category of genetic systems, covering all complex systems in which alternation between inbreeding and outbreeding exists. Mother-son and sibling mating in *C. dactyliperda* are far from being anomalous and are common among other haplodiploids (Borgia, 1980). We suggest that further studies on species showing high inbreeding level through mother-son behaviour and other mating systems that include mating among genetically related parents, would be rewarding as a means of testing the evolutionary stability of mixed-mating systems.

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