Flying the nest: male dispersal and multiple paternity enables extrafamilial matings for the invasive bark beetle *Dendroctonus micans*

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Abstract

There is an evolutionary trade-off between the resources that a species invests in dispersal versus in reproduction. For many insects, reproductive success in patchily-distributed species can be improved by sibling-mating. In many cases, such strategies correspond to sexual dimorphism, with males – whose reproductive activities can take place without dispersal – investing less energy in development of dispersive resources such as large body size and wings. This dimorphism is particularly likely when males have little or no chance of mating outside their place of birth, such as when sperm competition precludes successful fertilisation in females that have already mated. The economically important bark beetle pest species *Dendroctonus micans* (Coleoptera: Curculionidae, Scolytinae) has been considered to be exclusively sibling-mating, with 90% of females having already mated with their brothers by emergence. The species does not, however, show strong sexual dimorphism; males closely resemble females, and have been observed flying through forests. We hypothesised that this lack of sexual dimorphism indicates that male *D. micans* are able to mate with unrelated females, and to sire some or all of their offspring, permitting extrafamilial reproduction. Using novel microsatellite markers, we carried out cross-breeding laboratory experiments and conducted paternity analyses of resulting offspring. Our results demonstrate that a second mating with a less-related male can indeed lead to some offspring being sired by the latecomer, but that most are sired by the first, sibling male. We discuss these findings in the context of sperm competition versus possible outbreeding depression.

Introduction

Local mate competition theory (Hamilton, 1967) predicts that, for non-random matings, females can maximise their fitness by producing broods with a female-biased sex ratio and just enough sons to fertilise all daughters. This strategy can be particularly favoured when opportunities for unrelated matings for offspring are low, such as for species living in confined habitats with patchily distributed broods. Hamilton (1967) suggests that under these circumstances, sibling-mating (henceforth 'sib-mating') males could be disinclined, or unable, to emigrate from the family and are often morphologically different from the females as a consequence of reduced investment in dispersal-related characteristics. Indeed, numerous insects with sib-mating strategies exhibit sexual dimorphism, such as fig wasps (Agaonidae), in which some species have flightless males that never leave the natal fig (Cook *et al*, 1997). There is, however, a trade-off between the reproductive benefits of dimorphism involving flightless males that mate with siblings, and the corresponding reduction in dispersal potential and possible fitness consequences of inbreeding. Thus, many insects have evolved polymorphism, with both flighted and flightless or flight-limited morphs (Harrison, 1980; Langellotto *et al*, 2000; Zera and Denno, 1997). Within the Scolytinae beetles, there are several examples of endogamous species with sexual dimorphism. In all Xyleborini, for example, unfertilized eggs develop into haploid males, which are smaller than the females and have reduced, nonfunctional wings (Kirkendall, 1993; Peer and Taborsky, 2004). In sib-mating *Ozopemon* beetles (Dryocetini), the males show an exceptional neotenic development with no wings (Jordal *et al*, 2002).

In the Great European Spruce Bark Beetle, *Dendroctonus micans* Kug. (Coleoptera: Curculionidae, Scolytinae, Hylurgini), significant sexual dimorphism does not occur (although males are on average slightly smaller than females; Robinson *et al*, 1984 – as for other *Dendroctonus* species; Foelker and Hofstetter, 2014). Both sexes have functional wings, and hence are able to disperse, yet sib-mating has been considered the rule for *D. micans* (Francke-Grossmann, 1950; Grégoire, 1988; Hamilton, 1967; Vouland *et al*, 1984). Gravid females bore solitary egg galleries beneath the bark of spruce trees, and the gregarious larvae initially feed together but, at the onset of pupation, separate into individual niches. As young adults they gather together again in the brood chamber to mate; 90% of females have mated and stored sperm by the time they leave the maternal gallery (Vouland *et al*, 1984). Except under outbreak conditions, brood chambers are isolated and rarely intersect; this system ensures that mating is purely among siblings. However, reproductively viable *D. micans* males have been observed flying through the forest (J-C Grégoire, pers. obs.); in passive traps fixed on trees, males have been recorded in comparable proportions to the ratio of males to females in the brood chamber (roughly 1:9 in traps, versus between 1:5 and 1:48 in the brood chamber: Francke-Grossmann, 1950; Grégoire, 1988; King and Fielding, 1989). The observation of flying males raises the question of whether exogamous matings can and do occur for *D. micans*; if not, what could be the evolutionary benefit of retaining wings and expending energy in flight if females have already mated with their brothers? The males might fly to seek out young, isolated females in the process of creating their own egg gallery; indeed, both Chararas (1962) and Jannin (pers. comm.) report separate cases of finding a male with a female in a new gallery. Alternatively, dispersing males may enter brood chambers still containing one or several young, pre-emergent females. In both cases, the females found by these outgoing males are likely to have already

mated with their own brothers, and sperm competition may occur. For evolution to have favoured the retention of flight in males, these secondary matings are, however, likely to be at least partially successful.

Dendroctonus micans is an economically important pest, capable of destroying hundreds of thousands of hectares of spruce and pine forest plantations (Grégoire, 1988). In the internal parts of its distributional range (i.e. away from the expanding range edges in Central France, the United Kingdom, Georgia and northeastern Turkey: Grégoire, 1988), the density of *D. micans* in the phloem of spruce generally remains low, with few trees affected and those hosting only one or two brood chambers, insufficient to cause tree mortality. Biological control by a specific predator, the Monotomid beetle *Rhizophagus grandis* Gyll. is largely responsible for this situation (Grégoire, 1988). However, under outbreak conditions, which are particularly common at the expanding edge of the species' range, density can increase to such levels that heavily-infested and weakened trees are killed (Grégoire, 1988). Active control measures for the pest are being sought and implemented, including introduction of *Rhizophagus grandis* to outbreak regions (Grégoire *et al*, 1992; Grégoire *et al*, 1985), and attempts to isolate other potential biocontrol tools (e.g., Yaman and Radek, 2005). The sporadic nature of outbreaks makes control difficult, however, particularly in the face of key gaps in our knowledge of the reproductive and dispersal strategies employed by *D. micans*.

Here we used laboratory experiments to test the hypotheses that: i) multiple matings can occur for *D. micans*; ii) such matings lead to some or all progeny being sired by the less-related latecomer (e.g., via sperm competition). To address these hypotheses, we

developed and used novel microsatellite loci that allowed us to assess paternity in the offspring of females that had mated with both their brother/s and unrelated male/s. This research provides insights into the evolutionary trade-off between reproductive and dispersal capacity, and inbreeding mitigation strategies in a sib-mating insect.

Materials and methods

Collection and culture of live beetles for crossbreeding

Wild *D. micans* were collected as pre-emergent adults and larvae from Sitka spruce (*Picea sitchensis*) trees from sites in Brittany in France (forêt de La Hardouinais: 2°21'47.03"W, 48°13'13.56"N, on 5 July 2011) and from Norway spruce (*Picea abies*) in the Ardenne in Belgium (Nassogne: 4°54'36.82" E, 50°4'31.92" N, on 17 August 2011, and again on 15 May 2012). Beetles were kept alive in the laboratory with freshly-cut pieces of spruce phloem and fine sawdust.

Genotyping

Development of novel microsatellite primers

An initial attempt was made to isolate polymorphic microsatellite primers for population genetic analyses in 2004, using traditional bacterial cloning approaches. Due to the low yield of polymorphic loci from this work, a second attempt was made using 454 pyrosequencing approaches in 2011.

For the first attempt, microsatellites were isolated following a biotin-enrichment protocol modified from Kijas *et al.* (1994). The genomic DNA library was constructed from DNA extracted from 20 pooled *D. micans* individuals from each of five populations across France (Aisne, Orne, Tarn / Hérault, and Aveyron) and Belgium

(province of Luxembourg) (Supporting Information Table S1). Polymorphism was assessed for the nine most promising microsatellites using ten *D. micans* from each population.

For the second attempt, in 2011, 1 ug of pooled genomic DNA from 12 *D. micans* individuals from various populations in France, the Russian Far East, Sweden, Denmark, Romania, Germany, Poland and Austria (Supporting Information Table S1) was sent to the company GenoScreen in France for preparation of a microsatellite library via 454 next generation sequencing. Primers for the most promising 43 loci were tested on an initial seven samples of *D. micans* from various regions. Those that showed any indication of poymorphism were further tested on 95 samples from various regions throughout Europe (15 samples from Northern France, 23 from Southern France, 10 from Belgium, eight from the Russian Far East, 15 from Denmark and Sweden, 19 from the Baltic, and five from central Europe; these regions were selected based on COI sequences from phylogeographic analysis, with each region exhibiting distinct haplotype groups: F. Mayer, unpublished data). In some cases more than one primer pair was tested for a single locus.

Summary allelic diversity statistics were calculated using GenAlEx v. 6.5 (Peakall and Smouse, 2012).

DNA extraction for cross-breed experiments

Rather than genotype each egg and larva, a pooled approach was adopted, with calibration following Bretman *et al* (2009) (see below). Offspring were separated into groups according to stage and size: eggs, larval size classes 1 (1-2 mm long), 2 (2-3

mm), 3 (3-4 mm) and 4 (4-5 mm), and pupae. For larvae of size class 4 and pupae, only half of each individual was used in extractions to reduce the quantity of tissue. Tissue from the entire pooled sample from each size class for each brood was homogenised using a Retsch Mixer Mill MM301. For adult *D. micans,* tissue from the abdomen was removed for DNA extraction, with the head and thorax preserved as vouchers. Genomic DNA was isolated from all samples (pooled offspring, by stage / size class, and adults) using the High Pure PCR Template Preparation Kit (Roche) and eluted in 100 µl of elution buffer.

Allele strength calibration

To enable assessment of the relative strength of alleles from parents in pooled offspring samples, allele calibrations were established following Bretman *et al* (2009), with quantified DNA from French males mixed with that of Belgian females, and the proportion of the male DNA for each calibration comprising 6.25%, 12.5%, 25%, 50%, 75%, 87.5% and 93.75%, respectively. Two sets of calibration mixes were made from four adults (not those used in cross-breeding experiments, but from the same populations) for each of the two loci (Dm-AG51 and Dm-36), and the proportions of the area under the parental allele peaks compared to the actual proportions of the mixtures by linear regression in R (R Development Core Team, 2013). As found by Bretman *et al* (2009), the relative proportion of the alleles closely predicted the relative areas under the allele peaks for male and female alleles (R^2 between 0.74 and 0.97, P between 0.010 and 0.000), indicating no strong difference in peak size among alleles within loci, and permitting direct comparison of allele peak area in pooled samples to roughly estimate the relative proportion of sibling- versus foreign-male sired offspring in broods.

DNA amplification

PCRs were performed in a 10 µl volume using a MJ Research PTC 1000 or PTC 2000 Thermal Cycler. An initial denaturing step of 4 min at 95 °C was followed by 30 PCR cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s and finished with a 30 min extension step at 72 °C. Each reaction consisted of approximately 30 ng genomic DNA, 0.25 µM of combined primers (with a 2:1 ratio of the unlabelled reverse primer to the M13-labelled forward primer), 0.5 mM of each dNTP, 1X buffer, 1.5 mM $MgCl_2$, 0.5 uM dye conjugate (FAM-M13, following Schuelke, 2000, with modifications) and 1 U of HotStarTaq DNA polymerase (Qiagen). Primers for locus Dm-AG51 were: 5' M13-TATGTCCAGCAGTGGATGAA (forward) and 5' GGTTCGGAGTCTTTCCTTG (reverse), and for Dm-36 were: 5' M13-TGCGTAAAATCAACAAAAATCG (forward) and 5' ATGCCAAGGAGGAATTTCAA (reverse). For PCRs involving sperm, conditions were as above except that PCR volume was adjusted to 20 µl, and the maximum possible amount of extracted DNA was added (i.e., no water was used to dilute the reaction). Amplified products were electrophoretically separated with an automated sequencer (Applied Biosystems 3730), and peaks were visualised using Peak Scanner version 1.0 (Applied Biosystems) for allele calling.

Cross-breeding experiments

Cross-breeding experiments were carried out using males from France and females from Belgium. Ten females from the first collection of Belgian samples were chosen at random for dissection, and sperm from the spermatheca removed to assess the paternal genotype. All females had sperm in their spermathecae, indicating that mating had occurred within the brood chamber (these females were, however, observed to have immature ovaries). Groups of live Belgian females were introduced into polystyrene

boxes. Foreign (French) males were added to these boxes, and left for seven to ten days to allow possible mating (see Table 1). Experiments C1 – C3 were carried out using males and females collected from the wild in late 2011. Experiments C4 and C5 were conducted in May-June 2012, using adult females collected from the wild in May 2012, and virgin foreign males reared from larvae collected in France in 2011. Individual broods from each experiment were labelled with a unique code, e.g. broods within experiment C1 were coded C1.1, C1.2, C1.3, etc.. After having been given the opportunity to mate with foreigners, all adults were individually introduced into 40 cm high freshly cut spruce logs, by positioning each beetle over pre-established entry holes and preventing rapid escape by attachment of a small polythene cap over the entry site (Grégoire and Merlin, 1984). More than one beetle was introduced to each log, but entry holes were positioned such that sub-cortical brood chambers would be unlikely to intersect, and only beetles from the same crossing (Table 1) were inoculated into the same log. Logs were isolated from each other by placement in large, sealed plastic tubs with small mesh windows for aeration.

For experiments C1-C3, phloem from logs was removed 27 days after inoculation with the cross-mated females. No females were found to have laid eggs or created brood chambers. Dissection of one female from crossing C1 showed that the ovaries had still not matured, suggesting females were still in a state of developmental diapause (Grégoire, 1984). All surviving females were introduced into new spruce logs in sealed containers (as above), and these containers were then placed in climate-controlled chambers (one half of the logs were in a Sanyo versatile environmental test chamber MLR-351H, made by the Sanyo Electronic Co., Ltd., Japan; the other half were in a Binder climate chamber KBWF 720, Binder, D-78532 made in Tuttlingen, Germany)

with daily climate cycles as shown in Supporting Information Fig. S1 to simulate accelerated winter and spring in an attempt to break the diapause. After 52 days, the logs were removed from the climate chamber and stripped of phloem. Surviving beetles had produced larval broods. Mothers and broods were collected, placed directly in 96% ethanol, and stored in a freezer at -20°C until DNA extraction. For experiments C4 and C5, no diapause-lifting treatment was necessary, and eggs were removed from brood chambers 34 days after females were introduced to logs.

Results

Isolation and characterization of microsatellite sequences

Isolating polymorphic microsatellite loci in highly inbred organisms can be extremely challenging (Berg *et al*, 2003; Buehler *et al*, 2011; Keller *et al*, 2011), and *D. micans*, a sibling-mating beetle, would be expected to exhibit little genetic variation across large geographic regions. Given the low expected genetic variation, and the focus of this current study on paternity rather than population genetic analyses, we chose to maximize the number of populations sampled at the cost of within-population sample size. Consequently, population sizes were too small for meaningful comparisons of observed and expected heterozygosities, but were adequate for discerning which microsatellite loci showed any polymorphism, and which could potentially distinguish among populations in distinct geographic regions.

For the cloning approach to isolate microsatellites, polymorphism was found for five of nine amplified markers, but with no more than two alleles at any locus (Supporting Information Table S2). No heterozygote was found except in the Belgian population (Table 2). Near-neglible heterogeneity was observed within populations; however, it

was possible to distinguish among some of the populations: 1) Dm-AG31 characterised one of the French populations (Orne) with the unique allele 211; 2) Dm-AC60 characterised a region of southern France (Aveyron, Tarn and Hérault) with allele 145; and 3) Dm-AG51 characterised the Belgian population with allele 102 (Table 2).

For the 454 approach to isolate microsatellites, initial tests on seven samples indicated polymorphism at 11 loci. Further tests of these 11 loci on 95 samples from seven regions indicated that for most, genetic diversity was low, with an average of 4.3 alleles per locus (see Supporting Information Table S2), but some showed moderate levels of diversity (four loci had five or more alleles), and some could apparently distinguish among geographic regions (Table 3). For the locus Dm-36, only allele 136 was found in samples collected from northern France, and only allele 138 was detected in samples collected from Belgium, allowing us to use this locus to assess paternity in cross-breeding experiments between beetles from the Ardenne (Belgium) and Brittany (north-west France), along with locus Dm-AG51. As in the previous attempt to isolate loci, population sizes of samples tested in this trial were too small for robust comparison of observed and expected heterozygosities within populations; however, summary allelic diversity statistics for each locus across all populations are given in Supporting Information Table S3. For most loci, the fixation index indicated a high level of inbreeding.

Cross-breeding experiments

Analysis of the sperm from ten random females from the Belgian population confirmed that each female had already mated with a male which shared her genotype, being homozygous for the only alleles detected for these loci from any *D. micans* in Belgium;

i.e. the sole alleles in the sperm were allele 102 for locus Dm-AG51, and allele 138 for Dm-36.

Living mothers were retrieved from 42 of the 52 brood chambers in the cross-breeding experiments, but ten females were missing, most probably due to death and corporal decay; mothers were not retrieved from broods C1.3, C3.1, C3.2, C4.1, C4.8, C4.9, C4.24, C4.25, C5.11 and C5.3. All retrieved mothers were confirmed to be homozygous for the only alleles that have been detected in the Belgian populations. Experimental broods from C1, C2 and C3 comprised between 54 and 189 eggs, larvae and pupae, and broods from C4 and C5 had between 7 and 191 eggs (Table 4). Some brood samples failed to amplify, but those that did demonstrate that the second mating, by the non-related, foreign male, can potentially influence the genetic structure of the brood, with sperm from both the first and second males being used to fertilise eggs (Fig. 1). In all but two broods from experiments C1-C3, offspring yielded both Belgian (allele 102 for locus Dm-AG51, and allele 138 for Dm-36) and French (allele 108 for locus Dm-AG51, and allele 136 for Dm-36) alleles. French alleles were found at all larval size classes in broods with any French influence, suggesting that sperm from the foreign male was used to fertilise eggs reasonably consistently thoughout oviposition. For locus Dm-AG51, the relative proportion of the allele from the first sire to the second sire ranged from a mean within broods of 1.5 to 20. This result indicates that the first male always contributed more than the second male to the brood, in some cases up to twenty times as much (Fig. 1). For locus Dm-36, the relative contributions of the first to second males ranged from 1 – 5.5. The differences in proportions among loci may be due to error associated with the calibrations; the regressions, while indicating a good fit of allele peak strengths to parental proportions, were slightly more variable for Dm-AG51 (R^2

from 0.74 – 0.92, P from 0.01 – 0.001) than for for Dm-AG36 (R^2 from 0.93 – 0.97, P < 0.001). Nonetheless, the relative proportions within loci were similar; for example, the samples that showed the least and greatest difference in paternal contribution were the same, respectively, for both loci. For experiments C4 and C5, only Belgian alleles were found in the eggs; no contribution from the French sires was detected, although these males were retrieved and confirmed to have alleles distinct from the Belgian females (allele 108 for locus Dm-AG51, and 136 for Dm-36).

Discussion

Multiple paternity in *D. micans*

Our results provide strong support for both of our hypotheses, demonstrating that i) multiple matings can occur for *D. micans*, and ii) a second mating can lead to some offspring being sired by the latecomer. There is thus an evolutionary driver for the maintenance of flight capacity in male *D. micans*, as dispersal can allow males that have already mated with their sisters in the natal chamber to seek out less related females and sire more offspring.

Despite evidence that multiple matings can occur in *D. micans*, allowing beetles to mate with less related individuals, the species nonetheless engages in widespread sib-mating. This strategy may safeguard and maximise the reproductive success of the pest. Under endemic conditions, the population density of *D. micans* is low (Grégoire, 1988), as is the consequent likelihood of individuals encountering each other in the forest. Females are nonetheless ensured some reproductive success, as they leave the natal chamber with a spermatheca containing sperm from their brothers. Should they then fortuitously find and mate with one or more non-related males, their eggs can be fertilised using

sperm from multiple sires. Other sib-mating insects have also developed strategies that permit a degree of extrafamilial mating; for example, in fig wasps more than one female may lay eggs in a single fig (allowing mixing of broods) and some species have winged males that can disperse (West and Herre, 1998). Where extrafamilial mating is possible, there is predicted to be a weakening of the strong female-biased sex ratio that can be driven by Local Mate Competition in situations where males can only mate with siblings (West and Herre, 1998). Likewise, where males are winged, smaller broods are expected than where males are wingless, as reproduction is not solely limited to the local patch (Cook *et al*, 1997; Hamilton, 1979). In *D. micans* there is, however, a contrast between the ability of males to disperse and the strong female-biased sex ratio (up to 1:48 male:female: Grégoire, 1988) and large brood size (up to 191 eggs were collected from a single brood during this study), suggesting that extrafamilial mating, though possible, may be relatively rare for this species.

Sperm competition is well documented in insects (Parker, 1970; Simmons and Siva Jothy, 1998). In sperm displacement, for example, males use their reproductive organ to displace stored sperm; e.g., the spermatophore of the male rove beetles *Aleochara curtula* expands into the spermatheca, forcing out previously stored sperm before delivering its own (Gack and Peschke, 1994). Other mechanisms of sperm competition include repeated copulations, physiological incapacitation of rival sperm (Simmons and Siva Jothy, 1998), and pre-copulatory mechanisms such as mate guarding, as in some strictly monogynous scolytine beetle species (Kirkendall, 1983). Parker (1970) predicted that evolution should drive sperm displacement strategies when multiple matings occur, but that when such matings are common, selection should favour strategies that enable the first-mating male to reduce competition from latecomers. Our

results indicate that for *D. micans*, the second, less related mating is less successful, in terms of number of offspring sired, than the first mating of the female with her brother. More offspring in the broods that showed any evidence of a successful second mating were found to be sired by the first male (Fig. 1). Although for most of our experimental crossings the second mating appears to have had no influence at all, with the first male siring all offspring, in all but two cases these were from experiments C4 and C5, in which the 'foreign' males were reared from larvae, and it is possible that these males were unable to mate for some reason; perhaps, like the females, sexual maturity in males can be stalled by seasonal diapause. Nonetheless, our results from experiments C1-C3 indicate that a second mating, while possible, is likely to be less successful than the first. This result may indicate sperm competition, with the first male to mate somehow limiting the success of latecomers (perhaps through their sperm dominating the available volume of the spermatheca; or through a mating plug: see Boomsma *et al*, 2005). Whether or not sperm competition from the first-mating male occurs could be tested by introducing virgin females to a foreign males first, and then to a sibling, to assess whether it is the relationship of the male to the female or the order of mating that is most important.

As an alternative to sperm competition, our results may represent reduced fitness of offspring sired by less related males. A study by Peer and Taborsky (2005) of inbreeding depression in sib-mating ambrosia beetles found lower hatching rates for eggs fertilised by less-related males than by a female's brother. The authors concluded that the extreme levels of inbreeding in this species had purged most deleterious alleles, such that inbreeding was less damaging to an individual's fitness than outbreeding. There was no evidence from our results that the proportion of eggs fertilised by the

foreign male was greater than the proportion of larvae he sired, but our study did not set out to directly test offspring fitness via hatching success or other measures, and future research is needed to ascertain whether sperm competition or outbreeding depression is at the root of the lower success of second, less-related matings in *D. micans*. Grégoire (1988) suggested that low genetic diversity as a result of inbreeding may not be particularly problematic for *D. micans*; as the species often does not kill its host, the host may not exert strong selection pressure on the parasite. Thus, there may be little evolutionary pressure for *D. micans* to mate beyond the immediate family, although the investment of males in dispersal would, in this case, be puzzling. *D. micans* may be an evolutionarily young species, and the male phenotype may thus reflect an ancestral tendency for males to disperse to find mates, the change to which has not yet resulted in a loss of wings. Large-scale population genetic analyses could help to shed light on the full extent of outbreeding in this species.

Our research demonstrates that a second mating can occur and be successful for *D. micans*, but does not reveal whether dispersing males seek out solitary, gravid females in the forest, or broods of pre-emergent young adults. Future research should utilise field and behavioural experiments to test which category of females (pre-emergent or solitary individuals) are most attractive for these less related males.

Novel microsatellite markers for *D. micans*

One objective of this research was to characterise microsatellite loci that would allow us to determine brood paternity among *D. micans* colonies. The difficulty of finding large numbers of polymorphic microsatellite loci in the Scolytinae is well known (Davis *et al*, 2009; Gauthier and Rasplus, 2004; Kerdelhué *et al*, 2003; Sallé *et al*, 2003), and our

initial trials, using traditional cloning techniques, demonstrate that *D. micans* is no exception, with only nine microsatellite loci amplifying, only five of which showed any (and limited) polymorphism. Our second attempt, using outsourced 454 pyrosequencing approaches, was more successful, yielding 11 polymorphic microsatellite loci, several of which showed regional differences that could indicate population structure within Europe. In particular, samples from the Russian Far East had unique alleles for many loci (Table 3). Our goal in this study was not, however, to provide a comprehensive study of genetic structure among and within populations (indeed, our within-population sample sizes were inadequate for such assessments), but to identify useful loci for paternity analyses in our cross-breeding experiments, in which we were successful. Future studies, using a larger number of samples from each population, could further test these microsatellite loci, and use the most informative to characterise population genetic structure and heterozygosity, as well to identify large-scale dispersal pathways in *D. micans* and the source/s of new invasions.

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Tables

Table 1: Origins and numbers of female (f) and male (m) *D. micans* used in cross-breeding experiments. Some females died during the experiment, and some failed to produce eggs. The number of surviving females that oviposited is indicated in parentheses in the final column. Females were collected from Nassogne (Belgium) in August 2011 (experiments C1-C3) and May 2012 (experiments C4-C5). Males were collected from France in July 2011 as pre-emergent adults (experiments C1-C3) and larvae (experiments C4-C5).

Exp.	**Male origin**	**# m**	**Female origin**	**# f (surviving)**
C1	France (wild-caught adults)	2	Belgium (wild adults)	6 (3)
C2	France (wild-caught adults)	1	Belgium (wild adults)	3 (3)
C3	France (wild-caught adults)	1	Belgium (wild adults)	3 (2)
C4	France (reared from larvae)	9	Belgium (wild adults)	30 (26)
C5	France (reared from larvae)	9	Belgium (wild adults)	30 (18)

Table 2: The microsatellite loci isolated via cloning and alleles from the populations sampled in 2003-2004. Populations: *Lux*: Province of Luxembourg (Belgium); *Ais*: Département of Aisne (France); *Orn*: Département of Orne (France); *Tar*: Départements of Tarn and Hérault (France); *Ave*: Département of Aveyron (France): see Table S1 for collection details. Alleles which characterise a population (found throughout but only within that population) are shown in bold; note that Tarn and Aveyron are both in the same region (south-western France) and are indistinguishable using these genetic markers, suggesting they comprise a single population.

	Population				
Locus	***Lux***	***Ais***	***Orn***	***Tar***	***Ave***
Dm-AC13	87	87	87	87	87
Dm-AG14	116/**170**	116	116	116	116
Dm-AG31	218	218	**211**	218	218
Dm-AG41	**132**/134	134	134	134	134
Dm-AG45	153	153	153	153	153
Dm-AC60	141	141	141	**145**	**145**
Dm-AG51	**102**	108	108	108	108
Dm-AG55	165	165	165	165	165
Dm-AG69	120	120	120	120	120

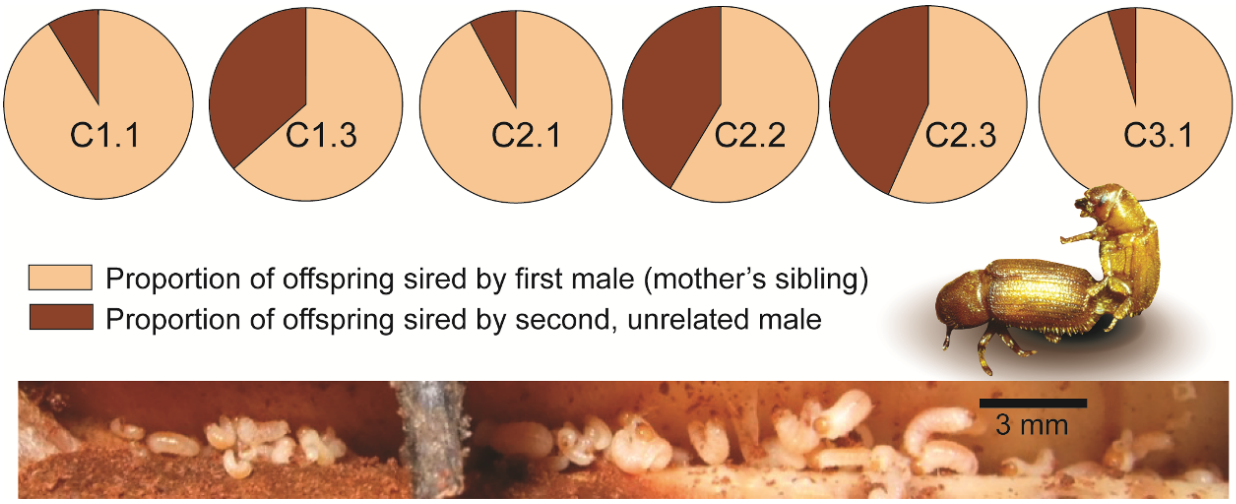
Table 3: The microsatellite loci isolated via 454 sequencing and alleles from the populations throughout Europe. Populations: *NFr*: Northern France (n=15); *SFr*: Southern France (n=23); *Be*: Belgium (n=10); *Ru*: the Russian Far East (n=8); *Sca*: Scandinavia (Denmark and Sweden) (n=15); *Ba*: eastern Baltic (n=19); *CEu*: Central Europe (Austria and neighbouring regions) (n=5). See Table S1 for collection details. Alleles which might characterise a population are shown in bold. Rare alleles (those that were only detected in one or two individuals in a given population) are marked with asterisks.

	Population						
Locus	***NFr***	***SFr***	***Be***	***Ru***	***Sca***	***Ba***	***CEu***
Dm-8	166/169*	166	166	163*/166	163	166	166
Dm-10	208*/233	233	233	233/236*	221*/233	224*/233	221*/233
Dm-16	118	118	118	**115**	118	118	118
Dm-19	154	154	154	154/158	154	154	154
Dm-20[#]	155/157	155/157	155/157	**147**/149*	155/157	155/157/159	157
Dm-30	179/259	179/259	181/183*/259	212*/214/216	179	187/195/197*/259*	179/191*/259
Dm-36	136	136/138*	138	136/138	136	136	136/138*
Dm-43	207/209	209	207	203*/**205**/207	207/209	207	207/209*
Dm-45[#]	310	310	310	316*/325*/343*/347*/349	310*/312	310	310
Dm-46	144*/150	144/150	144/150	142*/146*/148/152	144/150	144/150	144*/150
Dm-47[#]	157	157/159*	159	153/157	157	157/159*	157*/159

Table 4: Number of eggs, larvae (by size class) and pupae for each brood in experiments C1-C3. All broods in experiments C4 and C5 were terminated at egg stage, with between 7 and 191 eggs collected per brood, and means of 88 eggs for C4, and 77 for C5. DNA extractions were carried out on pooled samples from each size class in each brood.

Brood	**C1.1**	**C1.2**	**C1.3**	**C2.1**	**C2.2**	**C2.3**	**C3.1**	**C3.2**
Eggs	0	0	0	51	44	48	0	0
Larvae: size class 1	0	16	23	36	84	69	0	0
Larvae: size class 2	29	8	14	0	0	0	0	0
Larvae: size class 3	47	15	26	0	0	0	0	0
Larvae: size class 4	13	15	0	0	0	0	147	6
Pupae	0	0	0	0	0	0	42	75
Totals	*89*	*54*	*63*	*87*	*113*	*117*	*189*	*81*

Figure 1: Proportion of offspring sired by related (first mating) and unrelated (second mating) males. Results are shown for offspring of broods where any evidence of paternity by the unrelated male was found (broods 1 and 3 in experiment C1, broods 1-3 in experiment C2, and brood 1 from experiment C3) for locus Dm-AG51. Inset: photograph of young adult *D. micans* mating (J-C Grégoire), and larvae from an experimental brood as the phloem was peeled from a spruce log – note that the larvae vary considerably in size.



Supplementary Information:

Table S1. Microsatellite loci developed for *D. micans*. The observed size range and the number of alleles (N_a) are based on 42 individuals from five populations (three regions) in the case of microsatellites isolated via cloning, and on 95 individuals from seven regions in the case of microsatellites isolated via 454 sequencing. The annealing temperature (T_{ann}) is given for each primer pair. PCR product sizes for alleles read using PeakScanner include the M13 sequence on the forward primer; thus if an M13-labelled primer is not used, allele sizes should be adjusted by -18 bp. *for some loci isolated via cloning, allele size was calculated by estimation on a gel; these are marked with an asterisk. For all others, allele size was calculated using PeakScanner software.

Isolation	**Locus**	**Primers (5'→3')**	**T.ann (°C)**	**Core repeat**	**PCR product size range**	**N_a**
Cloning	Dm-AC13	F: CACTGGCGGTATATCC R: CAGAGGGAATTGGTTTCAA	55	(TG)10	87*	1
Cloning	Dm-AG14	F: CACTGGCGGTATATCC R: CAGAGGGAATTGGTTTCAA	60	(AG)8	116-170*	2
Cloning	Dm-AG31	F: CAATTAACTTACGCAACCTGT R: CTCCAAATTCCTTTCGTT	60	(TC)4(CTCTT)5	211-218	2
Cloning	Dm-AG41	F: ATACAGGGATAATCATTAGAGGCATG R: CATAAAGAGAGCAGCATGACTGT	60	(TC)8(TA)5	132-134*	2
Cloning	Dm-AG45	F: GATGGGAGATTTGACAAAAACG R: ACTGAATGACCCTTACATACCAAA	60	(GA)7	153*	1
Cloning	Dm-AC60	F: GATCGGTCGATCAAACTTT R: CATTCAGTGGTGTTCTAGTTT	58	(GT)12	141-145	2
Cloning	Dm-AG51	F: TATGTCCAGCAGTGGATGAA R: GGTTCGGAGTCTTTCCTTG	58	(GA)16	102-108	2
Cloning	Dm-AG55	F: CGCAATCTTCCGTGTCGTAT R: GAATAGATCGGAATGGAGAA	60	(TC)14	165*	1
Cloning	Dm-AG69	F: CAGTAATGAGAGAACGACGAT R: ATACAGGAAAGGAAGGTCG	60	(AG)8	120*	1

Isolation	Locus	Primers (5'→3')	T.ann (°C)	Core repeat	PCR product size range	N_a
454	Dm-8	F: GCTCCTGTTGCAATTAGGATATACA R: TGTCGATAGCCTTTTCGATG	58	(ACA)10	163-166	2
454	Dm-10	F: AGGGTTTTGATCTGGGGATT R: CAGGAGTGGATGAATACGGG	58	(TTC)8	208-236	5
454	Dm-16	F: ATCCAGGAATGGATGGACAA R: AATTGCCCTCGAAATCTGTG	58	(AAG)8	115-118	2
454	Dm-19	F: AGATGAGATTTCAGTTGCTGTTAAT R: TCCCATAACCGCTTTGGTAG	58	(AC)14	154-158	2
454	Dm-20	F: TAATTTTCCGCACTGTCTCTGA R: ATCCTATGTGGAATACCGGG	58	(TG)14	147-157	4
454	Dm-30	F: CGAAACCATCACGGGAAATA R: TGTCCAGGAGTGGATGAACA	58	(TC)12	179-216	10
454	Dm-36	F: TGCGTAAAATCAACAAAAATCG R: ATGCCAAGGAGGAATTTCAA	58	(TG)10	136-138	2
454	Dm-43	F: ATTTAGTCAATTCTTGGCTTGC R: ATTCTTTGTTCATACAGCTGGC	58	(TG)9	203-209	4
454	Dm-45	F: AAGCGGCCACTTTCACCA R: AGTTTGAGGCAACCAAAACG	58	(CT)9	310-349	7
454	Dm-46	F: TGAGTGGTCTATTTGGTGCAA R: CGCAATCAAGACCCAAAGAT	58	(AC)9	142-152	6
454	Dm-47	F: AGGCCAGTATTAGCAAAACTTAACA R: ATCTGGCGATGGTCGAGAG	58	(GT)8	153-159	3

Table S2: Mean and standard error (SE) of allelic diversity statistics across all populations for each locus, for the microsatellite loci identified using 454 pyrosequencing. N_a: number of alleles. H_o: observed heterozygosity; H_e: expected heterozygosity; F: fixation index.

Locus		**Dm-8**	**Dm-10**	**Dm-16**	**Dm-19**	**Dm-20**	**Dm-30**	**Dm-36**	**Dm-43**	**Dm-45**	**Dm-47**	**Dm-46**
N_a	Mean	1.286	1.714	1.000	1.143	2.000	1.857	1.429	1.857	1.714	1.571	2.286
	SE	*0.184*	*0.184*	*0.000*	*0.143*	*0.218*	*0.340*	*0.202*	*0.261*	*0.565*	*0.202*	*0.286*
H_o	Mean	0.020	0.085	0.000	0.061	0.613	0.109	0.054	0.131	0.074	0.000	0.508
	SE	*0.020*	*0.036*	*0.000*	*0.061*	*0.158*	*0.071*	*0.054*	*0.052*	*0.050*	*0.000*	*0.083*
H_e	Mean	0.038	0.149	0.000	0.066	0.385	0.221	0.127	0.165	0.121	0.169	0.374
	SE	*0.024*	*0.066*	*0.000*	*0.066*	*0.083*	*0.090*	*0.072*	*0.051*	*0.096*	*0.081*	*0.049*
F	Mean	0.462	0.134	N/A	0.067	-0.558	0.486	0.709	0.197	0.205	1.000	-0.322
	SE	*0.288*	*0.184*	*0.000*	*0.071*	*0.203*	*0.231*	*0.190*	*0.172*	*0.163*	*0.000*	*0.087*

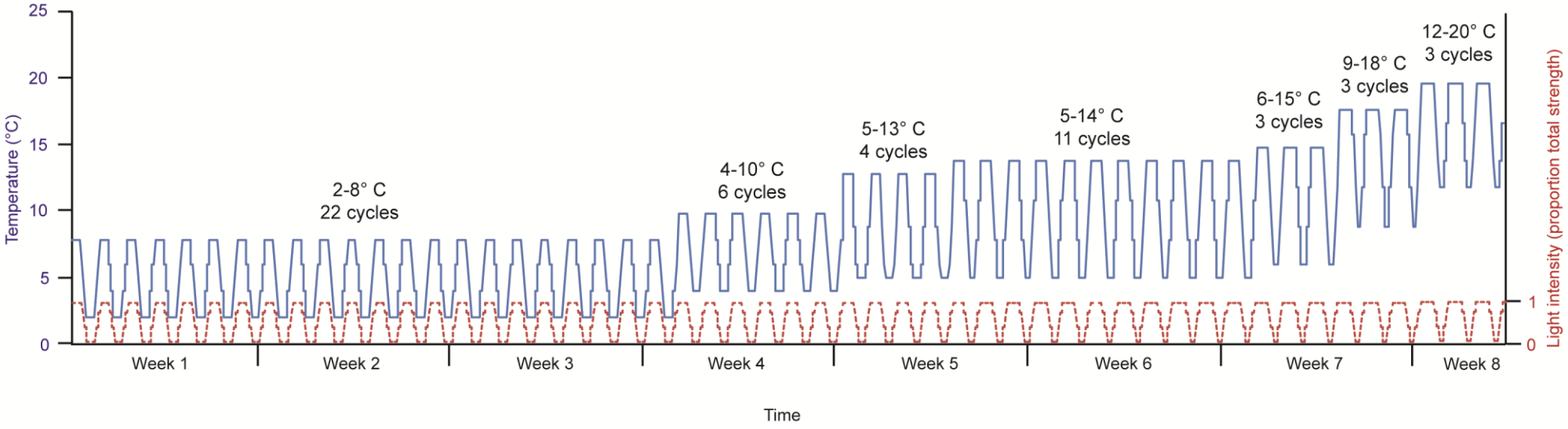


Figure S1: Chart showing temperature (solid line) and light (dashed line) regimes for breaking the diapause of *D. micans* female collected from Nassogne in Belgium in August 2011. The females had immature ovaries and, although they had mated, did not lay eggs until a winter – spring cycle had been simulated. Note that this diapause-lifting treatment has not yet been appropriately tested on a range of treatments or with controls, so should not be considered optimal or reliable, although in this case diapause in the cross-breeding experimental animals appears to have lifted successfully.