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Paternity analysis in the golden egg bug using AFLPs: do the males preferentially accept their true genetic offspring?

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Abstract. 1. The evolution of parental care and intraspecific parasitism involve conflicts of interest between mothers and other potential care givers who contribute to enhance offspring survival. In the golden egg bug, *Phyllomorpha laciniata* Villers (Heteroptera: Coreidae), females lay eggs on conspecifics and on plants. The adaptive significance of egg carrying in this species has been the subject of some controversy, which can only be resolved by determining the genetic relationship between the eggs and the adult who carries them. This study examined whether male acceptance of true genetic offspring occurs with a higher frequency than that expected from random oviposition on conspecifics.

2. Paternity analysis, using Amplified Fragment Length Polymorphism (AFLP) markers, was conducted on eggs carried by males housed with field-mated females.

3. Out of the total number of eggs sired by males in the experimental groups, the proportion of eggs carried by males that were their true genetic offspring was 30.8%.

4. Monte Carlo methods show that the probability of a male accepting an egg that is his true genetic offspring is higher than expected if females dumped eggs on males at random.

5. These results suggest that paternal care plays an important role in the maintenance of male egg carrying in this species. In addition, the methodology developed may become useful in determining true genetic parents in other species in which neither the father nor the mother can be determined by observational methods.

Key words. DNA fingerprinting, exclusion probability, genetic relatedness, golden egg bug, intraspecific brood parasitism, molecular markers, parental care, paternity, *Phyllomorpha laciniata*.

Introduction

The study of evolutionary ecology has been revolutionised in recent years by the introduction of molecular techniques. Our understanding of parental care, sexual conflict, mating strategies, and mating system evolution has been drastically altered by the determination of parentage and genetic relatedness (Gowaty & Karlin, 1984; Quinn *et al*., 1987; Burke, 1989; Queller *et al*., 1993; Avise, 1994; Hughes, 1998). The use of molecular techniques to determine paternity revealed that in populations with monogamous mating systems, so-called extra-pair copulations are frequent, and that in polygynous mating systems several males tend to fertilise the ova of each female. As a result of female polyandry, males often care for unrelated young (Birkhead & Møller, 1992; Møller & Birkhead, 1993; Westneat & Sargent, 1996). This unexpected finding has stimulated new theoretical models that attempt to

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explain under which conditions males should care for offspring given the risks of cuckoldry (Whittingham *et al*., 1992; Xia, 1992; Westneat & Sherman, 1993; Houston & McNamara, 2002; Webb *et al*., 2002), as well as experimental work aimed at understanding how males assess paternity certainty and how they react to a decrease in levels of paternity (see for example Sheldon & Ellegren, 1998; Wright, 1998; Neff & Gross, 2001). Among systems where parental care has already evolved, females not only manipulate males into providing care for offspring that are not their own, but they may also 'cheat' other females, by making them look after their offspring, a strategy known as 'intraspecific brood parasitism'. This behaviour entails laying eggs on another female's nest, thus manipulating the parasitised female into providing care for the parasitic female's offspring (Petrie & Møller, 1991; Field, 1992; Brockman, 1993; Arnold & Owens, 2002). For instance, females of the treehopper *Publilia concava* Say (Hemiptera: Membracidae) may exhibit a brood parasitism tactic in which they lay eggs in previously existing egg masses looked after by other females (Zink, 2003). By adopting this parasitic tactic females are able to lay a greater number of clutches along their lifetime, and females that exhibit a mixed strategy (maternal care and intraspecific brood parasitism) show the highest estimates of lifetime fecundity.

These findings reveal that the costs associated with the provision of care to offspring is a fertile ground for the appearance of conflicts of interest between mothers and males who may be willing to provide care despite some uncertainty about paternity (see Westneat & Sargent, 1996), as well as other females who may be manipulated into looking after other females' offspring. Understanding these conflicts between the true genetic mother and other potential caregivers is essential to explain the evolution of parental care and mating systems, but many questions are still unresolved.

The golden egg bug (*Phyllomorpha laciniata*) is a good model to study these issues because it shows a unique pattern of oviposition behaviour, which results in some eggs being carried by adults, and some being laid on plants where they develop unattended. Eggs derive a great benefit when carried by an adult because their survival rates improve considerably, reducing the rates of mortality caused by a parasitoid wasp (Reguera & Gomendio, 2002). Only a small proportion of the female population carries eggs, and females carry a small number of eggs. The scenario is different for males, as all males in natural populations end up carrying eggs, and males carry a greater number of eggs than females. Because females never carry their own offspring, the eggs they carry are likely to be the result of intraspecific parasitism by other females. The evolutionary significance of male carrying is the subject of an ongoing debate. Some authors believe that egg carrying by males is also likely to be the result of intraspecific parasitism (Kaitala *et al*., 2001), while other authors have argued that, while intraspecific parasitism may account for a small proportion of egg-carrying cases equivalent to the small proportion of females who carry eggs, it is unlikely to explain the much higher level of egg carrying observed among males (Gomendio & Reguera, 2001). These authors have suggested that, given the high costs of egg carrying for adults in terms of increased predation rates (Reguera & Gomendio, 1999; Kaitala *et al*., 2000), males are unlikely to accept eggs unless there are chances that at least some of the eggs will be their true genetic offspring. In addition, egg carrying maximises offspring survival rates, and therefore could be considered as a rudimentary form of parental investment, as the term refers to the effects on offspring survival and not to the degree of elaboration involved in the type of care provided (see Clutton-Brock, 1991). To elucidate the role of parental care and of intraspecific parasitism in this system it is crucial to determine with molecular methods the paternity of the eggs carried by males. Previous work has shown that between 13 and 22% of the eggs carried by males were sired by the carrying male (Tay *et al*., 2003). However, the last male to mate with a female fathers an average of 43% of the eggs laid during the following 5 days, and females lay eggs on males quickly after the end of the copulation (García-González *et al*., 2003). Furthermore, a low confidence of paternity does not mean that egg carrying has been driven by intraspecific parasitism (García-González *et al*., 2003), especially in this system where: (1) the survival of the offspring depends almost exclusively on egg carrying, (2) males cannot discriminate their own offspring, and (3) confidence of paternity is not expected to improve in future male breeding attempts because of the mechanism of sperm mixing operating in the species (Westneat & Sherman, 1993; García-González *et al*., 2003; García-González & Gomendio, 2004).

The aim of this study is to examine if male acceptance of true genetic offspring occurs with a higher frequency than that expected from random oviposition on conspecifics, as predicted by the hypothesis of male parental care. An experimental design in which neither the mother nor the father was known *a priori* was conducted and a new methodology was developed to assign paternity estimating the putative mother. For paternity determination Amplified Fragment Length Polymorphisms (AFLPs) was used, a technique based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos *et al*., 1995). Recent studies have shown that AFLPs can be useful for an estimation of paternity in the golden egg bug and other insects (García-González *et al*., 2003; Simmons *et al*., 2004).

Materials and methods

Study animals

Forty-five individuals of *P. laciniata* (29 males and 16 females) were collected in El Espinar (Segovia, Central Spain) at the end of May, when they are at the peak of their reproductive activity (monitoring of the population from the beginning of the reproductive cycle allowed us to determine this particular stage). Data collected throughout

a 5-year study and a series of preliminary studies examining sperm contained in the females' spermatheca showed that at this stage of the reproductive cycle all females in the population have mated at least once (García-González, 2002). Individuals were transported in individual plastic vials to the laboratory and placed in small Petri dishes (5.5 cm diameter). Prior to the experiment, eggs were removed from carrying males as well as from carrying females. Individuals were kept in constant conditions during the experimental period (25 °C, lights on from 08.00 hours to 21.00 hours). One day after their capture, four groups of different sex ratios and densities were established with individuals taken at random (Table 1).

Individuals in the high-density groups were kept in small plastic containers (16.5 cm × 16.5 cm × 10.5 cm) whereas individuals in the low-density groups were kept in large glass containers (1 m × 0.5 m × 0.5 m). Throughout the experimental period, individuals were provided daily with fresh branches of the host plant *Paronychia argentea* Lam. (Caryophyllaceae). Inside the containers enough plant was provided to ensure that it always represented a greater surface area than that represented by individuals, to make sure that plant availability (to feed or to lay eggs) was not a limiting factor.

Individuals were allowed to mate and to oviposit freely and after 8 days they were isolated and eggs were removed carefully from their backs. The thorax of the adults were extracted, discarding the digestive tract, and frozen to −80 °C. Each egg was placed in an Eppendorf tube and was checked daily until hatching. Emerged nymphs were also frozen to −80 °C.

Eggs on plants were collected on the fourth and eighth day of the experiment. Under the experimental conditions, nymphs hatched around 11 days after oviposition. Therefore no egg, either laid on plant or laid on a conspecific, could have hatched without being noticed. Only eggs laid on males were analysed.

To keep the conditions of sex ratio and densities in each group constant, if an individual died before the sixth day of the experiment it was replaced by another individual of the same sex. Dead individuals were frozen after removing the eggs that they were carrying, and the procedure described above was followed to obtain the nymphs. In group C, one male died on the fourth day and one female died on the first day of the experiment and they had to be replaced (male C1 replaced by male C4, female C1 replaced by female C3). In group D, two males died on the third and fourth days (D1 and D2 replaced respectively, by D7 and D8). Only one individual died after the sixth day (D3).

Non-virgin females were employed in the experiment. So far, all attempts to rear this insect in captivity have been unsuccessful. Although virgin females were obtained from the field at the very beginning of the reproductive season (March to April), these females do not lay eggs when placed in captivity although they do engage in copulations (García-González, 2002). Thus, it is not possible to carry out studies using virgin females, which adds further complexity to the study of paternity in this insect.

AFLP analysis, fragment scoring, and correlation in migration

AFLPs were resolved for 36 adults and for nymphs that had emerged from eggs laid on males ($n = 44$) using the AFLP Plant Mapping Protocol (Perkin-Elmer Applied Biosystems, 1996, Perkin-Elmer Corporation, Foster City, California). Thirty different AFLP selective primer combinations were tested and two primer pairs were identified as the most polymorphic, and therefore informative, and were the ones that offered the most clean and reproducible patterns: *Mse*I-CAT and *Eco*RI-AAG (JOE-Green), and *Mse*I-CAG and *Eco*RI-AAC (NED-Yellow). Further details have been given elsewhere (García-González *et al.*, 2003).

Each fragment was considered as a dominant locus with two states: presence or absence. AFLP profiles were scored for the presence/absence of fragments in the 60–300 bp range. Only unambiguous AFLP markers that were easily scored were used. The size in base pairs was given by the comigration of a size standard. Two peaks were considered of the same size if they differed by less than 0.5 bp. Scoring was carried out without knowing the identity of the individuals or the potential relatedness between them. One individual did not amplify with any primer combination and hence it was excluded from the analyses.

Six different samples chosen at random were replicated for the two primer pairs to make an estimate of the error percentage of the AFLP analyses. AFLPs were conducted for each set of six samples separately. The rate of repeatability, expressed as the number of repeatable peaks out of the total number of peaks reached 96.6% for the combination of both *Mse*I-CAT – *Eco*RI-AAG (JOE-Green) and *Mse*I-CAG – *Eco*RI-AAC (NED-Yellow) primer pairs, for the two sets of six samples processed independently.

Table 1. Sex ratio and density of the experimental groups established with 32 individuals of *Phyllomorpha laciniata.*

Group	Sex ratio (males:females)	Density
A	4:4	High (eight individuals per 0.0272 m^2; 293.8 individuals per m^2)
B	6:2	High (eight individuals per 0.0272 m^2; 293.8 individuals per m^2)
C	4:4	Low (eight individuals per 0.5 m^2; 16 individuals per m^2)
D	6:2	Low (eight individuals per 0.5 m^2; 16 individuals per m^2)

An index of correlation in migration (IC) was used for all loci i and j, with $i \neq j$, to check for correlation between loci (see Questiau *et al*., 1999; García-González *et al*., 2003). All pairwise comparisons between loci for all individuals (N) were calculated, using two states for each locus: one for presence of a peak, zero for the absence. An IC = 1 between two fragment positions means that when a peak appears at the ith position, another peak does not appear at the jth position, or vice versa. An IC = 0 means identical appearance or absence in both ith and jth position, which could indicate comigration of the two fragments. No correlation in the migration of bands was detected in the two primer pair profiles. A total of 121 660 pairwise comparisons were calculated for JOE-Green and 139 514 for NED-Yellow. No value of zero or one were detected for the sum of all individuals from 1 to N within each comparison for loci i and j, with the exception of comparisons between monomorphic loci (15 summatories equal to zero out of 1540 in Green due to comparisons between six monomorphic loci, and three summatories out of 1775 in Yellow due to comparisons between three monomorphic loci). The average index for JOE-Green was $IC_{Green} = 0.48 \pm 0.13$ (mean $\pm$ SD, $n = 1540$) and for NED-Yellow was $IC_{Yellow} = 0.42 \pm 0.20$ ($n = 1776$). This indicates the independence of the loci, a result in agreement with that obtained in a previous study (García-González *et al*., 2003).

Exclusion probability

Within the study population (79 individuals) two AFLP primer pairs generated a total of 116 fragments. The mean number of fragments generated per individual was 84.7 (SE = 0.86; $n = 79$; range = 59–98). A total of 107 fragments (92.2%) were polymorphic, as assessed by band absence in at least one individual.

Allele frequencies were calculated using the adult individuals of our population. Assumption of Hardy – Weinberg equilibrium is necessary to calculate the allele frequencies because AFLP markers are considered dominant. The proportion of individuals with no peak for a given locus is the genotypic frequency of the recessive homozygotes (q^2), with q being the estimation of the frequency of the allele absence in the population for that locus. The frequency of the allele presence was defined as p, with $p = 1 - q$. Only the polymorphic loci with $q^2 > 3/N$ were used, with N being the number of adult individuals, as recommended by Lynch and Milligan (1994).

The equation of Chakraborty *et al*. (1974) was used to compute exclusion probabilities (the probability that any one randomly chosen male can be excluded as the father of a chosen individual). An exclusion diagnostic marker (also termed diagnostic peak or diagnostic fragment) is defined by the situation in which, for a given locus, both a potential father and the true mother lack the allele (fragment absence in the AFLP profile) whereas the particular offspring has the allele (fragment presence in the AFLP profile). The existence of two or more exclusion diagnostic markers was used to exclude a male as the father of a given nymph, following Pena and Chakraborty (1994) and according to the previous study (García-González *et al*., 2003), thus allowing for one mutation in the AFLP profile of the true sire without exclusion.

Exclusion probabilities were calculated: (i) at the population level for loci with $q^2 > 3/N$, with N being the number of individuals, (ii) at the population level for all loci, and (iii) at the adult population level for loci with $q^2 > 3/N$, with N being the number of adults. For k markers, the cumulative probability of exclusion for at least one diagnostic marker (Q_{Exc}) and the exclusion probability P_{Exc} on at least two diagnostic markers were calculated, as shown elsewhere (García-González *et al*., 2003). A total of 76 polymorphic loci with $q^2 > 3/N$, with N being the number of adult individuals ($N = 35$), were used in order to calculate allele frequencies. Dominant allele frequencies (p), calculated from the frequency of the recessive phenotype q^2 (band absence) for each one of these polymorphic loci, varied from 0.014 (band absence in 34 of 35 individuals) to 0.66 (band absence in 4 of 35 individuals). The mean dominant allele frequency (p) over 76 polymorphic loci generated from 35 adults was 0.386 (SE = 0.019; mean p for JOE-Green = 0.392, SE = 0.029; mean p for NED-Yellow = 0.381, SE = 0.026). The global exclusion probability based on at least two diagnostic peaks (P_{Exc}), calculated for the combination of the two primer pairs, was 0.88 for: (i) all the population for loci with $q^2 > 3/N$, with N being the number of individuals, (ii) all the population for all loci, and (iii) for the adult population for loci with $q^2 > 3/N$, with N being the number of adults. The global exclusion probability considering a single diagnostic peak in order to exclude paternity by a carrier male (Q_{Exc}) was 0.98 for (i) (ii), and (iii).

Paternity of eggs carried by males

Paternity assignment in *P. laciniata* is difficult to carry out because it is not possible to conduct studies with virgin females, which implies that the eggs laid may have been fertilised by sperm from males who copulated with the females in the field before the experiment started. Furthermore, under the conditions of this study each experimental group has several possible mothers for any given nymph (from two to five, depending on the number of females in each group), which adds complexity to the determination of the true genetic parents. The procedure used here to determine paternity was based on the determination of exclusion diagnostic markers (diagnostic peaks in the AFLP profile). The existence of two or more diagnostic peaks is used to exclude a non-sire. This procedure was applied with some modifications (see below) to allow paternity exclusions when multiple females are potential mothers. The steps to exclude paternity were as follows.

Step 1. AFLP fragments that were present in the nymph but absent in the carrier male as well as in all the potential

mothers of the nymph (all the females in the group) were looked for, comparing together the AFLP profiles of all females in each group. A carrier male was excluded as the father of the nymph when at least two diagnostic peaks were found in the profile comparison, i.e. when at least two peaks were present in the nymph and absent in the male as well as in the set of potential mothers.

Step 2. For the study of relatedness among individuals the similarity index (Lynch, 1990; Lynch & Milligan, 1994) was not used, but a pairwise genetic dissimilarity matrix was calculated for all individuals based on Euclidean distance (Krauss, 1999) using NTSYSpc 2.02i software (Exeter Software, New York). Euclidean distance between individuals i and j for all loci x (where x_{ki} and x_{kj} are equal to either 1 or 0) is:

$$E_{ij} = \sqrt{\sum_{k=1}^{n} (x_{ki} - x_{kj})^2}.$$

Step 3. Values of genetic relatedness estimated from Euclidean distance were used to identify a putative mother from all the potential mothers of a given nymph (all the females in each group). For the 15 nymphs that were not excluded as sired by the carrier male in step 1, the female with the highest degree of relatedness among the females within the nymph's group was selected as the putative mother.

Selection of the putative mother relies on a higher relatedness between nymph and putative mother than expected by chance. At the end of the paternity exclusion process this assumption was verified exploring differences between (i) genetic similarity of nymph x_i and putative mother and genetic similarity of nymph x_i and females of the same group of the nymph, excluding the putative mother (individuals with probability $P > 0$ of being the mother of the nymph), and (ii) genetic similarity of nymph x_i and putative mother and genetic similarity of nymph x_i and females of the other groups (individuals with probability $P = 0$ of being the mother of the nymph), for x from $i = 1$ to $i = n$, with n being the number of nymphs that were not excluded on step 1 as sired by the male who carried them during egg stage.

In these comparisons the data were not independent because for a given nymph there was only one value of relatedness with the putative mother and a series of values for the relatedness with other females (Danforth & Freeman-Gallant, 1996). Thus, it was necessary to control by nymph when analysing differences in genetic relatedness. A mixed model ANOVA (Statsoft, 1996) was carried out, in which nymph was entered as a random factor and parentage as the fixed factor with the following levels: (A) nymph – putative mother, (B) nymph – females with probability $P > 0$ of being the nymph's mother, and (C) nymph – females with probability $P = 0$ of being the nymph's mother. This analysis was preferred over a t-test for dependent samples because the latter uses the mean genetic value for each group of values for a nymph and does not consider variance of data in levels B and C. The dependent variable (genetic dissimilarity) was transformed using logarithmic transformation (Sokal & Rohlf, 1981).

Step 4. Once a putative mother for each nymph was selected, exclusion diagnostic peaks were sought between nymph and carrier male and putative mother for the 15 nymphs for whom the carrier male was not excluded as the father in step 1. A carrier male was excluded as the nymph's father when two or more diagnostic peaks were found in the profile comparison of these three individuals. A nymph possessing none or only one peak that was not present in both putative mother and carrier male, i.e. a nymph with none or only one diagnostic peak, was assumed to be sired by the carrier male. Profiles of nymph – putative mother – non-carrier males within the nymph's group were also compared to verify assignment of paternity. To do this, it was examined if there were any nymphs possessing none or only one diagnostic marker with another male within the nymph's group. In this case, the male having a higher genetic relatedness with the nymph was assumed to be the father. This ambiguity was found on three occasions, with non-carrier males having a higher genetic relatedness with the nymphs than carrier males.

Step 5. As an alternative to steps 2–4, the minimum rate of intraspecific parasitism experienced by carrier males irrespectively of the determination of the putative mother was calculated. One way to calculate this is to determine, for each nymph, the exclusion of the carrier male as the father comparing the profiles of nymph – carrier male – female x_i, for x from $i = 1$ to $i = n$, with n being the number of females within the nymph's group. Two or more diagnostic peaks in all the profiles between nymph and carrier male with each one of the females in the group were indicative of non-paternity by the carrier male. The minimum rate of intraspecific parasitism was thus established without carrying out identification of putative mothers.

Step 6. Finally, for nymphs that were not sired by the males who carried them, it was determined whether the father was a male within the nymph's group or a male with whom the mother mated in the field. Exclusion diagnostic peaks were looked for among nymph – putative mother – male x_i, for x from $i = 1$ to $i = n$, with n being the number of males in the nymph's group. A male from the nymph's group was regarded as the nymph's father when none or only one diagnostic peak was found in this profile. When, for a given nymph, two or more diagnostic peaks were found for all the profiles concerning the males in the nymph's group, the nymph was assumed to be fathered by a male who mated with the female before the experiment started (i.e. in the field). In the case of a nymph having none or only one diagnostic peak with more than one male within the nymph's group, the male with the greater genetic relatedness with the nymph was assumed to be the father.

Finally, the procedure of paternity assignment for those nymphs for whom it was concluded that they were sired by the carrier male or by another male in the nymph's group

was verified. A mixed model ANOVA (see step 3) was carried out to check the existence of differences between genetic similarity of nymph x_i and putative father and genetic similarity of nymph x_i and other males (in this case all males in the experiment are individuals with probability $P > 0$ of being the nymph's father because the nymph's mother could have mated with any of them in the field), for x from $i = 1$ to $i = n$, with n being the number of nymphs fathered by males from the nymph's group. Each individual nymph was entered as a random factor and parentage as fixed factor with two levels: (A) nymph – putative father and (B) nymph – other males. The dependent variable (genetic dissimilarity) was transformed using logarithmic transformation.

Expected probability of a male carrying eggs fertilised by him by chance

Monte Carlo methods (Manly, 1991) were used to simulate the random distribution of eggs within groups. Probabilities for the allocation of eggs among males were calculated from the simulated distribution frequencies for the number of eggs fertilised divided by the number of eggs carried (taking into account the number of eggs fertilised by each male, the number of eggs carried by each male, the number of males, and the total number of eggs within the treated group). Random distribution of eggs assumes no differential female selection of males to oviposit and no differences among males in accepting or rejecting eggs, this being the null hypothesis. The alternative hypothesis states that a bias in the male acceptance of fertilised eggs following paternity certainty and paternal investment predictions will be observed (see for example Westneat & Sherman, 1993; Wright, 1998).

Results

In all experimental groups eggs were laid preferentially on males (54.5–95%). Females laid eggs to a lesser extent on other females (5.0–39.4%), and even fewer on plants (0.0–15.8%) (Table 2). From a total of 72 eggs carried by males, 44 nymphs (61.1%) could be analysed, with 75%, 58%, 50%, and 60% being analysed in groups A to D respectively (Table 3). In total, 79 individuals (35 adults and 44 nymphs developed from eggs carried by males) were analysed in 18 subgroups of carrying males – nymphs – potential mothers distributed among four different sex ratio and density groups (A – D, Table 3).

Table 2. Number and proportion of eggs laid on the three different substrates (male, female, or plant) by the females in the experimental groups.

Group	Eggs on males	Eggs on females	Eggs on plants	Total
A	20 (60.6)	11 (33.3)	2 (6.1)	33
B	19 (95.0)	1 (5.0)	0 (0.0)	20
C	18 (54.5)	13 (39.4)	2 (6.1)	33
D	15 (78.9)	1 (5.3)	3 (15.8)	19
Total	72 (68.6)	26 (24.8)	7 (6.7)	105

AFLP profiles were resolved for a total of 42 nymphs and for the males who carried them as eggs (see Table 3). These 42 nymphs possessed between 0 and 10 fragments present in the nymph and not present in both the carrier male and the set of potential mothers, i.e. diagnostic peaks (Fig. 1). Twenty-seven nymphs possessed two or more diagnostic peaks when the profiles of the nymph, the carrier male, and the set of potential nymph's mothers (all the females in the group) were compared. Thus, on this first step of exclusion of paternity by the carrier male, 64.3% of nymphs were regarded as not fathered by the carrier male (Table 3, step 1). In 15 nymphs out of 42 (35.7%) there was none or a single mismatched peak. Thus, after step 1 of paternity exclusion, and based on the level of acceptance defined in materials and methods these nymphs were considered as possibly fathered by the male who carried them.

A putative mother for each nymph was determined among the females of the nymph's group. The female with the highest genetic similarity with the nymph was assumed to be the nymph's mother because (1) genetic distance for the relationship nymph – putative mother (A) was significantly lower than that for nymph – females of the same group of the nymph excluding the putative mother (B) (mixed model ANOVA, $F_{1,14} = 53.13$; $P < 0.001$; $n_A = 15$, $n_B = 41$), and (2) genetic distance for the relationship nymph – putative mother (A) was significantly lower than that for nymph – females of the other groups (C) (mixed model ANOVA, $F_{1,14} = 131.28$, $P < 0.001$; $n_A = 15$, $n_C = 139$) (Fig. 2).

Once the putative mother was assigned, exclusion diagnostic peaks among nymph – carrier male – putative mother were analysed. After this procedure, seven nymphs exhibited none or only one diagnostic peak. However three of them exhibited none or only one diagnostic peak with another male from the nymph's group when comparing the profiles of nymph – putative mother – other males from the nymph's group. These three nymphs have a higher genetic relatedness with the non-carrier males, thus they were excluded as fathered by the carrier male. Therefore, after step 4 of paternity exclusion, four nymphs were considered to have been sired by the males who carried them at the egg stage.

The minimum rate of intraspecific parasitism experienced by carrier males calculated as described in step 5 of methods, without identification of putative mothers for each nymph, was 83.3% (35/42 eggs were carried by males that did not sire them) (Table 3, step 5). Therefore, the different steps based on the estimation of the putative mother to assign paternity raised the percentage of eggs not sired by the male who was carrying them from 83.3% to 90.5% in the overall study population (Table 3).

For those nymphs not sired by the carrier male it was determined whether their father was among the other males

Table 3. Females and males in each group, potential mothers of the nymphs in each group, number of eggs carried by each male, number of eggs analysed, and number of nymphs excluded as sired by the carrier male after different steps of determination of paternity: step 1, exclusion of paternity based on profiles nymph – carrier male – 'all potential mothers for the nymph together'; step 4, exclusion based on profiles nymph – carrier male – putative mother; step 5, exclusion based on profiles nymph – carrier male – 'all potential mothers, one each time' (see Materials and methods for details). In some groups there are more individuals than the initial numbers established because some individuals died during the experimental period and they were replaced by other individuals (see Materials and methods). Values in parentheses are percentages.

Group (potential mothers)	Male	No. of eggs		No. of eggs excluded as sired by the carrier male after different steps			
		carried	analysed	Step 1	Step 4	Steps 1 and 4	Step 5
A	A1	7	5 (71.4)	0 (0)	3 (60)	3 (60)	1 (20)
(A1, A2,	A2	2	2 (100)	0 (0)	1 (50)	1 (50)	1 (50)
A3, A4)	A3	6	5 (83.3)	1 (20)	4 (80)	5 (100)	5 (100)
	A4	5	3 (60)	1 (33.3)	1 (33)	2 (66.7)	2 (66.7)
Subtotal A		20	15 (75)	2 (13.3)	9 (60)	11 (73.3)	9 (60)
B	B1	4	4 (100)	4 (100)	–	4 (100)	4 (100)
(B1, B2)	B2	5	1 (20)	0 (0)	1 (100)	1 (100)	1 (100)
	B3	2	1 (50)	1 (100)	–	1 (100)	1 (100)
	B4	3	1 (33.3)	1 (100)	–	1 (100)	1 (100)
	B5	1	1 (100)	1 (100)	–	1 (100)	1 (100)
	B6	4	3 (75)	2 (66.7)	1 (33.3)	3 (100)	2 (66.7)
Subtotal B		19	11(57.9)	9 (81.8)	2 (18.2)	11 (100)	10 (91)
C	C1	1	0 (0)	–	–	–	–
(C1, C2, C3,	C2	1	1 (100)	1 (100)	–	1 (100)	1 (100)
C4, C5)	C3	4	3 (75)	3 (100)	–	3 (100)	3 (100)
	C4	12	5 (41.7)	5 (100)	–	5 (100)	5 (100)
	C5	0	0	–	–	–	–
Subtotal C		18	9 (50)	9 (100)	0 (0)	9 (100)	9 (100)
D	D1	0	0	–	–	–	–
(D1, D2)	D2	1	1 (100)	1 (100)	–	1 (100)	1 (100)
	D3	0	0	–	–	–	–
	D4	1	1 (100)	1 (100)	–	1 (100)	1 (100)
	D5	2	2 (100)	2 (100)	–	2 (100)	2 (100)
	D6	7	2 (28.6)	2 (100)	–	2 (100)	2 (100)
	D7*	3	2 (66.7)*	–	–	–	–
	D8	1	1 (100)	1 (100)	–	1 (100)	1 (100)
Subtotal D		15	7 (46.7)	7 (100)	0 (0)	7 (100)	7 (100)
Total	22	72	42(58.3)*	27 (64.3)	11 (26.2)	38 (90.5)	35 (83.3)

*It was possible to determine paternity in only 42 nymphs out of 44 analysed because PCR did not amplify DNA from male D7.

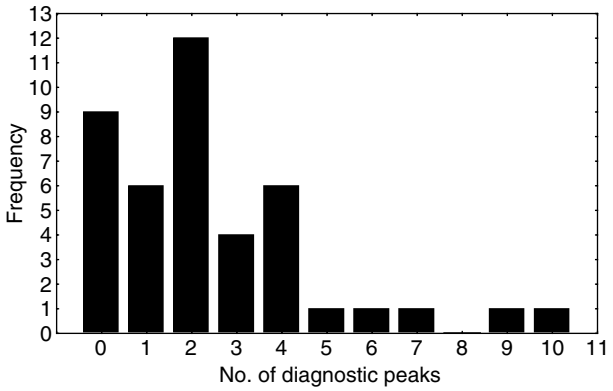

Fig. 1. Distribution of the diagnostic markers between the profiles for sets of nymph – carrier male – potential mothers of the nymph.

of the group or whether he was a previous partner of the mother with whom she mated in the field (i.e. before the female was brought to the laboratory), as described in step 6. Genetic similarity between nymph and putative father (A) was significantly higher than that for nymph and other males (B) (mixed model ANOVA, $F_{1,12} = 17.17$; $P = 0.0014$; $n_A = 13$, $n_B = 280$, see step 6 in Materials and methods) (Fig. 3). This was calculated for 13 nymphs that were fathered by any male in the nymph's group (see below).

Out of 42 nymphs for which paternity could be assigned, four (9.5%) were sired by the male carrying them, nine (21.4%) were sired by another male from the group, and 29 (69%) were sired by males with whom females mated before the start of our experiment (Fig. 4). Thus, the first term can be considered paternity by the carrier male, the second one can be considered intraspecific brood parasitism experienced by the carrier male, which is due to eggs fertilised by males from the same group, and the third term

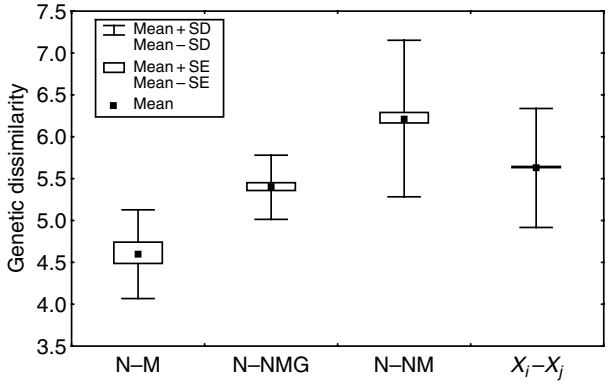

Fig. 2. Genetic dissimilarity for different relationships between: N – M, nymph and mother (for 15 nymphs that were not excluded as sired by the carrier male in step 1, 15 pairwise comparisons); N – NMG, nymph and non-mother, with females other than the putative mother, from the same group of the nymph (15 nymphs, 41 pairwise comparisons); N – NM, nymph and non-mother, females from groups other than the nymph group (15 nymphs, 139 pairwise comparisons); $X_i - X_j$, all relationships between all individuals i,j, $i \neq j$ from $i = 1$ to $i = 79$ (3081 pairwise comparisons).

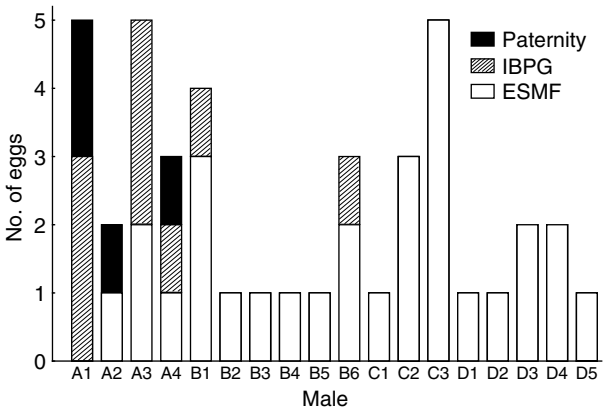

Fig. 4. Number of eggs carried by each male discriminating between eggs sired by the male who carried the eggs ('Paternity'), sired by other male of the same group (Intraspecific brood parasitism due to eggs fertilised by males in the group, IBPG), or sired by other males not present in the experimental group, i.e. males who mated with females before they were brought into the laboratory (eggs sired by other males in the field, ESMF).

being eggs fertilised by sperm from males that mated with the female in the field.

To determine whether the probability of carrying fertilised eggs was due to random egg allocation, the scenario of egg carrying in group A, the group where we detected paternity of carried eggs, was simulated 2000 times. The real scenario was as follows: from 15 eggs laid by females in group A, male A1 carried two eggs fertilised by him and three others non-fathered by him, male A2 carried one egg fertilised by him and another egg non-fathered by him, male A3 carried five unrelated eggs, and male A4 carried one egg fertilised by him out of three carried eggs (see Fig. 4). Knowing from the paternity analyses that males A1, A2, A3, and A4 fertilised each a total of five, three, one, and two eggs, respectively, a random distribution of these 11 eggs plus four other eggs fathered by males other than the males in the group was generated. This process was repeated 2000 times and the frequencies of eggs carried by the father were recorded.

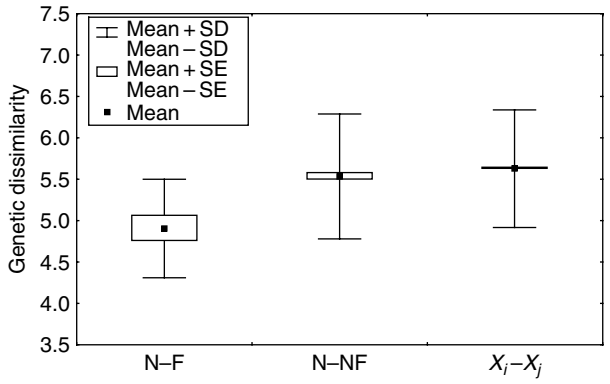

Fig. 3. Genetic dissimilarity for the relationships between: N – F, nymph and putative father (for 13 nymphs sired by males from the nymph's group, 13 pairwise comparisons); N – NF, nymph and other males (13 nymphs, 280 pairwise comparisons); $X_i - X_j$, all relationships between all individuals i,j, $i \neq j$ from $i = 1$ to $i = 79$ (3081 pairwise comparisons).

The probability of obtaining our real scenario for all males taken together was $P = 0.048$. This probability is similar to that obtained using permutations with repetition (for n elements with n_1 elements of one type, eggs fertilised by the male x_i, and n_2 elements of another type, eggs non-fertilised by the male x_i) for each male separately, and then calculating the probability of the event for all males ($P = 0.046$). For each particular male, the confidence of carrying eggs from the predictions derived from paternity certainty and paternal investment (1 − probability of the male carrying eggs fertilised by him at random) was as follows. For male A1 the confidence of that carrying two eggs fertilised by him is not the result of a random egg distribution was between 43% and 84%. For male A2, the confidence of that carrying one egg fertilised by him is not the result of a random distribution (with identical conditions mentioned above) was between 63% and 97.5%. Finally, for male A4, the confidence of that carrying one egg fathered by him is not the result of a random distribution (with identical conditions mentioned above) was between 65.2% and 97.6%.

Discussion

The AFLP technique detected high levels of polymorphism in *P. laciniata*. Given the estimated allele frequencies, the

theoretical expected percentage of offspring for which all males except the true genetic father can be excluded (exclusion probability) was 88%. This expected exclusion probability implies a number of assumptions including random mating and random extraction of individuals from the population (Lewis & Snow, 1992). Another assumption is that closely related males do not compete for paternity, a fact that has been seen to overestimate the probability of excluding a randomly chosen non-father (Double *et al.*, 1997). In this study, care was taken to ensure random collection of individuals from a natural population, and there is no evidence of non-random mating (Reguera, 1999).

In the present study it was not possible to determine by direct observation which female was the mother of a given nymph. There were, for each nymph, between two and five potential mothers, depending on the experimental group. This difficulty was solved by the statistical determination of the putative mother among all the potential mothers of a nymph. Differences were found for the genetic similarity between nymphs and putative mothers (the latter selected as the female with the highest degree of relatedness among the females with a probability $P > 0$ of being the mother of the nymph) and nymphs and non-putative mothers. A second difficulty relates to the fact that the study could not be carried out with virgin females (see Materials and methods). This implies that for each nymph there were multiple potential fathers – males from the nymph's experimental group and males from the natural population who could have copulated with the nymph's mother before the start of the experiment. To solve this problem a procedure was developed to exclude non-fathers, based on the determination of exclusion diagnostic markers. More specifically, it was possible to determine that a nymph was fathered by a male outside the nymph's experimental group when two or more diagnostic peaks were found for all profiles of nymph – putative mother – male x_i, for x from $i = 1$ to $i = n$, with n being the number of males in the nymph's experimental group.

The exclusion of the egg-carrying male as the father of the nymph reached 83.3% of cases (without identification of putative mothers, as indicate in step 5 of Materials and methods), or 90.5% of nymphs (assessing first the putative mother as described in Materials and methods). However, these results show that there was a high prevalence of eggs fertilised by sperm from males not present in the experimental groups (i.e. that mated with the female in the field before females were captured). In group B, 10 out of 11 eggs carried by experimental males were fathered by males from the field, and in groups C and D all nymphs were fathered by males not present in the experimental groups. Because these two are the low density groups, encounter rates are expected to be low, thus one possibility is that in groups C and D experimental males did not copulate with females during the experiment, or that copulations did not involve sperm transfer, a relatively common phenomenon in this and other species (García-González, 2004; García-González & Gomendio, 2004).

In order to analyse whether males are more likely to accept carrying their true genetic offspring, only eggs that are fathered by males from the experimental group should be considered. The reason for this is that when eggs are fertilised by males outside the experimental group, fathers do not have the opportunity to accept their own eggs (due to the obvious restrictions imposed by the experimental conditions). Taking all experimental groups into account, only 13 out of 42 (31%) of all eggs laid on males were fathered by a male within the group. Out of these 13 eggs sired by males in the experimental groups, four (30.8%) were sired by the carrier male, and nine (69.2%) were carried by a male other than the father. Group A showed a moderate rate of paternity by egg carrying males in relation to eggs sired by others males from the group: four nymphs out of 11 sired by males in the group had been sired by the carrier male; this represents 36.4% paternity in carrier males (Fig. 4, Table 3).

The densities that were set up in experimental groups were much higher than those found in natural populations. Densities in the experimental groups reached 16 individuals per m^2 in groups C and D, and 293.8 individuals per m^2 in groups A and B, whereas data gathered from natural populations in an area of 400 m^2 in 44 different days throughout three reproductive periods (years 1998–2000; García-González & Gomendio, 2003), showed densities ranging from 0.005 to 0.265 individuals per m^2 (mean density = 0.042 individuals per m^2, SE = 0.007). Natural densities are therefore difficult to reproduce in studies carried out in the laboratory to ascertain whether males accept eggs that they have fathered. Because high densities promote egg laying in conspecifics (Reguera, 1999), it is likely that the experimental conditions also made it easier for females to lay eggs on conspecifics that were not the true genetic father because under such conditions the opportunities to reject egg-laying attempts by moving away are limited. A recent study by Richardson and Burke (2001) showed that, for an avian species, high breeding densities were associated with an increase in the frequency and extent of extra-pair paternity. The fact that a very low proportion of eggs were laid on plants supports the view that the high densities in the experimental groups favoured egg laying on conspecifics. Therefore, the null hypotheses in this study, namely that males do not carry eggs that they have fathered and that females dump eggs randomly, was conservatively strengthened by the experimental set up, which favoured intraspecific brood parasitism. Despite this, a larger proportion of eggs were laid on males than on females. Furthermore, if only those cases in which eggs were fathered by experimental males and thus had chances of being accepted by the genetic father were taken into account, 30.8% of those eggs were laid on fathers even under high densities that favour intraspecific parasitism. In addition, Monte Carlo simulations show that males are more likely to accept eggs that they have fathered. This evidence suggests that paternal care plays a role in this system.

The golden egg bug together with the giant water bugs of the subfamily Belostomatinae (Heteroptera: Belostomatidae) are the only well documented cases of insects in which females glue eggs on the backs of conspecific males (Ridley, 1978; Zeh & Smith, 1985; Clutton-Brock, 1991; Smith, 1997). In all belostomatid species male care is obligatory because eggs that are not looked after by males die. Similarly, golden egg bug eggs that are not carried by a conspecific but are laid on the vegetation suffer high parasitoid and predation pressures that yields a mortality rate of 97% (Reguera & Gomendio, 2002). However, the low levels of paternity in the golden egg bug contrast with the mechanisms of paternity assurance in giant water bugs and the assumed high levels of paternity for offspring carried by males in belostomatinae species (Smith, 1979a, b). The rates at which males carry eggs that are not their true genetic offspring demands an explanation concerning the reasons why this occurs. Whereas last male sperm precedence in giant water bugs means that mating males sire the majority of eggs laid by the female, the mechanism of sperm competition operating in the golden egg bug seems to be one of sperm mixing (García-González *et al.*, 2003; García-González & Gomendio, 2004). This mechanism accounts for the fact that males in this species do not enjoy high levels of paternity and confidence of paternity. More importantly, it implies that the process by which sperm from different males fertilise a female's eggs is not predictable by males. As males are incapable of discriminating their own eggs, and cannot predict when their own eggs will be laid, but eggs suffer very high mortality rates if they are not carried by adults, males face the dilemma of not accepting eggs and running the risk of allowing their own offspring to die on plants, or accepting eggs even though they can only ensure that a proportion of the eggs they carry are their true genetic offspring. A male will have to make this decision many times over the breeding season, since, in contrast to giant water bug females, females of the golden egg bug tend to lay one egg at a time, and they continue to lay eggs over several months.

Finally, egg-carrying males in the golden egg bug do not suffer a reduction in mating opportunities, which is perhaps the main cost preventing the evolution of paternal care in other species (Zeh & Smith, 1985; Clutton-Brock, 1991; Smith, 1997). *Phyllomorpha laciniata* males that carry eggs are not restricted to stay in a particular place looking after the offspring because eggs are glued to their back, egg carrying does not affect mate searching and copulation, and in natural populations back space does not become a limiting factor over the breeding season (Gomendio & Reguera, 2001).

Further molecular analyses addressing the genetic relationship between egg carriers and eggs are needed to fully understand egg-carrying behaviour in *P. laciniata*. A recent study showed that males father an average of 43% of the eggs laid by females, and that females lay eggs soon after copulation on the mating male's back (García-González *et al.*, 2003). In addition, Tay *et al.* (2003) recently used microsatellite markers to look at the proportion of eggs carried by males that were sired by the carrying male in *P. laciniata*. They concluded that the rate of paternity obtained (ranging between 13% and 22% depending on the methodology to determine paternity) did not support the view of male egg carrying functioning as paternal care. However, showing low paternity rates is not enough to solve the evolutionary puzzle posed by the golden egg bug system because paternal care may evolve even when confidence of paternity is not high, depending on trade-offs between current benefits and costs, as well as present and future reproduction (Whittingham *et al.*, 1992; Westneat & Sherman, 1993; Mauck *et al.*, 1999; García-González *et al.*, 2003). The results presented in this paper show that the proportion of eggs carried by males that are their true genetic offspring is greater than expected by chance. This is probably achieved by accepting eggs preferentially after copulations end. In addition, previous studies have shown that males extend the duration of copulation and transfer more sperm in the presence of rival males (García-González & Gomendio, 2004). Thus, males try to ensure the paternity of the eggs they carry, which may be considered as a rudimentary form of parental care with a component of deceit on the part of females. These findings show that paternal care may evolve under conditions in which certainty of paternity is low, provided the benefits in terms of offspring survival are high enough. *Phyllomorpha laciniata* females cannot lay eggs on themselves, and eggs laid on plants are unlikely to survive. Thus, to enhance offspring survival they have no other choice but to lay eggs on conspecifics. Females respond by rejecting most egg-laying attempts. However, male reproductive success would suffer from high offspring mortality rates if eggs are laid on plants, and for this reason males are more willing to accept eggs as long as a proportion of them are their true genetic offspring.

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