RESEARCH ARTICLE

Mechanisms of inbreeding avoidance in the one-piece drywood termite *Neotermes chilensis*

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Abstract Inbreeding depression refers to a decrease in fitness components in the offspring of closely related sexual pairs. Given the disadvantages of inbreeding depression, it is of interest to study the mechanisms involved in its avoidance, particularly in social insects. In termites, colonies are founded by dispersing individuals. Two types of mechanisms may account for inbreeding avoidance: indirect mechanisms that occur before the dispersing individuals come into contact (sex-biased production at colony level, sex-biased emergence timing, dispersal, and sex-biased dispersal), and an active mechanism (kin recognition) that occurs when dispersing individuals come into contact. We have used ecological, behavioral and genetic approaches to study the mechanisms of inbreeding avoidance along the complete process of colony foundation by *Neotermes chilensis*, i.e., from the production of dispersing individuals in the colony to the production of siblings by the newly formed reproductive pair. This is the first report to address both indirect and active mechanisms of inbreeding avoidance in the same study, in the same termite species, and through the complete process of colony foundation. The results show that dispersal is the main indirect mechanism of inbreeding avoidance and that kin recognition is unlikely as an active mechanism of inbreeding avoidance.

Keywords Termites · Kin recognition · Inbreeding avoidance · Microsatellites

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Introduction

Inbreeding refers to the mating between closely related individuals (Kristensen et al. 2010); ensuing increased homozygosity of deleterious recessive alleles in the offspring, known as inbreeding depression (Pusey and Wolf 1996; Charlesworth and Willis 2009), may lead to the decrease in one or more components of fitness such as reproduction, survival and growth (Pusey and Wolf 1996). In social species, the study of mechanisms of inbreeding avoidance is particularly important because close relatives live in frequent contact (Tabadkani et al. 2012).

Termites are eusocial insects. Reproduction normally occurs between primary reproducers but may also involve secondary reproducers within a mature colony. A reproductive behavioral repertoire is displayed by the newly formed couple outside the colony prior to the founding of a new colony; this repertoire is similar in all termites (Eggleton 2011). During the reproductive period, the dispersal of alate individuals of both sexes produced inside the colony occurs as swarms; upon landing, alates loose their wings and give rise to de-alates. Once de-alates of different sexes meet, they engage in a nuptial promenade (the male walks behind the female in a tandem behavior) after which the couple searches for a nesting substrate. When such substrate is found, the couple builds a copularium and mates; shortly

after mating, the female starts oviposition. The founding couple is referred to as the primary reproductor pair (PRP).

Reproduction within a mature colony involves the formation of neotenics (secondary reproducers) from pseudoworkers; they can mate with the primary reproductors or among themselves. Additionally, in three *Reticulitermes* species, *R. speratus* (Matsuura et al. 2009), *R. virginicus* (Vargo et al. 2012) and *R. lucifugus* (Luchetti et al. 2013), the queen has been shown to reproduce parthenogenetically to produce female neotenics which can mate with the king (Matsuura et al. 2009).

Deleterious effects of inbreeding in termites have been shown through the comparisons of worker bodyweight (Husseneder et al. 2005) and colony size (Husseneder et al. 2007) between natural colonies with or without neotenics, through comparison of fecundity (Fei and Henderson 2003) and immunocompetence (Calleri et al. 2006) of colonies produced artificially in the laboratory from inbred and outbred de-alate pairs, and through determining the survival of members of colonies formed from reproducers with different degrees of relatedness (DeHeer and Vargo 2006).

Molecular studies in natural colonies have shown that the colony-founding individuals in a broad range of species are not closely related genetically (review: Vargo and Husseneder 2011). This implies the occurrence of inbreeding avoidance in the establishment of new colonies, i.e., in the part of the reproductive cycle occurring outside the colony. Both indirect and active mechanisms of inbreeding avoidance have been described for that part of the reproductive cycle (Tabadkani et al. 2012; Vargo and Husseneder 2011). Indirect mechanisms refer to those exhibited up to the formation of de-alates with the overall effect of decreasing the probability of encounter of individuals of different sex from the same colony; they are: sex-biased production of alates at the colony level, i.e., some colonies may produce one sex in preference to the other (Lenz and Runko 1993; Roisin and Lenz 2002; Husseneder et al. 2006), sex-biased emergence timing, i.e., one sex may emerge from a given colony earlier than the other (Morbey and Ydenberg 2001), dispersal, i.e., as alates fly away from their colony during swarming the chance of encountering a nestmate decreases as the distance flown increases (Messenger and Mullins 2005; Husseneder et al. 2006; Vargo et al. 2006; Hu et al. 2007), and sex-biased dispersal, i.e., one sex may disperse to longer distances than the other (Shellman-Reeve 1996). On the other hand, an active mechanism which may take place during mate choice by de-alates is kin recognition, i.e., the capacity of an individual to discriminate between conspecifics differing in genetic relatedness (reviews: Michener and Smith 1987; Smith and Breed 1995).

Few reports have addressed in termites the mechanisms of inbreeding avoidance in the colony-founding pair (Shellman-Reeve 2001; Husseneder et al. 2006; Husseneder

and Simms 2008; Vargo and Husseneder 2011) and no studies have evaluated the mating choice process from dispersal of alates to the formation of a colony in any one termite species. We have undertaken such studies with *Neotermes chilensis* (Blanchard, 1851) (Kalotermitidae), a monogamous one-piece termite (i.e., individuals nest and forage in a single piece of wood) endemic to Chile between ca. 26–33°S whose reproductive behavioral repertoire is similar to that of other termites (Rippa and Luppichini 2004). Mature colonies usually contain fewer than 400 individuals; no neotenics have been described for this species. *N. chilensis* represents a good model for inbreeding avoidance studies because complete colonies can be conveniently followed, emergence of alates from the colony can be controlled in the laboratory, and the PRP is easily accessible. Using ecological, behavioral and genetic approaches, we have explored for the first time in a single species of termite the complete spectrum of inbreeding avoidance mechanisms during the founding of a new colony; additionally, we have studied the mating of de-alates and subsequent oviposition in an artificial arena. Our results show that dispersal is the main mechanism of avoiding inbreeding and that kin recognition is most likely not involved in the process.

Materials and methods

Species and study area

Studies were carried out in a population of *N. chilensis* found at Las Chilcas (32°52′S; 70°52′W) inside dry scapes (floral stems) of *Puya berteroniana* Mez (Bromeliaceae). The site is within the sclerophyllous shrub community of central Chile (Gajardo 1994) where the predominant species are *P. bertereroniana*, *Adesmia arborea* Bert. ex Savi (Fabaceae), *Colliguaya odorifera* Mol. (Euphorbiaceae) and *Echinopsis chiloensis* (Colla) H.Friedrich & G.D.Rowley(Cactaceae).

Inbreeding avoidance

The degree of relatedness was compared between: (a) nestmates (before swarming), (b) alates caught during a swarm (i.e., after eventual indirect mechanisms of inbreeding avoidance had taken place), and (c) PRP (i.e., after the eventual active mechanism of inbreeding avoidance had taken place). Correspondingly, three experimental groups of individuals were confirmed as follows: (a) nestmates (pseudo-workers and soldiers) contained in six dry scapes of *P. berteroniana* (between six and twenty-seven individuals were found inside each colony; total $N = 74$); (b) swarming alates collected using light traps between 21:00 and 23:00 h during February and March (two traps during six nights in

2012 and seven traps during eight nights in 2013), the distance between them being 1.45 km in 2012 and ranging from 0.075 to 2.35 km in 2013. The alates collected (75 in 2012 and 270 in 2013) were brought to the laboratory, sexed and separated by trap and collection event; and (c) PRP extracted from different colonies ($N = 19$).

The mean relatedness within the three experimental groups of individuals was determined using microsatellite markers. Ten microsatellites were designed, eight of which were successfully amplified in all individuals studied (for details on PCR conditions, see Electronic supplementary material); they were used to determine the relatedness between all possible pairs of nestmates, between pairs of alates of different sex collected in a given day and a given trap within a swarm, and between members of each PRP, using the Queller and Goodnight index (Queller and Goodnight 1989) in the KINSHIP 1.3.1 software (Goodnight and Queller 1999). Mean values for nestmates, alates and PRP (Fig. 1) were compared using a Kruskal–Wallis test followed by Newman–Keuls a posteriori tests (Siegel and Castellan 1988).

Additionally, within each experimental group two different approaches (likelihood analysis and direct comparison of experimental and simulated relatedness values) were independently used to assign the pairs described in the preceding paragraph to either the full sibling or the unrelated categories; only these categories were used because the PRP is monogamous (Thorne 1997; Vargo and Husseneder 2011). In the first approach, the likelihood function implemented in the KINSHIP software was used.
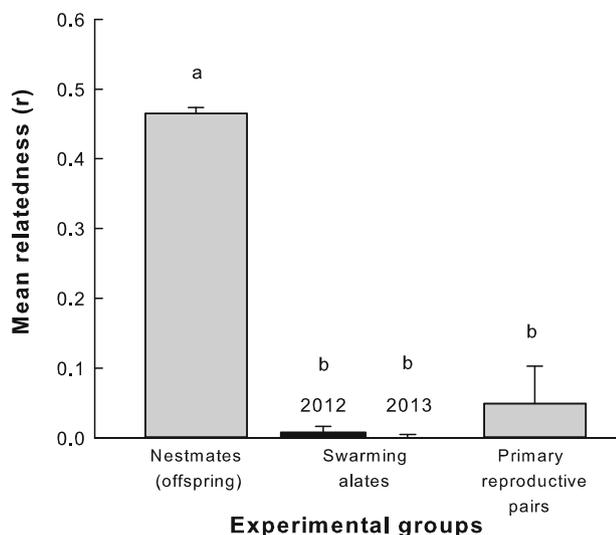
In the second approach, ten thousand unrelated and ten thousand full sibling pairs were simulated using the KINSHIP software; two distribution curves were generated with the simulated relatedness values and the point of intersection of these curves projected to the x-axis (relatedness equal to 0.26) was used to assign individuals in the experimental groups to the two kinship groups (Fig. 2). Finally, a Fisher exact test was used to compare the three experimental groups in terms of assignments to kinship groups made by the likelihood and simulation approaches.

Testing for sex-biased emergence of alates and for sex-biased production as indirect mechanisms of inbreeding avoidance

Fifty dry scapes of *P. berteroniana* were surrounded by a mesh, brought to the laboratory in Santiago and maintained exposed to natural environmental conditions on top of a roof. Only 14 colonies gave rise to swarms. The sex of individuals which emerged and were caught in the surrounding mesh was registered daily at midnight during 2 months. The count started when the first alate emerged. To ensure that scapes studied contained a single nest and that all alates had emerged, the scapes were dissected after the period of observation; if scapes were multinest or alates remained in them, such scapes were not taken into consideration in further analyses. Consequently, the final number of scapes used in this experiment was seven.

The mean emergence day was calculated for each sex within each scape and the statistical difference between these parameters was tested with a Mann–Whitney U test. Sex-biased production of alates at the colony level was tested by comparing with a binomial test (http://vassarstats.net/) the total number of male and female alates which emerged from each colony.

Testing for dispersal and for sex-biased dispersal during a swarming event as indirect mechanisms of inbreeding avoidance

The genotyped alates caught in seven traps during the 2013 swarm were used in these analyses on account of the larger number of traps set that year and the larger number of individuals collected.

To test for dispersal, individuals from both sexes were used together in an isolation by distance analysis, i.e., the correlation of the among-traps genetic distance (F_{ST}) matrix with the among-traps geographical distance matrix; a Mantel test was performed using the GenAlEx v.6.5 software (Peakall and Smouse 2012). The genetic distance (F_{ST}) was calculated between pairs of traps as described by Weir and Cockerham (1984) using the Genetix software (Belkhir et al. 1996).

Fig. 1 Mean relatedness between nestmate pairs, male–female alate pairs from any given day in a swarming event (*bars* for 2012 and 2013 seasons), and primary reproductive pairs. Significant differences were found among groups ($H_3 = 1109.3$; $P < 0.001$). *Different letters* show significant differences at $P = 0.05$

Fig. 2 Distribution of 10,000 simulated pairs of unrelated and 10,000 pairs of full siblings generated with the KINSHIP 1.3.1 software (*upper curves*) and of three experimental groups (*bars*): nestmates (N = 572), swarming alates during the 2012 (N = 460) and 2013 seasons (N = 2074), and primary reproductive pairs (N = 19). Frequencies were determined using a relatedness interval of 0.04 units. For visual simplicity, lines linking frequency values are shown for the simulated pairs. The intersection between the curves is denoted by a *vertical dotted line* in the five distributions shown
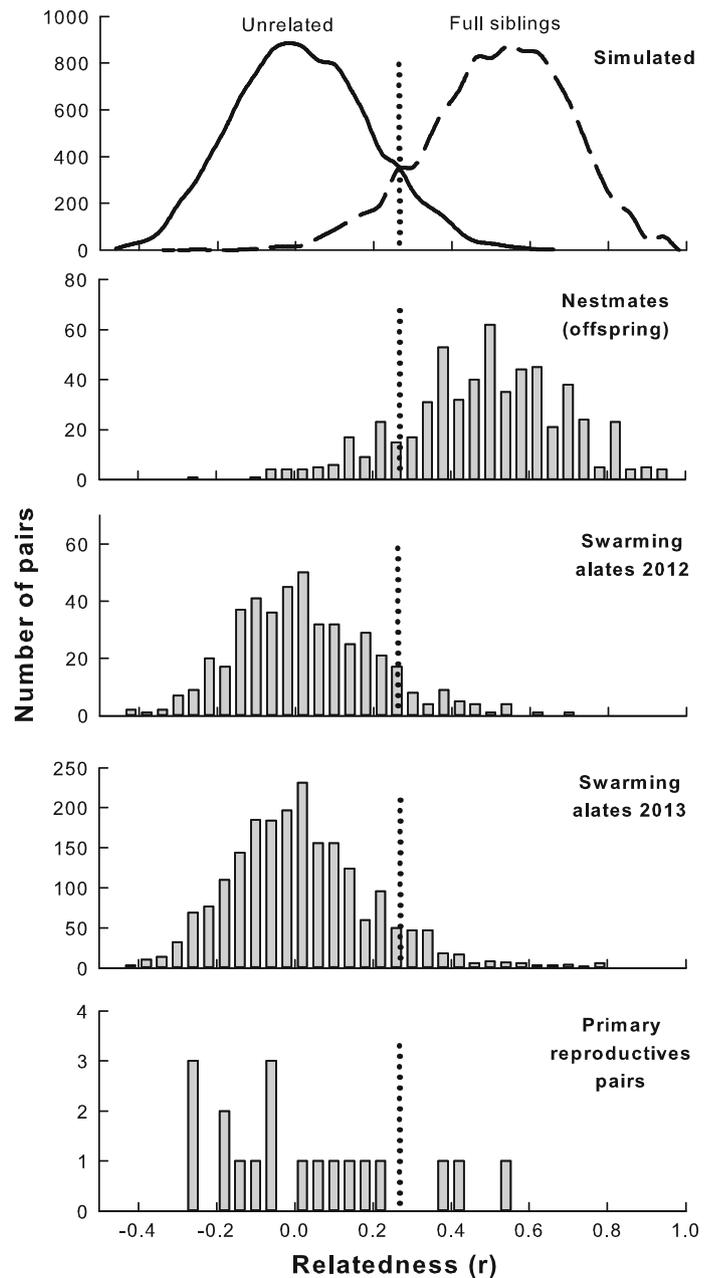
To test for sex-biased dispersal, four different parameters were determined independently for males and for females in each trap and then compared between sexes: (1) genetic differentiation (F_{ST}) (Goudet et al. 2002), performed with the GenAlEx v.6.5 software; (2) inbreeding index (F_{IS}) (Goudet et al. 2002), calculated with the Genetix software; (3) mean relatedness degree (r) (Goudet et al. 2002), calculated with the KINSHIP software, and (4) assignment index correction (Favre et al. 1997) obtained with the GenAlEx v.6.5 software. To test for significant differences between males and females, F_{ST}, F_{IS}, r and assignment index correction values were compared using a permutation test (5000 permutations) coded in R software (R

Development Core Team 2013). When sex-biased dispersal occurs, the sex showing the least dispersal (philopatric sex) is expected to show a significantly higher F_{ST} and r values but a lower F_{IS} value compared with the sex showing the most dispersal (dispersing sex). Furthermore, the dispersing sex will show negative mean assignment index correction.

Testing for overall efficiency of indirect mechanisms of inbreeding avoidance

To determine the overall consequences of indirect mechanisms of inbreeding avoidance, alates were allocated separately into colony groups (fullsibs) in the 2013 swarm

using the COLONY 2.0.4.0 software (Jones and Wang 2010). The number of all possible inbreeding (from the same colony) and outbreeding (from different colonies) intersexual pairs was determined in each trap for each day. The results were added up for all traps and days and an overall expected inbreeding/outbreeding ratio was calculated.

Testing for an active mechanism of inbreeding avoidance in the laboratory

To test the occurrence of an active mechanism of inbreeding avoidance in the laboratory, 60 scapes of *P. berteroniana* were brought to the laboratory, surrounded by a mesh and kept in the darkness at 16 °C. Alate emergence was stimulated and synchronized by raising the temperature to 30 °C and providing light with a 14L:10D photoperiod. Alates emerged from 19 scapes and became trapped in the mesh; they were separated by sex and scape of origin, and were individually placed in Petri dishes lined with filter paper to facilitate movement and wing shedding, thus giving rise to virgin de-alates. A preference bioassay was performed in which a virgin focal de-alate was enclosed in a Petri dish lined with filter paper, with two virgin de-alates of the opposite sex, one from the same scape as the focal individual (sibling) and the other from a different scape (unrelated). The bioassay arena was video-recorded during 20 min. The behaviors registered were: time to first proctodeal palpation, time to first allogrooming, time spend in and frequency of occurrence of giving (males as focal individual) or receiving (females as focal individual) proctodeal palpation and allogrooming. Data were analyzed with JWatcher v1.0 (Blumstein et al. 2006); times spent in different behaviors and frequencies of occurrence of behaviors were compared between non-nestmate and nestmate pairs using the *t* test or the Mann–Whitney *U* test, depending on the data following or not normality and homoscedasticity conditions, respectively. Once the bioassay was finished, each individual was used for genetic analyses. Additionally, at the end of the experiment the scapes were dissected and only data from those which proved to be monocolonial (a single PRP was found in them) were used in the analyses. Fifteen replicates were finally retained with the focal de-alate being a male and another fifteen with the focal de-alate being a female. Males and females were used as focal individuals because previous studies have shown that both males and females are involved in mate choice (Shellman-Reeve 1999; Husseneder and Simms 2008).

To study the relatedness and kinship relationships among the 90 individuals used in the behavioral bioassays, their DNA was extracted and amplified with eight microsatellite markers. The relatedness and kinship relationships between individuals from each bioassay replicate were determined using the KINSHIP 1.3.1 software. Values for pairs that mated were compared with values of pairs which did not mate using a one-tailed *t* test (Siegel and Castellan 1988).

Mating in artificial colonies

A virgin male and a virgin female de-alate from the same or different nests were obtained as explained above and were introduced in a Petri dish containing sawdust of *P. berteroana* (80 %) and cellulose (20 %) and kept in the dark. Six pairs of nestmates and seven of non-nestmates were formed. They were observed to check for the occurrence of mating; subsequently, the number of eggs laid by each mated pair was registered and compared with a two-tailed *t* test.

Results

Inbreeding avoidance

The mean relatedness between nestmate pairs (offspring), female–male alate pairs in the 2012 and 2013 swarms, and PRP is shown in Fig. 1. The mean relatedness of nestmate pairs (0.465 ± 0.0085, mean $\pm$ standard error) closely corresponded to that expected for full siblings (0.50). The statistical analysis showed that the mean relatedness of nestmate pairs (excluding PRP) was significantly higher than that of PRP ($P < 0.001$) and of pairs of different sex among alates from a swarming event ($P < 0.001$ for both years). The mean relatedness of female–male alate pairs did not differ between the 2012 and 2013 swarms ($P = 0.834$). Moreover, the mean relatedness of female–male alate pairs in the 2012 and 2013 swarms was not statistically different from that of PRP ($P = 0.196$ and $P = 0.289$, respectively). The above comparisons are supported by the distribution of kinship groups between the different experimental groups (Table 1).

Testing for sex-biased emergence of alates and for sex-biased production as indirect mechanisms of inbreeding avoidance

The duration of the emergence period of alates from escapes brought from the field was 33.9 ± 3.2 days (mean $\pm$ ES, range 22–48 days); 82.7 ± 9.4 alates of both sexes emerged from each scape (mean $\pm$ ES, range 41–105 alates). Sex proportion between males and females did not depart significantly from 1:1 and only one scape showed significant protandry (Table 2).

Table 1 Assignment to full sibling and unrelated categories of all possible pairs of nestmates, pairs of alates of different sex collected in a given day and a given trap within a swarm (2012 and 2013 seasons), and members of each PRP, based on two different approximations: likelihood analysis and direct comparison of experimental and simulated relatedness values

Approximation	Kinship relationship of pairs formed	Experimental groups (N)			
		Nestmates (74)	Swarming alates		Primary reproductive pairs (19)
			2012 (75)	2013 (270)	
Likelihood	Full siblings/unrelated	489/83 a	34/426 b	189/2518 b	3/16 b
Simulation	Full siblings/unrelated	483/89 a	37/423 b	210/2497 b	3/16 b

Significantly different proportions in rows are depicted by different letters (Fisher exact test, $P < 0.05$)

Table 2 Summary of tests for sex-biased production of alates (i.e., deviation from 1:1 sex ratio) and sex-biased emergence of alates (i.e., differences in the mean day of emergence of males and females) from 7 scapes collected in the field and kept on a roof in Santiago

Scape	Production of alates		Day of emergence of alates		
	m/f ratio (n)	Deviation from 1:1	Males (mean ± SEM)	Females (mean ± SEM)	P value
1	1.10 (105)	n.s.	5.5 ± 0.44	5.4 ± 0.66	0.207
2	0.76 (104)	n.s.	17.9 ± 0.88	16.85 ± 0.76	0.352
3	1.34 (61)	n.s.	15.2 ± 1.36	16.3 ± 1.68	0.607
4	1.14 (77)	n.s.	10.6 ± 1.44	10.8 ± 1.97	0.756
5	1.39 (86)	n.s	18.1 ± 1.46	17.4 ± 1.72	0.498
6	0.95 (41)	n.s.	5.7 ± 1.16	13.8 ± 2.83	0.027
7	0.81 (105)	n.s.	11.8 ± 1.25	13.5 ± 1.12	0.408

The deviation from 1:1 sex ratio was determined with a binomial test and the emergence timing of males and females was compared with a Mann–Whitney U test (except for scape 3, which could be analyzed with a t test)
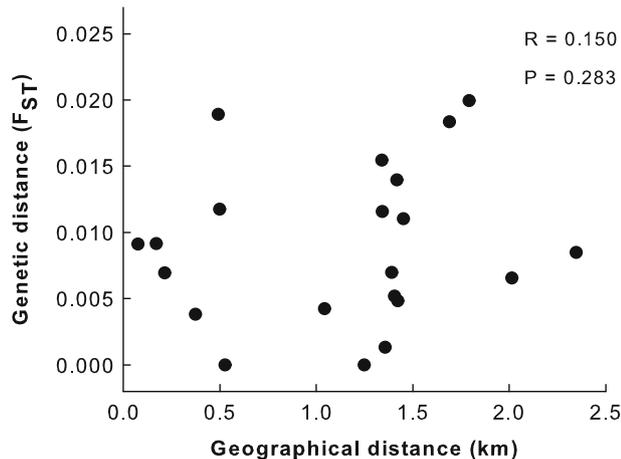

Fig. 3 Isolation by distance among alates from seven traps in the 2013 swarming event for males and females together. Non-significant isolation by distance was found. The P and R values were obtained from a Mantel test

Testing for dispersal and for sex-biased dispersal during a swarming event as indirect mechanisms of inbreeding avoidance

Siblings were found in traps as distant as 2.35 km, a distance comparable to that flown by alates of other termite species (Vargo and Husseneder 2011). Differences in mean genetic distance between pairs of traps were found not to be significantly correlated with their geographical distance (Mantel test: $R = 0.150$; $P = 0.283$; Fig. 3).

Neither genetic differentiation (F_{ST}), inbreeding index (F_{IS}), relatedness degree (r) nor assignment index correction (AIc) showed differences between sexes (Table 3).

Testing for overall efficiency of indirect mechanisms of inbreeding avoidance

The overall expected inbreeding to outbreeding ratio was 0.057, i.e., for each one hundred putative encounters between de-alate pairs only five are expected to be inbreeding encounters. Additionally, alates collected during the 2013 swarm (270 individuals) were inferred from allele data to come from 105 different colonies.

Active mechanism of inbreeding avoidance

The analysis of bioassays showed non-significant differences in the behavior of a de-alate towards a nestmate or a non-nestmate de-alate in all behavioral variables assessed, both when the focal de-alate was a male or a female (Table 4). Moreover, genetic relatedness of pairs which

Table 3 Assessment of occurrence of sex-biased dispersal through various parameters: mean genetic differentiation (F_{ST}) between traps, inbreeding index (F_{IS}) and relatedness degree (r) within traps, and corrected assignment index (AIc) among traps are given as the expected values for the philopatric and dispersing sexes, and the observed values for females and males

	Philopatric sex	Dispersing sex	Females (mean ± SE)	Males (mean ± SE)	P value
F_{ST}	Higher	Lower	0.009 ± 0.001	0.011 ± 0.003	0.543
r	Higher	Lower	0.010 ± 0.016	−0.002 ± 0.012	0.545
F_{IS}	Lower	Higher	0.033 ± 0.025	0.045 ± 0.022	0.724
AIc	Positive	Negative	0.129 ± 0.147	−0.094 ± 0.126	0.236

Statistical differences between sexes were established using a permutation test (5000 permutations) coded in R software

Table 4 Behavioral bioassay involving a focal de-alate (male or female) confronted simultaneously with a nestmate de-alate and a non-nestmate de-alate

Focal de-alate	Behavioral parameter	Nestmate	Non-nestmate	Statistical parameter	P
Male	Time to first proctodeal palpation	11.0 ± 3.46	17.9 ± 1	$t = 1.18$	0.291
Male	Frequency of proctodeal palpations	2.57 ± 0.95	1.67 ± 0.67	$U = 8.5$	0.667
Male	Time spent in proctodeal palpations	0.30 ± 0.13	0.066 ± 0.052	$U = 4$	0.183
Male	Time to first allogrooming	5.93 ± 2.41	3.32 ± 2.10	$t = -0.83$	0.425
Male	Frequency of allogrooming	3 ± 0.93	4 ± 1.11	$U = 21$	0.463
Male	Time spent in allogrooming	1.12 ± 0.59	1.04 ± 0.50	$U = 24$	0.694
Female	Time to first proctodeal palpation	7.96 ± 4.23	7.44 ± 3.84	$t = -0.091$	0.931
Female	Frequency of proctodeal palpations	1 ± 0	1.5 ± 0.29	$U = 2$	0.533
Female	Time spent in proctodeal palpations	0.14 ± 0.12	0.24 ± 0.092	$t = 0.64$	0.560
Female	Time to first allogrooming	4.14 ± 1.36	3.39 ± 1.47	$U = 20$	0.662
Female	Frequency of allogrooming	4.8 ± 1.50	3.86 ± 1.60	$U = 12.5$	0.432
Female	Time spent in allogrooming	1.96 ± 0.9	1.26 ± 0.34	$t = 0.63$	0.543

Times in min, frequencies in min^{-1}. P values are given for the comparison between behaviors involving the nestmate and the non-nestmate

mated was not significantly different from that of pairs which did not mate (mean ± s.e.: mating pairs = 0.203 ± 0.052, non-mating pairs = 0.302 ± 0.49; $t_{58} = 1.401$; $P = 0.167$).

Nestmate or non-nestmate male and female pairs of *N. chilensis* de-alates were introduced into an artificial arena simulating their host. They all mated and laid eggs. Three months after the experiment was set up, non-significant differences were found in the number of eggs laid between nestmate (4.5 ± 0.96; mean ± SE) and non-nestmate (4.6 ± 0.81; mean ± SE) pairs ($t_9 = -0.078$; $P = 0.94$).

Discussion

In the inbreeding avoidance analyses, the significantly lower level of relatedness in PRP than in nestmate pairs shows that inbreeding avoidance mechanisms are operating in *N. chilensis*, consistent with findings on most termite species where inbreeding has been estimated through genetic analysis (Vargo and Husseneder 2011). The significantly higher relatedness of nestmates with respect to alates from both swarming events shows that indirect mechanisms of inbreeding avoidance are taking place, consistent with the inbreeding/outbreeding ratio of 0.057 found in swarming individuals caught in traps; furthermore, the non-significant differences between alates from both swarming events and PRP show that active mechanisms of inbreeding avoidance are not taking place. The non-significant differences between both swarming events studied suggest that the mean relatedness in a swarm is a phenomenon which occurs independent of the year, number of traps set and number of alates caught in them.

These preliminary conclusions were further explored. Data on emerging alates showed the absence of sex-biased production at colony level and the occurrence of male-biased emergence (protandry) in only one of the seven colonies analyzed. Furthermore, the analyses of genetic differentiation, degree of relatedness, inbreeding index and assignment index correction performed for each sex separately showed that sex-biased dispersal of alates was non-significant or at least not strong enough to be detected by these analyses (Favre et al. 1997); thus, inbreeding avoidance cannot be achieved on the basis of males from a given colony being able to disperse differently in terms of distance from females of that colony, and viceversa. Finally,

the lack of isolation by distance for both sexes together showed that alates were able to disperse for distances at least equal to the maximal distance between traps; in other words, swarming individuals within the study area were able to meet, independent of the distance between their colonies of origin. Hence, this dispersion capacity constitutes the main mechanism of inbreeding avoidance.

On the other hand, the genetic and behavioral analyses of bioassays performed in the laboratory showed conclusively the absence of nestmate recognition, and since genetic relatedness of pairs which mated was not significantly different from that of pairs which did not mate, absence of kin recognition during mating could also be concluded. Moreover, in colonies formed in the laboratory mating occurred between all pairs of de-alates, whether they corresponded to nestmates or non-nestmates, and non-significant differences were found between the number of eggs laid in the artificial colonies formed by nestmates and by non-nestmates. Thus, these laboratory experiments support the notion that recognition during swarms is not important for inbreeding avoidance.

The present results agree with those of DeHeer and Vargo (2006) who showed that the likelihood that the termites *R. flavipes* and *R. virginicus* paired with siblings was inversely related to their inferred dispersal abilities. Furthermore, Husseneder et al. (2006) found low mean relatedness of PRP of the separate-piece termite *Coptotermes formosanus*, thus suggesting inbreeding avoidance; in addition, they found that mean relatedness among alates was not different from that of PRP, and concluded that indirect mechanisms are sufficient to explain the inbreeding avoidance observed and that it is not necessary to postulate an active mechanism to explain it.

The present study explored for the first time the occurrence of all described indirect and active mechanisms of inbreeding avoidance in the founding of a new colony in a single species of termite using a variety of approaches. It showed that dispersal of alates as indirect mechanism is sufficient to explain the low levels of inbreeding found in PRP of *N. chilensis* and provided strong support to the idea that kin recognition is not involved in inbreeding avoidance during the founding of a new colony in a single-piece termite.

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