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Clinal variation in colony breeding structure and level of inbreeding in the subterranean termites *Reticulitermes flavipes* and *R. grassei*

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Abstract

Social insects exhibit remarkable variation in their colony breeding structures, both within and among species. Ecological factors are believed to be important in shaping reproductive traits of social insect colonies, yet there is little information linking specific environmental variables with differences in breeding structure. Subterranean termites (Rhinotermitidae) show exceptional variation in colony breeding structure, differing in the number of reproductives and degree of inbreeding; colonies can be simple families headed by a single pair of monogamous reproductives (king and queen) or they can be extended families headed by multiple inbreeding neotenic reproductives (wingless individuals). Using microsatellite markers, we characterized colony breeding structure and levels of inbreeding in populations over large parts of the range of the subterranean termites *Reticulitermes flavipes* in the USA and *R. grassei* in Europe. Combining these new data with previous results on populations of both species, we found that latitude had a strong effect on the proportion of extended-family colonies in *R. flavipes* and on levels of inbreeding in both species. We examined the effect of several environmental variables that vary latitudinally; while the degree of inbreeding was greatest in cool, moist habitats in both species, seasonality affected the species differently. Inbreeding in *R. flavipes* was most strongly associated with climatic variables (mean annual temperature and seasonality), whereas nonclimatic variables, including the availability of wood substrate and soil composition, were important predictors of inbreeding in *R. grassei*. These results are the first showing that termite breeding structure is shaped by local environmental factors and that species can vary in their responses to these factors.

Keywords: Isoptera, microsatellites, neotenics, population genetics, Rhinotermitidae, social organization

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Introduction

Societies of eusocial insects are characterized by a reproductive division of labour, in which one or few individuals specialize in reproduction (queens in the Hymenoptera, that is, ants, bees and wasps; kings and queens in termites) while being supported by nonreproducing workers who tend the reproductive castes and rear the brood. Genetically, the simplest colonies consist

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of families headed by a single queen mated to a single male. The high degree of relatedness in such simple-family colonies maximizes indirect fitness benefits among colony members, thereby promoting highly cooperative behaviour and reproductive altruism (Hamilton 1964a,b). However, many social insect species deviate from simple families in ways that profoundly affect the genetic structure of colonies. In fact, there is a tremendous variation within and among social insect species in all components of colony breeding structure: the number of reproducing individuals, the number of mates they have, the degree of relatedness among them and their relative reproductive output (Ross & Keller 1995; Ross 2001). Documenting the variation in social insect breeding structure and identifying its underlying causes has been a major goal of students of social evolution.

Colony breeding structure in social insects is jointly shaped by intrinsic social forces related to group living and extrinsic ecological pressures (Hölldobler & Wilson 1977; Herbers 1993; Bourke & Franks 1995; Ross & Keller 1995; Foster *et al.* 2006; Korb & Heinze 2008). Most studies of colony breeding structure have focused on kin composition and its consequences for cooperation and conflict among group members, primarily in the Hymenoptera (reviewed in Bourke & Franks 1995; Ross & Keller 1995; Pamilo *et al.* 1997; Queller & Strassmann 1998; Ross 2001). The role of ecological factors in shaping social insect breeding structure has received far less empirical study.

The two breeding structure components that have received the most study from an ecological perspective are queen number in ants and multiple vs. single mating in ants and bees. Constraints on the ability of dispersing queens to successfully found independent colonies are a primary factor promoting polygyny (the presence of multiple cohabiting queens) within ant colonies by favouring queens to join established colonies or to remain in the natal nest. The ecological constraints hypothesis is supported by both theoretical work (Pamilo 1991; Nonacs 1992) and empirical studies showing that the number of queens within colonies is greater in areas with higher population densities and/or limited nest sites (Herbers 1986; Heinze 1993; Bourke & Heinze 1994; Pedersen & Boomsma 1999; Molet *et al.* 2008).

Multiple mating (polyandry) by hymenopteran queens increases intracolonial genetic diversity. It has been hypothesized that greater genetic diversity among the worker force may be beneficial because it dilutes the negative effects of genetically incompatible matings, increases the efficiency of division of labour in colonies and improves resistance to pathogens and parasites (Boomsma & Ratnieks 1996; Crozier & Fjerdingstad 2001; Oldroyd & Fewell 2007). Recently, Corley & Fjerdingstad (2011) found a latitudinal cline in European populations of the ant *Lasius niger* with southern populations exhibiting higher levels of polyandry and greater levels of genetic diversity than northern populations. These results show a clear effect of environment on mating frequency and are consistent with a model predicting higher levels of multiple mating in more mild, predictable environments (Rueppell *et al.* 2008).

Termites (Isoptera), although much less studied than the Hymenoptera, also show exceptional diversity in colony breeding structure (reviewed in Vargo & Husseneder 2011). Most of this diversity depends on the number of reproductives (queens and kings) within colonies (Thorne 1985). The presence of multiple reproductives within termite colonies either increases or decreases within-colony genetic diversity, depending on whether multiple same sex reproductives originate from inside or outside the colony. Multiple reproductives are common in the subterranean termites (Rhinotermitidae) where neotenics (nonwinged precocious reproductives) are widespread (Myles 1999). In many species, colony breeding structure changes as the colony ages (Shellman-Reeve 1997; Thorne *et al.* 1999; Vargo & Husseneder 2009). Colonies generally start as simple families headed by monogamous pairs of male and female primary (winged adult) reproductives which pair together during nuptial flights. As colonies age, the primary reproductives are succeeded by their neotenic offspring who engage in inbreeding. In addition, colonies can sometimes form mixed families containing offspring from multiple unrelated same sex reproductives which may come together through fusion of two or more colonies (DeHeer & Vargo 2004; Perdereau *et al.* 2010). Despite the high level of variation within subterranean termites, the factors shaping colony breeding structure in this group are poorly known.

Studies of two species of *Reticulitermes* suggest local environmental conditions may affect the numbers and types of breeders within colonies. Results on the North American species, *R. flavipes,* indicate possible clinal variation in breeding structure. Bulmer *et al.* (2001) studied populations in Massachusetts (42.45°N) located near the northern edge of the range of this species. Further south, *R. flavipes* has received extensive study where colony breeding structure has been particularly well characterized (reviewed in Vargo & Husseneder 2009). These latter studies, which involved some 150 colonies from several populations in central North Carolina (35.82°N) and one population in South Carolina (32.80°N), showed remarkable uniformity in colony breeding structures and high proportions of simple-family colonies. Compared to the populations studied in Massachusetts, the North and South Carolina populations were less inbred. The southern colonies are

characterized by higher frequencies of simple-family colonies (75% compared to 45% in Massachusetts), fewer neotenic reproductives in the extended-family colonies, and a much smaller percentage of mixed-family colonies (2% compared to 14%). In Europe, there is evidence of potential latitudinal variation in the colony breeding structure of *R. grassei*. Among three populations studied in France (DeHeer *et al.* 2005), the northernmost population (La Coubre Forest, 45.78°N) was composed exclusively of extended families, whereas more southerly populations (44.34 and 44.29°N) had between 26.7 and 43.7% simple families. Even further south in Portugal (38.11°N), Nobre *et al.* (2007) found that 46.7% of colonies were simple families.

In this study, we tested the hypothesis that colony breeding structure exhibits latitudinal variation in *R. flavipes* and *R. grassei*, two closely related subterranean termites that occur on separate continents. To accomplish this, we characterized the breeding structure in six populations of *R. flavipes* spanning a large part of the range of this species in the eastern USA. We also included data from previous studies of four populations in other parts of its range. In addition, we studied three populations of *R. grassei* from Spain and combined these data with results of previous studies on populations from France (DeHeer *et al.* 2005) and Portugal (Nobre *et al.* 2007). We found strong clinal variation in colony breeding structure and levels of inbreeding in both species. We further investigated the relationship between breeding structure and several environmental factors that vary geographically. Our results show that moist, cool conditions were associated with greater degrees of inbreeding in both species but also show differences between species in response to other variables such as seasonality.

Materials and methods

Sample collection

Samples of *Reticulitermes* spp. workers and soldiers were taken from natural wood debris (downed limbs, tree trunks and stumps) in undisturbed and managed forests from the eastern USA and Spain. Each sample consisted of at least 20 workers (but generally many more) and a few soldiers (and in one case a primary queen), taken from a small section of decayed wood, usually no more than a few square centimetres in size. Immediately upon collection, each sample was placed in a vial containing 95% ethanol. The ethanol in each vial was replaced and any debris removed 24–48 h after collection. Vials were then kept at 4 °C until DNA extraction.

In general, sampling was conducted along a more or less linear transect up to 1 km in length with at least 15 m between collection points. This 15-m spacing between samples was based on the results of previous studies showing that samples of *R. flavipes* in central North Carolina were nearly always from different colonies (Vargo 2003b; DeHeer & Vargo 2004; DeHeer *et al.* 2005; Vargo & Carlson 2006). Similarly, collections of *R. grassei* in France at this interval have been found to be largely different colonies (DeHeer *et al.* 2005).The number of collections per site was generally between 20 and 30, yielding between 14 and 37 colonies of the target species (Table 1). The number of colonies of each target species sampled was usually lower than the number of samples collected due to individual colonies being represented across multiple collection sites or the presence of other species of *Reticulitermes* (see below).

Reticulitermes flavipes *collection sites*

Samples of *Reticulitermes* spp. were collected at six locations between northern Delaware and southeastern North Carolina (Fig. 1; GPS coordinates given in Table 1), representing the three major biogeographic regions in the mid-Atlantic and Southeastern USA: the Blue Ridge Mountains, the Piedmont and the Coastal Plain. Our northernmost samples came from White Clay Creek State Park and Lums Pond State Park in northern Delaware and were collected in September 2002. The two parks are located approximately 25 km apart. Samples from these two sites were not genetically differentiated [F_{ST} not significantly different from zero, as determined by the program Genetic Data Analysis v. 1.1 (Lewis & Zaykin 2000; available at http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php)] and were therefore combined into a single population designated DE.

We collected samples from three sites in Virginia in September and October 2002, spanning a large geographic area. The western most samples came from Fenwick Mines (FM) located in the mountainous area near the West Virginia border. The northernmost site was in Mason Neck State Park (M) located along the Potomac River approximately 35 km south of Washington, DC. The third site was located in the Coastal Plain near the southeastern city of Suffolk (S).

We sampled two sites in North Carolina. In the Blue Ridge Mountain region, we collected samples in August 2000 and September 2002 from the forests surrounding North Carolina State University's Mountain Horticultural Crops Research and Extension Center located near Fletcher (FT). In the Coastal Plain region, we collected samples in July 2000 from Bladen Lakes State Forest located near Elizabethtown in the southeastern corner of the state (BF).

Table 1 Locations, colony compositions and levels of inbreeding for the study populations of *Reticulitermes flavipes* and *R. grassei*, including both new newly studied populations characterized here and previously studied populations incorporated in the analyses

Location	Population designation	Latitude	Longitude	No. colonies	No. simple families	No. extended families	No. mixed families	Prop extended families	F_{IT}	Reference
USA populations of *R. flavipes*										
Bladen Forest, North Carolina	BF	34.75	−78.543117	20	17	3	0	0.150	0.037	This study
Fletcher, North Carolina	FT	35.429414	−82.566506	37	28	9	0	0.243	0.112	This study
Fenwick Mines, Virginia	FM	37.619267	−80.261942	14	9	5	0	0.357	0.110	This study
Suffolk, Virginia	S	36.7282	−76.584169	20	14	3	3	0.150	0.051	This study
Mason Neck State Park, Virginia	M	38.639914	−77.192208	18	9	9	0	0.500	0.060	This study
Lum's Pond and White Clay Creek State Park, Delaware	DE	39.663714	−75.757681	29	16	12	0	0.414	0.086	This study
Raleigh, North Carolina	R	35.817939	−78.724264	319	250	63	6	0.190	0.037	Vargo (2003a,b), DeHeer & Vargo (2004), Vargo & Carlson (2006) and Parman & Vargo (2008)
Middlesex Fells, Massachusetts	MF	42.450703	−71.104872	22	6	13	3	0.590	0.289	Bulmer *et al.* (2001)
Charles Towne Landing State Historic Site, South Carolina	CT	32.801811	−79.983136	18	13	4	2	0.222	0.030	Vargo *et al.* (2006)
Lincoln, Nebraska	LN	40.742	−96.708	7	0	5	2	0.714	0.443	DeHeer & Kamble (2008)
European populations of *R. grassei*										
La Coubre Forest, Dépt. Charente-Maritime, France	A	45.78225	−1.230166	24	0	24	0	1	0.294	DeHeer *et al.* (2005)
Ychoux, Dépt. Landes, France	B	44.345669	−1.01996	15	4	11	0	0.733	0.306	DeHeer *et al.* (2005)
Pissos, Dépt. Landes, France	C	44.299306	−0.78112	32	14	18	0	0.562	0.211	DeHeer *et al.* (2005)

Table 1 *Continued*

Location	Population designation	Latitude	Longitude	No. colonies	No. simple families	No. extended families	No. mixed families	Prop extended families	F_{IT}	Reference
Embalse de la Torre de Abraham, Cuidad Real Prov., Spain	D	39.3715	−4.217759	28	23	5	0	0.179	0.007	This study
Granada, Granada Prov., Spain	F	37.316762	−3.389801	27	10	16	1	0.593	0.177	This study
Cantalejo, Segovia Prov., Spain	G	41.217171	−3.935397	30	9	20	1	0.667	0.202	This study
Beja, Portugal	BP	38.11	−8.04	15	5	8	2	0.533	−0.020	Nobre *et al.* (2008)

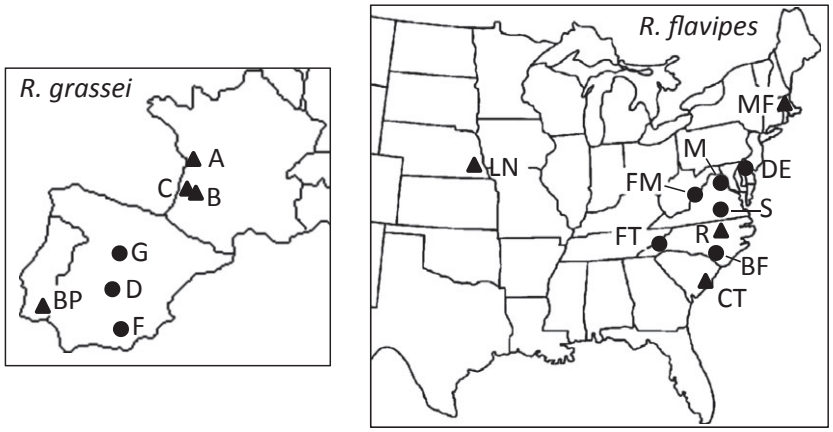

Fig. 1 Location of *Reticulitermes grassei* and *R. flavipes* populations sampled. Circles show populations sampled for the present study; triangles show populations from previous studies included in the geographic analyses. Population abbreviations are given in Table 1.

Reticulitermes grassei *collection sites*

Samples of *R. grassei* were collected from three locations in Spain during May and June 2004 (Fig. 1; see Table 1 for GPS coordinates): Site D was located near the Embalse de la Torre de Abraham, in the Province of Cuidad Real, some 55 km SSW of Toledo; site F was situated in the mountains between Granada and Guadix in the Province of Granada; and Site G was located about 5 km SSW of the town of Cantalejo in the Province of Segovia.

Genotyping

DNA was extracted from individual workers using either the Qiagen DNeasy kit or the Puregene DNA isolation kit (Gentra Systems, Inc. Minneapolis, MN, USA). For the USA samples, at least one individual per sample was used for species identification according to the PCR-RFLP method of Szalanski *et al.* (2003). For the samples from Spain, we determined species identity by comparing microsatellite alleles known to be diagnostic for *R. grassei* with those known to be diagnostic for *R. banyulensis*, the only other *Reticulitermes* species reported to occur in this region (Kutnik *et al.* 2004; DeHeer *et al.* 2005). For *R. flavipes*, we genotyped 20 individuals per collection site at each of eight microsatellite loci, six from Vargo (2000) (*Rf1-3*, *Rf5-10*, *Rf6-1*, *Rf15-2*, *Rf21-1* and *Rf24-2*) and two from Dronnet *et al.* (2004) (*Rs10* and *Rs15*). For *R grassei*, we also genotyped 20 workers per collection site, and these were scored at 10 microsatellite loci: *Rf6-1*, *Rf15-2*, *Rf24-2* and *Rf21-1* from Vargo (2000); *Rs10*, *Rs15*, *Rs62*, *Rs76* and *Rs78* from Dronnet *et al.* (2004); and *Rs1* from DeHeer *et al.* (2005). Our PCR protocols followed those of Vargo (2000), Dronnet *et al.* (2004) and DeHeer *et al.* (2005) in which PCR products are labelled with either IRD 700 or IRD 800 fluorescent dye. We determined genotypes by running the PCR products on 6% polyacrylamide gels on a Li-Cor 4300 DNA analyser and scoring allele sizes relative to a 50- to 350-bp standard.

Determination of colony affinity and measures of genetic diversity

To determine the colony identity of samples within a given site, workers from each collection point were tested against workers from all other collection points for genotypic differentiation using the program GENEPOP on the Web (Raymond & Rousset 1995; available at http://wbiomed.curtin.edu.au/genepop/). Pairs of collection points that were not significantly differentiated were grouped together into the same colony. Of the 113 *R. flavipes* samples collected, in only three cases were different collection points grouped into the same colony; in all three cases, adjacent collection points had identical alleles and genotypes at all eight loci. Thus, between 14 and 37 different colonies were characterized per population (Table 1). Similarly, the 90 samples of *R. grassei* collected were determined to belong to 85 colonies (27–30 colonies per site; see Table 1). The numbers of colonies of each species from the study sites are given in Table 1. All samples collected from a given study site were considered part of the same population. Basic descriptive statistics of the microsatellite markers (mean number of alleles per locus, mean allelic richness and mean expected heterozygosity) were estimated for each population using FSTAT v.2.9.3.2 (Goudet 2001) from the genotype of one individual per colony.

Determination of colony breeding structure

Analysis of colony breeding structure was performed in two stages. First we determined colony family type through pedigree analysis of worker genotypes. Colonies were considered 'simple families' if the worker genotypes at all loci conformed to those expected among the offspring of a single monogamous pair of reproductives and if the frequencies of the genotypes did not deviate significantly from expected ($P < 0.05$). Significance of genotype frequencies was determined by performing *G*-tests comparing expected and observed frequencies for each locus and then summing these locus-specific values to obtain an overall *G*-value. Colonies were considered 'extended families' after Vargo *et al.* (2003) and DeHeer & Vargo (2004) if they met the following criteria: there were no more than four alleles at a locus and they either contained genotypes inconsistent with a single pair of parents (e.g. five genotypes or three classes of homozygotes) or the frequencies of genotypes deviated significantly from those expected in simple families. Extended-family colonies were consistent with inbred colonies descended from simple-family colonies, presumably headed by multiple inbreeding neotenics (by themselves or together with one or more of the original colony founders). Finally, a few colonies of each species had >4 alleles at a locus indicating a more complex genetic structure than would be possible in colonies consisting of the inbred descendants of a monogamous pair. Such colonies can arise through fusion of one or more colonies and were considered 'mixed families' (DeHeer & Vargo 2004, 2008).

The second step in the analysis of colony breeding structure was to estimate the components of genetic variation partitioned among three levels (Thorne *et al.* 1999; Bulmer *et al.* 2001; Vargo & Husseneder 2009, 2011): the individual (I), the colony (C) and the total population (T). F_{IT} is a measure of the degree of inbreeding within individuals relative to others in the population (i.e. observed heterozygosity in individuals compared to the expected heterozygosity based on the allele frequencies in the population at large). In social insects such as termites, it is equivalent to the standard inbreeding coefficient F_{IS} in solitary species (Vargo & Husseneder 2011). F_{IC} is a measure of the level of inbreeding in individuals relative to others in the same colony and is very sensitive to the colony breeding structure, especially in extended-family colonies (Thorne *et al.* 1999). F_{CT} is similar to F_{ST} but represents the degree of differentiation among colonies rather than between populations. For this analysis, we used FSTAT to estimate the various *F*-components for all colonies within a site and for simple and extended families separately. Standard errors were generated by bootstrapping over loci.

Effect of latitude and bioclimatic variables on breeding structure and inbreeding

To expand the geographic range in these analyses, we included data for previously studied populations of *R. flavipes* and *R. grassei*. We included data for *R. flavipes* from Raleigh, North Carolina (Vargo 2003a,b; DeHeer & Vargo 2004; Vargo & Carlson 2006; Parman & Vargo 2008), Charleston, South Carolina (Vargo *et al.* 2006), Middlesex Fells, Massachusetts (Bulmer *et al.* 2001) and Lincoln, Nebraska (DeHeer & Kamble 2008). Together, these samples cover much of the latitudinal range of this species along the east coast of the USA, although *R. flavipes* does occur as far south as the tip of Florida (7° latitude south of our southernmost collection point in Charleston, South Carolina). Previous data for *R. grassei* came from three populations from southwestern France, one from La Coubre Forest in the Département of Charente-Maritime, and two from the Département of Landes (DeHeer *et al.* 2005), as well as one population from Beja, Portugal (Nobre *et al.* 2008). Altogether, these sites spanned nearly the entire natural latitudinal range of *R. grassei* (GPS coordinates are given in Table 1). From these previous studies, we obtained the proportion of extended-family colonies

and the F_{IT} values. In cases where multiple values were reported, either because more than one study had been conducted on the same population or results from two nearby sites were reported separately, we combined the results into a single data set. In such cases, F_{IT} was calculated by averaging across studies (or sites) and weighted by the number of colonies in each study or site. The values used in the analyses are given in Table 1.

We examined several bioclimatic variables for each of the study sites (Table 2). At the location where samples for each species were collected, we estimated the climatic variables of mean annual temperature, temperature annual range, temperature seasonality (the average intra-annual variation in mean monthly temperature), and annual precipitation, by extracting them from the WorldClim Global Climate Data (http://www.worldclim.org/) at 10-by-10 degree grid cell resolution (Hijmans *et al.* 2005). We also investigated potential and actual evapotranspiration, the aridity index (Trabucco & Zomer 2009, 2010), net primary productivity, water balance (Foley *et al.* 1996; Kucharik *et al.* 2000), standing plant biomass (Ruesch & Gibbs 2008) and soil characteristics (% sand, % silt and % clay; FAO/IIASA/ISRIC/ISSCAS/JRC 2012).

To investigate the relationship between breeding structure and environmental variables, we used generalized least squares (maximum likelihood) to model colony breeding structure (proportion of extended families and overall level of inbreeding, F_{IT}) as a function of the various bioclimatic variables. We did not include the proportion of mixed-family colonies in our analysis because (i) such colonies were few in number in the studied populations and are relatively uncommon in subterranean termites and other termites generally (Vargo & Husseneder 2011); (ii) mixed-family colonies can be a temporary condition resulting from the fusion of two or more colonies with eventual reversion to either a simple-family or an extended-family colony (Fisher *et al.* 2004, unpublished data); and (iii) mixed-family colonies in subterranean termites most probably represent a second-order condition, arising through the fusion of two or more simple-family or extended-family colonies with little or no effect on the degree of inbreeding in individuals relative to their population, F_{IT} (Thorne *et al.* 1999). Models of breeding structure and inbreeding accounted for spatial autocorrelation by incorporating a Gaussian spatial correlation structure. When appropriate, response variables were transformed to satisfy model assumptions of normality: F_{IT} was arcsine square-root-transformed (with a +0.2 offset due to the presence of some negative values of F_{IT}). We used an information-theoretic approach to select the most probably models for predicting the breeding structure, given the available environmental and climatic data (Burnham & Anderson 2002).

The best-fitting models were selected on the basis of AICc (Akaike's information criterion corrected for small

Table 2 Bioclimatic variables investigated in this study to test for their association with colony breeding structure and levels of inbreeding

Variable	Reference	Notes
Annual mean temperature (°C)	WorldClim (http://www.worldclim.org/bioclim)	
Maximum temperature (°C)	WorldClim (http://www.worldclim.org/bioclim)	
Minimum temperature (°C)	WorldClim (http://www.worldclim.org/bioclim)	
Temperature annual range	WorldClim (http://www.worldclim.org/bioclim)	Minimum temperature minus maximum temperature
Temperature seasonality	WorldClim (http://www.worldclim.org/bioclim)	Coefficient of variation of mean monthly temperatures
Annual precipitation	WorldClim (http://www.worldclim.org/bioclim)	
Precipitation seasonality	WorldClim (http://www.worldclim.org/bioclim)	Coefficient of variation of mean monthly precipitation
Potential evapotranspiration	Trabucco & Zomer (2009)	
Actual evapotranspiration	Trabucco & Zomer (2010)	
Aridity index	Trabucco & Zomer (2010)	An index of the average water available in the soil, defined as the ratio between mean annual precipitation and mean annual evapotranspiration
Net primary productivity	Foley *et al.* (1996) and Kucharik *et al.* (2000)	
Water balance	Foley *et al.* (1996) and Kucharik *et al.* (2000)	
Vegetation biomass	Ruesch & Gibbs (2008)	
Soil composition	(FAO/IIASA/ISRIC/ISSCAS/JRC 2012)	Sand, silt and clay percentages are weighted averages

sample sizes). Models with ΔAICc's <7 (ΔAICc being defined as the difference between the AICc of the current model and the minimum AICc in the entire pool of models) comprised the best-fitting model subset. After identifying the best-fitting models, ΔAICc's were recalculated based on this subset; corresponding Akaike weights (the probability that a given model, m_i, out of i alternative models is the best model given the data; see Burnham & Anderson 2002) are therefore based on recalculated ΔAICc's. We accounted for uncertainty in the models in the best-fitting model subset by performing model averaging: estimates of each parameter were averaged across the best-fitting models (means were weighted by the Akaike weight of a given model). Unconditional standard errors were also computed for model-averaged estimates (Buckland *et al.* 1997).

For each of the two response variables (proportion of extended families and F_{IT}), model selection was performed on an initial pool of models potentially containing all combinations of the following terms, considered as fixed effects (including an intercept-only model): species, seasonality, vegetation biomass (a proxy for availability of wood substrate), mean annual temperature, aridity, soil composition (we focused on % sand as an estimate of soil water retention), and all two- and three-way interactions between species, seasonality and vegetation biomass. However, some higher-order interaction terms were dropped prior to model selection: initial parameter selection for inclusion in the model selection process was based on maximizing the strength of the relationship with breeding structure response variables while minimizing potential multicollinearity with other predictors. Marginality of interaction terms was conserved during model selection (main effects of all interaction term components were included). Given the substantial contribution of species identity to breeding structure (see Results), we subsequently performed model selection separately for each species on an initial pool of models containing all terms listed above (except species and its interactions with other terms). Model fitting and model selection were performed in R (R Development Core Team 2011).

Results

Descriptive statistics of microsatellite variability

Across the eight loci studied in *R. flavipes*, we detected a mean of 7.9 alleles per locus in the six newly studied populations (range = 6.0–8.9). Allelic richness averaged 6.8 alleles per locus with a range of 5.9–7.7 per population, and mean gene diversity was 0.699 with a range of 0.659–0.749. The mean values for the 10 loci used in *R. grassei* were slightly lower. Mean allele number for the three *R. grassei* populations was 4.7 alleles per locus (range = 4.4–5.3), and mean allelic richness was 3.9 alleles per locus (range = 3.4–4.4). Gene diversity averaged 0.624 with a range of 0.515–0.713.

Colony breeding structure

Across the six newly studied populations of *R. flavipes*, simple families were the most common, comprising from 55% of all colonies in the Delaware site to 85% in Bladen Forest, North Carolina (Table 1). All other colonies were extended families, except for four mixed-family colonies (three from the Suffolk, Virginia population, and one from the Lums Pond, Delaware population). Correspondingly, the levels of inbreeding of individuals varied from F_{IT} = 0.04–0.11 in the Bladen Forest, North Carolina population and both the Fletcher, North Carolina and FM, Virginia populations, respectively (Table 1). When these new data were combined with all previous data for this species, the new values were at the lower end of the complete spectrum of reported values, with the proportion of extended families ranging up to 0.71 and F_{IT} values up to 0.44 in a Lincoln, Nebraska population (DeHeer & Kamble 2008) located considerably west of the other populations investigated in the present study.

The three populations of *R. grassei* (D, F and G) also varied in colony breeding structure, ranging from 18 to 67% extended families in populations D and G, respectively, with the remaining colonies all simple families except for a single mixed-family colony in each of populations F and G (Table 1). Inbreeding levels differed strongly as well, ranging between F_{IT} = 0.01 and 0.20 in populations D and G, respectively (Table 1). These values were intermediate relative to those reported for the previously studied populations, which were primarily located north or west of the sites studied here (Table 1). More complete analysis of *F*-statistics and levels of within-colony relatedness are shown in Table S1 (Supporting information).

The proportion of extended-family colonies within a population was significantly correlated with F_{IT} values in both *R. flavipes* ($R^2 = 0.71$, $F_{1,8} = 19.17$, $P < 0.0025$) and *R. grassei* ($R^2 = 0.61$, $F_{1,5} = 7.68$, $P < 0.04$) (Fig. 2). As described in the 'Materials and methods', we accounted for spatial autocorrelation among these variables in subsequent analyses.

Effect of latitude and bioclimatic variables on breeding structure and inbreeding

To look for possible relationships between colony breeding structure and geographic location, we performed

regression analysis between latitude and the proportion of extended-family colonies in a population. As shown in Fig. 3, there was a strong and significant effect of latitude on the proportion of extended families across the 10 populations of *R. flavipes* ($R^2 = 0.73$, $F_{1,8} = 21.27$, $P < 0.002$). This relationship was weaker and not significant across the seven *R. grassei* populations ($R^2 = 0.39$, $F_{1,5} = 3.20$, $P > 0.13$). However, when the level of inbreeding within individuals relative to their populations, F_{IT}, was regressed against latitude, the relationship was significant in both species ($R^2 = 0.54$, $F_{1,8} = 9.25$, $P < 0.02$ and $R^2 = 0.57$, $F_{1,5} = 6.61$, $P < 0.05$, in *R. flavipes* and *R. grassei*, respectively), indicating a geographical gradient with increasing levels of inbreeding from south to north.

After accounting for spatial autocorrelation in models of the breeding structure, we found climatic and biotic variables to be important predictors of both the proportion of extended families and F_{IT} (Table 3, Fig. 4). In general, the degree of inbreeding was greatest in cool, moist environments. However, the degree of inbreeding was differentially associated with seasonality in the two species: *R. grassei* exhibited greater inbreeding in less seasonal environments (i.e. those showing less fluctuation in mean monthly temperature), whereas *R. flavipes* exhibited greater inbreeding in more seasonal environments (as detected by the presence of interaction terms with species in the best-fitting models, Table 3, Fig. 4; also compare coefficients for seasonality in separate models of inbreeding for *R. flavipes* vs. *R. grassei*, Tables S2 and S3, Supporting information). Furthermore, whereas inbreeding in *R. flavipes* was most strongly influenced by climatic variables (mean annual temperature and seasonality), additional nonclimatic variables, including soil composition (% sand) and vegetation biomass (the availability of wood substrate), were important predictors of inbreeding in *R. grassei*. In particular, inbreeding in *R. grassei* was greater in soils with lower sand content, that is, less well-drained soils. The relationship between inbreeding in *R. grassei* and vegetation biomass was more complex, and tended to depend on the degree of temperature seasonality in the region. The coefficient for the main effect of vegetation biomass was significant (the 95% confidence interval for the coefficient did not overlap zero) and negative in the model of F_{IT}, and trended negative in the model of the proportion of extended families. However, in the model of the proportion of extended families, the interaction between seasonality and vegetation biomass, while not significant, trended positive, suggesting higher inbreeding in regions that were both increasingly seasonal and contained greater vegetation biomass. Indeed, the proportion of extended families appeared to be greatest at intermediate combinations of seasonality and vegetation biomass (Fig. 4). The model of F_{IT} suggested a similar pattern; while not part of the best-fitting model subset, the interaction between vegetation biomass and seasonality trended positive, such that inbreeding was highest in more moderate environments (intermediate values of vegetation biomass and seasonality), and lowest in more extreme environments (high or low-valued combinations of vegetation biomass and seasonality). Together, these results suggest that while inbreeding in *R. grassei* tends to be higher in areas with lower availability of wood substrate, seasonal variation in temperature may impact the magnitude and/or direction of this relationship.

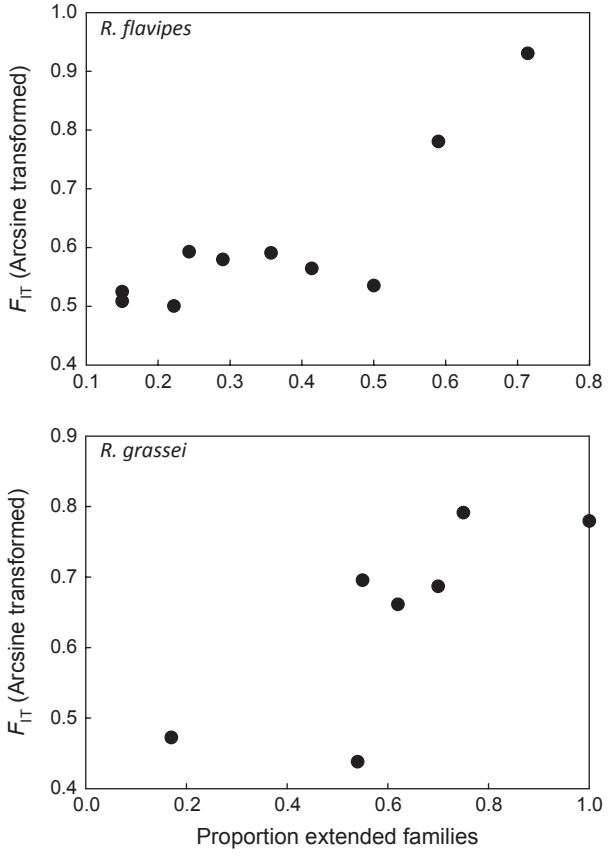

Fig. 2 Relationship between breeding structure and F_{IT} in the study populations of *Reticulitermes flavipes* ($n = 10$) and *R. grassei* ($n = 7$).

Discussion

In this study, we documented clinal variation in colony breeding structure and level of inbreeding in two widely distributed subterranean termite species occurring on separate continents. Our results, which combine new genetic data with information from several previous studies, show a strong association between bioclimatic variables and two key components of colony breeding structure: the numbers of reproductives within

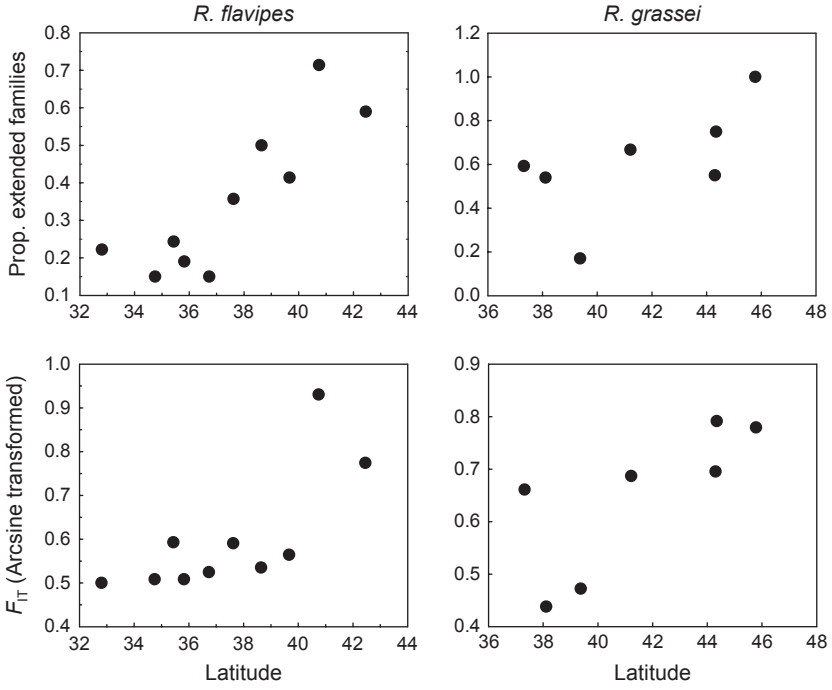

Fig. 3 Effect of latitude on proportion of extended families and levels of inbreeding (F_{IT}) in populations of *Reticulitermes flavipes* in the USA and *R. grassei* in Europe.

Table 3 Model-averaged parameter estimates, standard errors, and 95% confidence intervals based on the best-fitting model subset for colony breeding structure responses (proportion extended families and F_{IT})

Response	Parameter*	Relative importance†	β_{MA}	SE_{MA}	95% CI_{MA}
Prop. extended family	Intercept	1.00	−0.766	0.364	−1.60, 0.0644
	Seasonality	1.00	0.0129	0.00424	0.00332, 0.0225
	Soil comp.	0.86	0.00238	0.00281	−0.00399, 0.00876
	Species‡	1.00	1.31	1.34	−1.72, 4.34
	Veg. biomass	0.86	−0.0156	0.0124	−0.0431, 0.0118
	Species*Seasonality	1.00	−0.00820	0.0258	−0.0662, 0.0498
	Species*Veg. biomass	0.86	0.0535	0.0494	−0.0573, 0.164
	Seasonality*Veg. biomass	0.86	0.000198	0.000164	−0.000168, 0.000565
	Species*Seasonality*Veg. biomass	0.86	−0.000998	0.000969	−0.00318, 0.00118
F_{IT}§	Intercept	1.00	0.243	0.109	0.0298, 0.457
	Aridity	1.00	0.626	0.0785	0.472, 0.780
	Mean temp.	1.00	−0.0415	0.00471	−0.0507, −0.0323
	Species	1.00	0.0319	0.108	−0.181, 0.244
	Veg. biomass	1.00	0.000910	0.000330	0.000264, 0.00156
	Species*Veg. biomass	1.00	−0.00382	0.00176	−0.00727, −0.000362

*For each parameter, model-averaged coefficients (β_{MA}), standard errors (SE_{MA}), and 95% confidence intervals were based on weighted means from the best-fitting models (weighted by the Akaike weight (w_i) for each model, i, in which the term occurs); only terms from the best-fitting models are provided.

†Here and subsequently, relative importance values (the sum of Akaike weights (w_i) for each model, i, in which the term of interest occurs) range from 0 to 1, with values closer to 1, indicating greater influence of the term.

‡*R. flavipes* represents the baseline level for the species term and associated interaction terms

§For F_{IT}, a single best-fitting model was identified.

colonies and the degree of inbreeding. These findings offer the first clear demonstration of environmental conditions linked to colony breeding structure in termites, and one of the few such demonstrations in social insects.

Mean annual temperature and soil moisture (aridity index) were two major environmental factors associated with the level of inbreeding within colonies of both *R. flavipes* and *R. grassei*, with cool, moist conditions favouring higher levels of inbreeding (Tables S2 and S3,

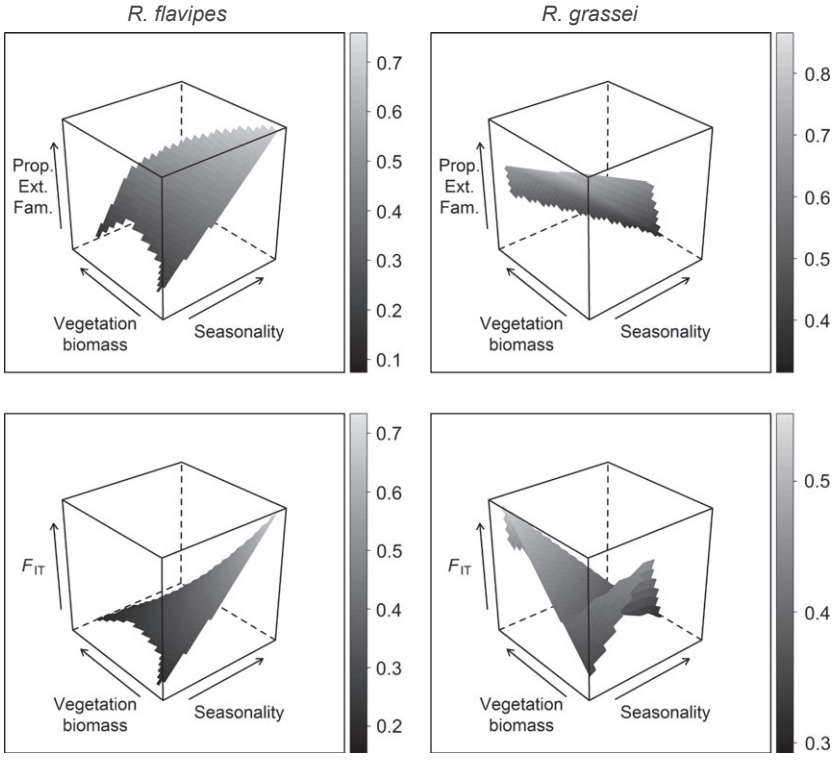

Fig. 4 Surface plots of the proportion of extended families and levels of inbreeding (F_{IT}) as functions of vegetation biomass and seasonality for *Reticulitermes flavipes* (left panels) and *R. grassei* (right panels). Greater proportions of extended families and levels of inbreeding are indicated with lighter shading of the surfaces.

Supporting information). The mechanisms by which temperature and moisture may affect colony breeding structure are not obvious, although both of these factors strongly influence foraging activity and wood consumption in subterranean termites (Haverty *et al.* 1974; Abensperg-Traun 1991; Haagsma & Rust 1995; Evans & Gleeson 2001; Houseman & Gold 2003; Su & Puche 2003; Fei & Henderson 2004; Nobre *et al.* 2008; Cornelius & Osbrink 2010, 2011; Wong & Lee 2010) and are therefore likely to be key determinants of colony growth rates and survival, especially at the colony foundation stage (Huang & Jung 1980; Fei & Henderson 2004). Temperature could possibly affect breeding structure if larger colony size (*i.e.* larger worker population) is favoured in colder climates, as demonstrated for ants (Kaspari & Vargo 1995), provided larger colonies can only be sustained by the combined egg production of multiple female neotenics (Thorne *et al.* 1999; Grube & Forschler 2004).

In the case of *R. grassei*, Clément and co-workers (reviewed in Clément *et al.* 2001) proposed an important role for temperature and moisture in determining colony breeding structure. Based on behavioural (Clément 1986) and allozyme (Clément 1981) data, these authors hypothesized that colonies in the cool, northern portion of this species' range in France and northwestern Spain, where soil moisture is high, formed 'open societies' with frequent exchange of workers and reproductives such that the termites in this area comprised one large super colony. In contrast, Clément *et al.* (2001) proposed that populations in the southern, warmer parts of its range in central and southern Spain were composed of localized colonies primarily headed by monogamous pairs of reproductives. Our results provide a measure of support for these conclusions with some important exceptions. There is indeed a trend from simple families in the southern parts of the range to extended families in the northern parts of the range. However, there is no evidence of a large super colony in the north; colonies throughout the range of this species show strong genetic differentiation from other nearby colonies (mean F_{ST} = 0.34), including three populations in France (DeHeer *et al.* 2005), the northernmost part of its distribution.

Whereas *R. flavipes* and *R. grassei* showed similar responses to mean temperature and moisture, these species evidently responded differently to other environmental and biological factors. *Reticulitermes flavipes* exhibited greater levels of inbreeding in more seasonal environments, while *R. grassei* exhibited greater inbreeding in less seasonal environments. This could reflect differences between the species in how temperature fluctuation throughout the year affects life history, for example, if more seasonal environments place greater constraints on the ability of *R. flavipes* alates to found colonies before the arrival of winter. If so, there could be greater pressure on individuals in northern populations to remain in the nest as actual or potential

neotenics than to leave the nest and attempt to found colonies independently. Alternatively, the apparent difference between the species in response to seasonality could be due to the fact that *R. grassei* has a much more limited latitudinal range and experiences only about half the seasonal variation that *R. flavipes* does. Thus, local processes may be more important in determining levels of inbreeding in *R. grassei* than in a species with greater latitudinal range such as *R. flavipes*. Also, inbreeding levels in *R. grassei* were negatively associated with wood availability (as measured by vegetation biomass) and with sand content in the soil. Low wood availability could favour larger, longer-lived colonies in *R. grassei* if the wood resource is clumped, perhaps in the form of large tree trunks and stumps. Such a situation could put a premium on holding on to resources for as long as possible, selecting for reproductive individuals to stay behind and inherit the colony and its food sources rather than disperse as alates. Soil composition could affect colony breeding structure if large colonies with numerous inbreeding neotenics require suitable moisture in the soil to maintain an extensive tunnel system. Soils with low sand content probably drain more slowly and stay moist longer, potentially promoting large colonies headed by neotenics over simple-family colonies. In contrast to *R. grassei*, neither vegetation biomass nor soil composition was a good predictor of inbreeding in *R. flavipes*.

Geographic variation in colony breeding structure has been previously reported in some other termites and in some ants. In the African termitid, *Macrotermes michaelseni*, which lacks neotenics but in which multiple primary queens (and less commonly multiple kings) can co-occur in established colonies (Darlington 1985), Brandl *et al.* (2001) found that polygynous colonies of this species were more common along the edges of its range than in the centre. These authors hypothesized that there may be selection for alates to join established colonies in marginal habitats where the chances of successfully founding new colonies is probably much lower than in more suitable habitats. However, colony breeding structure may not vary in response to ecological conditions in all termite species. For example, no variation was found in the breeding structure of the Australian mound building termite *Coptotermes lacteus* between two geographically separate and ecologically different populations (Thompson *et al.* 2007).

Studies of geographic variation in colony breeding structure in ants suggest that limitation of available nest sites or high population densities can shift colony breeding structure. Experimental manipulations demonstrated that limited nest sites favoured polygyny over monogyny in the North American species *Temnothorax* (=*Leptothorax*) *longispinosus* (Herbers 1986). Comparative studies of *Myrmica punctiventris* also indicate that polygyny is more common in populations with fewer nest sites (Banschbach & Herbers 1996), and work on *Myrmica sulcinodis* shows that queen number is higher in habitats with greater population density and nest site saturation (Pedersen & Boomsma 1999). Thus constraints on the availability of suitable nest sites to found new colonies appear to play an important role in determining colony breeding structure in some species of termites and ants. It remains to be determined whether such constraints play a role in the observed variation in breeding structure and levels of inbreeding in *R. flavipes* and *R. grassei*.

There are intriguing similarities between the latitudinal gradient in inbreeding in *R. flavipes* and *R. grassei* colonies found here and the pattern of geographic parthenogenesis reported for many plant and animal species with facultative asexual reproduction, in which asexual populations occur more often than sexually reproducing populations at higher latitudes and altitudes, in xeric environments, on islands or island-like habitats, and in disturbed habitats (Vandel 1928; Ghiselin 1974). This pattern was also evident in the present study when considering altitudinal variation in *R. flavipes*. The two populations that were near the mountains, Fletcher, North Carolina and FM, Virginia with altitudes of 646 and 457 m, respectively, had greater proportions of extended families (0.243 and 0.357, respectively), and those extended families were significantly more inbred (F_{IT} = 0.112 and 0.110, respectively), than the nearest Coastal Plain populations occurring at similar latitudes (Bladen Forest, North Carolina, altitude 41 m, prop. extended families = 0.15, F_{IT} = 0.037; and Suffolk, Virginia, altitude 5 m, prop. extended families = 0.15, F_{IT} = 0.051). Moreover, this pattern applies to the island-like populations of *R. flavipes* in France, where this species was introduced and where it forms exclusively highly inbred extended-family colonies that often undergo colony fusion (Dronnet *et al.* 2005; Perdereau *et al.* 2010). Thus, inbreeding in *R. flavipes* colonies varies geographically and in a manner very similar to that seen in facultatively parthenogenetic species.

Although the causes of geographic parthenogenesis remain controversial (see *e.g.* Lynch 1984; Peck *et al.* 1998; Haag & Ebert 2004; Kearney 2005), one of several proposed explanations has particular relevance concerning the observed clinal variation in colony breeding structure in *R. flavipes* and *R. grassei*. Various authors (Ghiselin 1974; Levin 1975; Glesener & Tilman 1978; Hamilton *et al.* 1981; Ladle 1992) have suggested that sexual reproduction may be most advantageous when pressure from biotic interactions is high due to co-evolutionary responses of predators, parasites and competitors. In contrast, abiotic interactions, according to this

scenario, are likely to dominate in sparsely inhabited environments, and in such situations asexual reproduction may have an advantage. To the extent that inbreeding depression may compromise the ability of subterranean termite colonies to cope with biotic pressures and to the extent that biotic pressures are weaker at higher latitudes, higher altitudes and in introduced habitats, then the same reasoning could apply to the pattern of geographic inbreeding observed in the present study. Recent studies have provided evidence of inbreeding depression in *R. flavipes* (DeHeer & Vargo 2006) and in the dampwood termite *Zootermopsis angusticollis* (Calleri *et al.* 2007), indicating that inbreeding can have important fitness consequences for termites.

Biotic pressure is difficult to assess, but it is reasonable to assume that the intensity of intraspecific competition is proportional to population density. Although there are few studies of population density of subterranean termites, recent studies conducted in Middlesex Fells, Massachusetts (Bulmer *et al.* 2001) and Raleigh, North Carolina (DeHeer & Vargo 2004) indicate dramatically higher population densities in the latter site; the average colony densities for two populations in Middlesex Fells, Massachusetts was 7.4 colonies per ha compared to an average of 194.4 colonies per ha in two Raleigh, North Carolina populations. In addition, it is certainly conceivable that pressure from pathogens could be higher in the south where they exert strong selection pressure against inbred colonies, although there is at present no data on relative abundance or virulence of pathogens for populations of *R. flavipes* or *R. grassei*. Similarly, the greater degrees of inbreeding observed in *R. flavipes* at higher altitudes and in the introduced populations in France could be associated with reduced intensity of biotic interactions. In the light of the present results, studies examining both geographic variation in biotic pressures, particularly intraspecific competition and pathogen load, as well as possible effects of inbreeding on competitive ability and disease resistance, could be especially fruitful in clarifying the role of specific ecological factors in shaping colony breeding structure in *R. flavipes* and *R. grassei*.

In conclusion, our results provide strong evidence that bioclimatic factors influence colony breeding structure in subterranean termites. There are a growing number of genetic studies characterizing the breeding structure of subterranean termites as well as other species from nearly all other isopteran families. These studies show remarkable variation in the composition of breeders in colonies and in the attendant levels of inbreeding, both within and among species (reviewed in Vargo & Husseneder 2009, 2011). The present findings not only suggest much of the observed variation may be due to environmental conditions, but also give clues about which climatic and ecological factors may be important in shaping breeding structures in termite colonies.

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E.L.V. designed the study, collected most of the samples, performed microsatellite genotyping, did much of the statistical analyses and took the lead role in writing the paper. L.L., L.E.S. and D.M.M. collected some of the samples and helped in writing the paper. S.E.D. performed statistical analyses and contributed to writing the manuscript. M.D.W. provided the bioclimatic data and helped with writing. A.-G.B. contributed to sample collection, data analysis and writing.

Data accessibility

Microsatellite data and data used in the regression analysis: DRYAD entry doi:10.5061/dryad.6c7v5.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Colony genetic structure of six populations of *Reticulitermes flavipes*.

Table S2 Model averaged parameter estimates, standard errors, and 95% confidence intervals based on the best-fitting model subset for colony breeding responses (proportion extended families and F_{IT}) for *Reticulitermes flavipes*.

Table S3 Model averaged parameter estimates, standard errors, and 95% confidence intervals based on the best-fitting model subset for colony breeding structure responses (proportion extended families and F_{IT}) for *Reticulitermes grassei*.