

Direct and indirect inferences on parasite mating and gene transmission patterns Pangamy in the cattle tick *Rhipicephalus (Boophilus) microplus*

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Abstract

Mating system plays a determinant role in the maintenance and distribution of genetic variation. Difficulties in applying standard methods of indirect inferences onto parasitic life-cycles partly explain the current lack of knowledge on parasite mating systems. The present study develops a combination of direct and indirect inference methods circumventing such difficulties, and illustrates in particular how such a combination modifies our understanding of the biology of the southern cattle tick, *Rhipicephalus microplus*. To directly assess how females and males pair in natural populations, we sampled 225 mating pairs, genotyped them at six microsatellite loci, and analysed the correlation between mating status and genetic relatedness. This analysis revealed pangamy, i.e. a random association of male and female genotypes. In addition, indirect methods inferred that sib-groups of ticks exploit distinct individual-cows, and hence that ticks probably move among cows during their parasitic lifetime. Altogether, these results negate the expectation of a high frequency of sib-mating, and show the coexistence of genetically diverging stocks within tick cohorts and populations. These results have several consequences for our understanding of *R. microplus*. For instance, while existing models of pesticide resistance management look perfectly applicable to this species, the epidemiological models of the micropathogens it vectors needs revision. More largely, the methods developed here would help clarify the evolutionary patterns of any dioecious parasite.

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1. Introduction

Mating patterns are pivotal for the evolution of any genetically encoded trait by determining the pattern of gene transmission and genotype formation across generations. Its evolutionary consequences are assessed with reference to pangamy, i.e. the random pairing of male and female genotypes with respect to their genetic relatedness. Molecular inferences on mating systems are usually indirect, and are performed according to two main indirect methods. Classically, deviation from pangamy is inferred from screening the genetic diversity within populations and performing a heterozygosity analysis

(e.g. Balloux, 2004). Gene transmission patterns can also be inferred by genotyping putative parents and offspring to perform paternity and/or pedigree analyses (Blouin, 2003; Araki and Blouin, 2005). Direct inferences are nonetheless possible with molecular tools in species for which scientists can identify the mating pairs (Prugnolle et al., 2004). In this latter case, the rationale is to test whether or not the genetic relatedness of copulating individuals is similar to that obtained by randomly pairing the sampled individuals (pangamy).

Focusing on parasites, a clear understanding of mating systems is essential for epidemiological understanding since traits such as virulence, drug-resistance and/or host-specificity are often genetically determined. Moreover, the interplay between mating system and among-host transmission of offspring is expected to be a key determinant in optimal parasite growth, virulence and/or manipulation of host

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behaviour (Frank, 1996; Brown, 1999; Parker et al., 2003) because it regulates the relatedness among co-infecting adults. However, the actual mating systems of parasites in natural populations remain very poorly known (Criscione and Blouin, 2005). This is mainly due to two complementary drawbacks. First, little attention had been paid to direct inferences on parasite mating systems (see Prugnolle et al., 2004 for an exception). Second, a prerequisite for accurate indirect inferences is difficult to fill for parasites: namely, the correct delimitation of the parasite population under study. This may strictly correspond to the individual-host for some parasite species, but for others, this may occur at the level of the host population (Criscione and Blouin, 2005; Criscione et al., 2005). The originality of the present study is to demonstrate the use of a new combination of direct and indirect inferences to confidently assess the mating system and gene transmission pattern in a tropical tick of veterinary importance.

Rhipicephalus (Boophilus) microplus is a major threat for tropical agrosystems because of its explosive demography (Frisch, 1999), its recurrent development of acaricide resistance (Foil et al., 2004), and because it can vector micro-pathogens virulent to both bovine and equine hosts (e.g. Uilenverg, 1976; Battsetseg et al., 2002). Considerable research has focused on *R. microplus* control strategies, the molecular changes involved in acaricide resistance, and on the agricultural risk factors involved in resistance evolution (e.g. He et al., 1999; Miller et al., 1999; Aguirre et al., 2000; Hernandez et al., 2000; Jamroz et al., 2000; Jonsson et al., 2000; Bianchi et al., 2003; Foil et al., 2004; Ducornez et al., 2005; Li et al., 2005; and references therein). More recently, the reproductive biology of acaricide-resistant and susceptible strains were compared to start to investigate whether resistance genes were at a selective disadvantage (fitness cost) in acaricide-free environments (Li et al., 2005; Davey et al., 2006). Interestingly, no study focused on *R. microplus* mating patterns, despite its importance in resistance evolution. Indeed, the earlier resistance is managed, the more efficient its control will be since the fitness cost of resistance genes tends to decrease with time (e.g. Cohan et al., 1994; Davies et al., 1996; Chevillon et al., 1999; Raymond et al., 2001; and references therein). Now, early in the evolution of resistance, acaricide-resistant ticks remain concentrated around their original population and are more genetically related to one another than to susceptible ticks. In addition, resistance management strategies use the geographical distribution of pesticides to find an optimal control of mating frequencies between resistant and susceptible genotypes through the interplay of random mating, selective advantage for resistant genotypes, fitness cost of resistance, and migration among pesticide-controlled and pesticide-free populations (e.g. Lenormand and Raymond, 1998; Vacher et al., 2003, 2004). Any deviation from pangamy thus affects the efficiency of pest management strategies. Such a deviation could result in an enhanced diffusion of resistance genes if kin are avoided in mate choice. Alternatively, if ticks tend to preferentially mate with relatives, the initial increase in frequency of resistance genes in the original population would be higher than that expected under pangamy.

Up to now the life-cycle of *R. microplus* was expected to result in high frequencies of sib-mating. Although eggs and young larvae are free stages, juveniles and adults were thought to spend their entire life-span on the same individual-host. Fully engorged females drop to the soil and die after egg-laying, so that large sib-groups ($N \approx 1800$, Barré et al., 2001) are present as clusters in the environment. This characteristic of sib-clustering is likely to last in subsequent life-stages as development seems to be synchronous among sibs, neighbour larvae tend to aggregate and seek a host on which they will complete their development and mate, and all parasitic stages of a given tick are considered to be completed on the same individual-host (Falk-Vairant et al., 1994; Morrel, 2000). In addition, mate-guarding apparently prevents females from mating more than once (Falk-Vairant et al., 1994). Therefore, mating between relatives seems difficult to avoid, unless mechanisms such as sex-biased dispersal (Prugnolle et al., 2003) and/or mate choice adjustment (Trouvé et al., 1999; Lucher and Millinski, 2003) have evolved in this species. In the present study, we specifically collected mating pairs on naturally infected cows to directly test whether or not the formation of mating pairs was independent of genetic relatedness in *R. microplus*. We also combined Bayesian and F_{IS} analyses (i.e. indirect inferences methods) in order to identify the most likely number of sib-groups sampled within herds, and to investigate the among-cows distributions of these sib-groups. The congruency of the overall pattern that emerged shed a new light on the basic biology of *R. microplus*, and hence on the biological causes behind previous results regarding its relative genetic differentiation within and among herds (Koffi et al., 2006a).

2. Material and methods

2.1. Tick sampling

In April 2003, the inspection of five cows within each of four New Caledonian herds led to an overall sample of 225 tick couples. Couples were individually collected on their hosts and preserved in 95% ethanol until genotyping. Table 1 describes the distribution of couples among cows and herds.

2.2. Tick genotyping

DNA extraction, polymerase chain reactions and genotype scoring were carried out as previously detailed (Koffi et al., 2006b). Ticks were genotyped at six microsatellite loci referred to as *BmA12*, *BmB12*, *BmC03*, *BmC07*, *BmD10*, and *BmD12* (respective GenBank accession numbers DQ001904, DQ001906, DQ001907, DQ001909, DQ001910 and DQ001911). The risk of error in genotype scoring was evaluated as detailed in Koffi et al. (2006a).

2.3. Direct inferences on mating system

The mating system was analysed by investigating the sign of the correlation between two descriptors of female–male pairs: the genetic relatedness and the mating status. Genetic relatedness between two individuals was computed using Wang's estimator,

Table 1
Observed and extreme estimates of the correlation r between genetic relatedness and mating status

	Observed		Minimal		Maximal	
	r	P -value	r	P -value	r	P -value
Individual-host						
LFB1 ($N = 6$)	0.029	0.86	-0.45	0.62	0.36	0.030
LFB2 ($N = 8$)	0.15	0.22	-0.41	8×10^{-4}	0.40	10^{-4}
LFB3 ($N = 6$)	0.17	0.30	-0.38	0.002	0.41	0.007
LFB4 ($N = 11$)	0.88	0.34	-0.39	10^{-4}	0.33	3×10^{-4}
LFB5 ($N = 6$)	0.25	0.13	-0.50	0.002	0.53	9×10^{-4}
CAB1 ($N = 6$)	-0.031	0.85	-0.30	0.077	0.56	10^{-4}
CAB2 ($N = 9$)	-0.13	0.14	-0.36	10^{-4}	0.33	2×10^{-4}
CAB4 ($N = 12$)	0.20	0.015	-0.32	4×10^{-4}	0.42	10^{-4}
CAB5 ($N = 11$)	0.037	0.68	-0.31	7×10^{-4}	0.31	7×10^{-4}
CAB6 ($N = 12$)	0.070	0.39	-0.34	10^{-4}	0.39	10^{-4}
LPB1 ($N = 17$)	-0.038	0.53	-0.24	10^{-4}	0.30	10^{-4}
LPB2 ($N = 15$)	-0.061	0.35	-0.26	10^{-4}	0.33	10^{-4}
LPB3 ($N = 17$)	0.047	0.42	-0.30	10^{-4}	0.35	10^{-4}
LPB4 ($N = 17$)	-0.056	0.36	-0.32	10^{-4}	0.30	10^{-4}
LPB5 ($N = 16$)	0.032	0.60	-0.22	3×10^{-4}	0.25	10^{-4}
BMB1 ($N = 12$)	0.0099	0.91	-0.32	10^{-4}	0.29	4×10^{-4}
BMB2 ($N = 12$)	-0.082	0.31	-0.26	0.002	0.32	10^{-4}
BMB3 ($N = 7$)	0.077	0.60	-0.36	0.006	0.36	0.001
BMB4 ($N = 12$)	0.050	0.55	-0.32	10^{-4}	0.35	10^{-4}
BMB5 ($N = 11$)	-0.11	0.24	-0.37	10^{-4}	0.35	2×10^{-4}
Herd						
LF ($N = 37$)	0.064	0.032	-0.23	10^{-4}	0.25	10^{-4}
CA ($N = 52$)	0.14	0.53	-0.20	10^{-4}	0.16	10^{-4}
LP ($N = 82$)	0.058	0.58	-0.16	10^{-4}	0.18	10^{-4}
BM ($N = 54$)	0.012	0.70	-0.20	10^{-4}	0.21	10^{-4}

The identification of either the individual-host or herd of origin is given with the corresponding number of tick mating pairs (N). Minimal and maximal correlation estimates were retrieved by rearranging data so that tick females were artificially paired with males that were, respectively, the least or the most genetically related to them. Significant P -values (bilateral tests) appear in bold characters.

an estimator shown to be unbiased and particularly robust for small sample sizes (Wang, 2002). Computations were performed with the MER V3 software developed by Wang (freely accessible at <http://www.zoo.cam.ac.uk/ioz/software.htm#MER>). The mating status of a given female–male pair was defined as 1 if it corresponded to a mating pair, and as 0 otherwise.

Pangamy corresponds to a situation where males and females mate independently of their genetic relatedness; i.e. where the two descriptors of female–male pairs are uncorrelated. Alternatively, a positive correlation between these descriptors would indicate a preference of kin as mates, and a negative correlation the avoidance of kin as mates. This was tested by comparing the observed correlation with its chance distribution obtained by randomising the mating status among female–male pairs (15,000 randomisations). This randomisation test was performed using F -stat version 2.9.3 (Goudet, 1995). This procedure was implemented either at the within cow or the within herd level (i.e. in the latter case, pooling the samples collected on different cows within herds). In each case this provided several independent P -values that we chose to combine using Fisher's procedure (Fisher, 1970). If there are n P -values to be combined, then the quantity

$$\chi^2_{\text{Obs}} = -2 \sum_{i=1}^{i=n} \log(P_i)$$

is a Chi-square variable with $2n$ degrees of freedom. We thus compared the value obtained for either the 20 individual-cows or the four herds to a 40-d.f. or a 8-d.f. Chi-square distribution, respectively.

The power of our genotypic data to detect deviation from pangamy was assessed by re-performing the randomisation tests on two artificial datasets per population. In the first dataset, we artificially paired the males and females the least genetically related to one another. In the second, we artificially paired the males and females the most genetically related to one another.

2.4. Complementary indirect inferences

Pangamy was indirectly tested using the unbiased F -statistics estimates developed by Weir and Cockerham (1984). F_{IS} measures the within-sample magnitude of deviation from Hardy–Weinberg expectations (HWE), and hence of pangamy. The significance of departure of F_{IS} from 0 was tested by randomising alleles between individuals within samples (15,000 permutations). Computations of estimates and randomisation tests were performed with F -stat version 2.9.3 (Goudet, 1995). Interestingly, the epidemiology of *Anaplasma marginale* with frequent transtadial but rare transovarian transmission in *R. microplus* suggests that the tick parasitic stages may move among individual-hosts (Uilenberg, 1976) despite what it is usually said for this species. Accordingly, we

performed our computations under two hypotheses. First, assuming that the individual-cow defined the tick breeding unit (i.e. assuming no tick movement among cows), we computed the average F_{IS} over the 20 cows on which tick mating pairs were collected. Second, assuming that the herd defined the tick breeding unit (i.e. that ticks do move among cows), we ignored the individual-host and computed the average F_{IS} over the four herds. Now, if one assumes that local deviation from random mating is entirely due to sib-mating, the rate of sib mating necessary to explain the F_{IS} estimate observed can be derived as follows. According to Crow and Kimura (1970) the inbreeding coefficient follows the recursive equation:

$$f(t) = \frac{1}{4}[1 + 2f(t-1) + f(t-2)]$$

If f_{bs} is the proportion of sib-mating events at each generation, and assuming a large population we then have:

$$f(t) = \frac{1}{4}[1 + 2f(t-1) + f(t-2)]f_{bs} + 0[1 - f_{bs}]$$

given the assumption of pangamy at herd scale. If p_i is the frequency of allele i in the herd then a mother will be homozygous for this allele with probability p_i^2 and its progeny will be heterozygous for allele i with probability $1 - p_i$ (the probability that a father provides a different allele). In the progeny, the expected frequency of allele i will be $(p_i + 1)/2$ and thus the expected heterozygosity is $2[(p_i + 1)/2][(1 - p_i)/2]$. Overall, the observed and expected heterozygosities in the progenies of homozygous females are thus, respectively:

$$H_{O/ii} = \sum_i p_i^2(1 - p_i)$$

$$H_{s/ii} = \sum_i p_i^2 \frac{(1 - p_i)(1 + p_i)}{2}$$

With a similar rationale, the observed and expected heterozygosities in the progenies of heterozygous females are, respectively:

$$H_{O/ij} = \sum_{i,j \neq i} 2p_i p_j \left[1 - \frac{p_i + p_j}{2} \right]$$

$$H_{s/ij} = \sum_{i,j \neq i} p_i p_j \left[\left(\frac{1 + 2p_i}{2} \right) \left(\frac{1 + 2p_j}{2} \right) + (1 - p_i - p_j)(1 + p_i + p_j) \right]$$

At equilibrium $f(t) = f(t-1) = f(t-2) = F_{IS}$ and thus rearranging the previous equation results in:

$$f_{bs} = \frac{4F_{IS}}{1 + 3F_{IS}}$$

The possibility of hidden genetic substructure of ticks within each herd was investigated with the Bayesian method developed by Corander et al. (2003). Unlike the F -statistics' analysis, this method does not treat the partition of individuals among samples as a given fact but as an unknown parameter to be estimated using the joint posterior distribution of the substructure and the allele frequencies within sub-populations. Computations of the optimal partition within herds was performed using BAPS version 4 (Corander et al., 2004) (freely downloadable at http://www.rni.helsinki.fi/~jic/baps_download.html) assuming a maximum of 20 partitions in each herd except PL (see below), and replicating the analysis 50 times per sampled-herd. In PL herd, we assumed a maximum of 50 partitions in the 50 replicates because the best partition appeared to be 20 (see Section 3). We investigated whether the best partition detected by BAPS re-united the sib-groups resulting from pangamy at the herd scale as follows. We computed the F_{IS} estimate observed with the best partition detected by BAPS for each locus and each herd, and looked at the sign of these estimates. It is indeed noteworthy that negative values are expected for the offspring of a single female–male pair, unless the parents have two identical heterozygous genotypes in which case a null value is expected. We brought further precision to this expectation by computing the exact F_{ISc} estimate expected within true sib-groups, noting that this estimate ultimately depends on allele frequencies within herds

Altogether, regardless of whether the progeny of females have been sired by one or several males, the F_{IS} expected within sib-groups resulting from pangamy at herd scale is calculated as

$$F_{ISc} = 1 - \left[\frac{H_{O/ii} + H_{O/ij}}{H_{s/ii} + H_{s/ij}} \right]$$

3. Results

R. microplus ticks find their mate on their individual-host. At that level, genetic relatedness and mating status was uncorrelated ($P > 0.1$), except for the female–male pairs collected on CAB4 ($r = 0.20$ and $P = 0.015$, Table 1). It is worth noting that among the 20 tick-collections, both negative and positive correlation estimates were found and that only a single test was significant at the 5% risk (i.e. 5% of all performed tests as expected under the null hypothesis). In contrast, the rearrangement of female–male pairs within individual-hosts aimed at either minimizing or maximizing such correlations resulted in significant correlations in all but two cases ($P > 0.05$ for the avoidance of relatives as mates on LFB1 and CAB1, Table 1). Finally, the Fisher's procedure for combining statistical tests confirmed that mating status and genetic relatedness were uncorrelated over the observed dataset ($P = 0.34$), but highly correlated over each set of rearranged data ($P \ll 10^{-5}$).

If *R. microplus* ticks move easily among cows during their parasitic lifetime, their mate choice could be adjusted at herd scale rather than at that of the individual-cow. We tested this possibility by re-investigating the correlation between genetic relatedness and mating status at herd scale. A weak positive

correlation was significant in LF herd ($r = 0.063$ and $P = 0.032$) whereas no correlation was evident in the three other herds ($P > 0.5$, Table 1). Re-arrangements of female–male pairs aimed at minimizing or maximizing the correlation between genetic relatedness and mating status always concluded in significant effects (Table 1). Finally, the Fisher's procedure for combining statistical tests concluded that genetic relatedness and mating status were uncorrelated over the observed dataset ($P = 0.26$), but highly correlated over each of the two rearranged datasets ($P \ll 10^{-5}$).

Indirect inferences on the *R. microplus* mating system determined using F -statistics were consistent with direct inferences at both the cow and herd scales. Indeed, the same average F_{IS} estimate within herds was obtained assuming the tick-breeding unit as defined by the individual-host ($F_{IS} = 0.047 \pm 0.019$) or by the herd it belongs to ($F_{IS} = 0.046 \pm 0.021$). In both cases, these estimates corresponded to a significant heterozygote deficit relative to HWE ($P < 2 \times 10^{-5}$). Assuming sib-mating as the only cause involved in the positive F_{IS} estimate, the frequency f_{bs} of brother–sister mating events would range from 0.10 to 0.22.

Bayesian analyses detected hidden genetic substructure within each herd. The most likely number of partitions (i.e. potential number of sib-groups) was 12 in the LF herd, 13 in the BM herd, 11 in the CA herd, and 20 in the PL herd. Interestingly, even if the optimal partition detected within herds by Bayesian analyses differed slightly between runs in BAPS, they always merged together ticks collected on different individual-cows. Most of the F_{IS} estimates (per locus and per herd) found within partitions were negative, supporting the assumption that the partitions could correspond to sib-groups (see means and standard errors in Fig. 1). Furthermore, the standard-errors of the per-locus averages of observed F_{IS} estimates overlapped, indicating good consistency among loci (Fig. 1). Finally, the observed F_{IS} estimates were in congruence with the expected F_{ISc} values (Fig. 1), indicating that the clusters recognised by BAPS are likely to mostly correspond to

sib-groups. Nevertheless, the observed F_{IS} estimates remained larger than the expected F_{ISc} values (Fig. 1), suggesting that other factors are involved.

4. Discussion

Given the minimal sample size of six mating pairs per individual-host, the set of loci used for computing genetic relatedness in this study was powerful enough to detect the possibilities of kin avoidance or kin preference in rearranged datasets. Therefore, our results demonstrate conclusively that the collected mating pairs were randomly formed without any oriented choice regarding the mate's genotype at neutral loci. Pangamy was observed both at the cow and herd scales, and the hidden genetic structure detected by Bayesian analyses within herds did not respect the sampling partition among individual-cows. This confirmed a previous result obtained by screening the genetic diversity of *R. microplus* populations within and among bovine herds with different tools and approaches (Koffi et al., 2006a): the individual-host is not a pertinent factor for gene transmission in this parasite species. In biological terms, these results strongly indicate that, contrary to expectations, *R. microplus* sibs frequently settle on distinct individual-cows, either as larvae seeking a new host, or as juvenile stages through movements among cows. This might imply revisiting the epidemiological models of diseases vectored by this tick. In particular, this result is pivotal for understanding outbreaks of micropathogens such as *A. marginale*, i.e. with frequent transtadial, but rare transovarian transmission (Uilenberg, 1976).

At first glance, the biological meaning of the significant deviation from HWE observed seems unclear given the random mating strategy of *R. microplus*. We cannot firmly rule out the possibility that this deviation could partly result from the occurrence of null alleles at each assessed locus within the sampled populations. Nevertheless, this putative bias, as well as other scoring sources of error, look very unlikely given among the loci congruency in F_{IS} estimates within the sampled tick collections (details not shown; see also Koffi et al., 2006a for details on larger datasets). Moreover, the consistency among loci was re-confirmed when investigating F_{IS} variation within the genetic clusters identified by Bayesian analyses (Fig. 1). By contrast, we can definitively rule out the hypothesis under which the observed deviation from HWE strictly results from sib-mating. Indeed, such a hypothesis would be in total contradiction with both the direct evidence for pangamy and the detected genetic sub-structure within herds given the F_{IS} values found within detected clusters. It is particularly noteworthy that significant sib-mating frequencies (estimated above 0.10) would have counted among the most plausible explanations if our investigation had been strictly based on standard indirect inference methods. This strongly argues for the necessity to use direct methods to make inferences about mate choice whenever possible (see also Prugnolle et al., 2004 for a complementary example on an indirectly transmitted parasite, *Schistosoma mansoni*), and to take into account the inherent difficulty in applying indirect methods to parasites (Criscione and Blouin, 2005; Criscione et al., 2005).

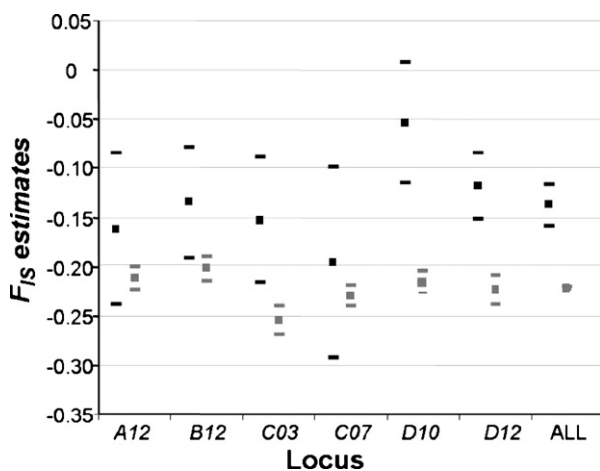


Fig. 1. Observed and expected F_{IS} estimates within the clusters detected by Bayesian analyses. The means, weighted by samples size, of the observed F_{IS} estimates (in black) and expected F_{ISc} values (in grey) were computed over the four herds for each locus and across all six loci.

Altogether, the significant deviation from HWE detected within the sampled tick collections is thus very likely to indicate admixture of genetically diverging stocks of *R. microplus* within herds. Such admixture could result from strong variance in the reproductive success of females, such that many sibs from a limited number of clutches survived within herds (Criscione and Blouin, 2005). The few clusters detected by Bayesian analyses within herds and the overall likelihood that these groups match with tick sib-groups support that hypothesis. Moreover, the segregation of pesticide resistance genes in these pesticide-controlled tick populations (Bianchi et al., 2003; Ducornez et al., 2005), and the high susceptibility of *R. microplus* free-living stages to micro-environmental variation in humidity (Corson et al., 2003, 2004) provide efficient functional explanations for that hypothesis. Nonetheless, the imperfect match between the F_{IS} values found among detected clusters and expected for sib-groups (Fig. 1) suggests the co-occurrence of secondary factors (e.g. a weak proportion of sib-mating events and/or another superposed genetic divergence among sympatric ticks) and/or the inability of BAPS to completely identify sib-groups. Further investigations of the genetic composition of the progenies of female ticks as well as theoretical investigations of Bayesian analyses are required to definitively settle this point.

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