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ISOLATION AND CHARACTERIZATION OF NEW POLYMORPHIC MICROSATELLITE MARKERS FOR THE TICK *IXODES RICINUS* (ACARI: IXODIDAE)

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ABSTRACT — Nine microsatellite markers were isolated from unfed larvae of *Ixodes ricinus* and were tested on two populations of nymphs collected on roe deer (N=21) and birds (N=39) in a French suburban forest. All markers were polymorphic, with limited evidence for deviations from linkage equilibrium. In accordance with previous markers developed for this species, we found large heterozygote deficits for six of the nine loci. Deficits were of the same order of magnitude within a tick infrapopulation, suggesting that population-level estimates were not due to a Wahlund effect among individual hosts, but more likely to technical problems (*i.e.*, null alleles due to mutations in the flanking regions of the microsatellites). Although micro-geographic substructure (e.g., homogamy within infrapopulations) can not be ruled out, it is possible that null alleles could be an inherent problem associated with this tick species and specific genome-level studies are called for. Despite the possible presence of null alleles, the precision of population genetic estimates was improved by the addition of the newly-developed markers making them a useful addition for studying the population ecology of *I. ricinus*.

KEYWORDS — Ectoparasite; Genetic markers; Population genetics; Tick-borne disease

Ticks are haematophagous ectoparasites of major importance as vectors of human disease (Parola and Raoult 2001). *Ixodes ricinus* (Arthropoda, Acari, Ixodidae) is the main vector species in Europe, transmitting numerous human and livestock diseases including Lyme disease, tick-borne encephalitis, anaplasmosis and babesiosis (e.g., Stanek 2009). Efforts to understand the ecology of this tick in relation to disease transmission is difficult under natural conditions. This is particularly true for estimating patterns of dispersal and host use, two essential factors for understanding disease risk (McCoy 2008). Indirect methods that employ genetic markers are currently one of the best options to overcome the inherent difficulty in studying parasitic organisms, but require certain assumptions in order to make robust inferences (De Meeûs *et al.* 2007). Microsatellite markers have been previously described and applied to populations of *I. ricinus* (Delaye *et al.* 1998, De Meeûs *et al.* 2002, 2004a, 2004b, Røed

et al. 2006, Kempf *et al.* 2009, 2010, 2011). However, analyses using these markers have revealed significant deviations from Hardy-Weinberg proportions within populations. Hypotheses to explain these heterozygote deficits are numerous and not mutually exclusive: null alleles, short allele dominance, Wahlund effects or homogamy (Kempf *et al.* 2009). From previous studies, it is clear that technical problems are frequent (De Meeûs *et al.* 2004a). However, even after accounting for these problems, deficits are still apparent within populations suggesting the presence of population substructure (De Meeûs *et al.* 2004a, Kempf *et al.* 2010). Here, we outline the development of additional microsatellite markers for *I. ricinus* in an attempt to improve the precision of population genetic estimates used to study the biological factors that may be behind these patterns.

New microsatellite loci were isolated from a microsatellite-enriched library according to Billotte *et al.* (1999). We extracted Genomic DNA from unfed larvae with DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's instructions. DNA was restricted by *Hae*III and fragments were ligated to Rsa21 and Rsa25 self-complementary primers (5′-CTCTTGCTTACGCGTGGACTA-3′ and 5′-TAGTCCACGCGTAAGCAAGAGCACA-3′) and amplified by Polymerase Chain Reaction (PCR). Products were hybridized to a biotin-labelled $I_5(GA)_8$ probe and Streptavidin MagneSphere Paramagnetic Particles (Promega). Enriched fragments were amplified by PCR, cloned in pGEM-T (Promega) and transformed in XL1-Blue competent cells (Stratagene). Recombinant colonies were randomly selected and amplified by PCR with Rsa21 primers. PCR products were run on a 1.1% agarose gel and transferred onto a Hybond N+ membrane (Amersham) which were hybridized with $[\gamma^{32}P]$dATP end-labelled $(GA)^{15}$ and $(GT)^{15}$ probes to verify amplification and improve fragment selection. Positive clones of differing fragment size were sent for sequencing (Beckman Coulter Ge-

TABLE 1: Characterization of nine microsatellite markers isolated in the present study for *Ixodes ricinus*.

Locus	Genbank Accession No.	Repeat motif	Primer sequence (5′-3′)	Size range	A_R
IRic04	JF724082	(AC)6(CA)7	F : ACGGGATGTTTAATTGG	164-208	18.09
			R : GATCGACGAATGATCTCTG		
IRic05	JF724083	(GA)8	F : CCTTACCAACCCTGTGTC	216-229	8.05
			R : GAGCCGAATTTTATGCAC		
IRic07	JQ349034	(CA)6(AC)7(ACAA)5	F : TATTTCTTCCGTGGTTCC	149-173	9.33
		(ACACAA)3	R : TGTTACCTTCGACAACGA		
IRic08	JF724084	(TG)9	F : TCATTGTCCCTTCCAGTACG	226-258	12.91
			R : AGAAAATAAGCGCCGAGAAA		
IRic09	JQ349035	(CT)10	F : AAAAGACCCCAGAAACAA	266-298	15.83
			R : GGGGAAGAAAATATGCTAA		
IRic11	JF724085	(AC)8	F : AGCTACGAGACTACATCAAAA	245-282	12.00
			R : TCAAAGACAGTGACGCTTA		
IRic13	JQ349036	(AC)8	F : AATGACGCCAGCGAGATAAT	156-170	7.47
			R : TCTATATAGGGGGTGGCGAAT		
IRic17	JQ349037	(CA)10	F : ATAGTGAGCGTTTGGACAAT	208-216	3.76
			R : CTCGCGTTTTAATGAAGTG		
IRic18	JQ349038	(CT)11	F : GTCCACGTCCTTTCACTCT	239-269	12.90
			R : GGAAACAAAAGACCAAGAAA		

A_R: allelic richness based on 19 diploid individuals

nomics). Sequences were analysed and primers were designed using the SAT software (Dereeper *et al.* 2007).

We chose 19 loci for preliminary tests after checking that they differed from those described in previous studies. We performed PCR amplifications following a M13 protocol where each forward primer is 5′-tagged with the M13 sequence (5′-CACGACGTTGTAAAACGAC-3′) and a 5′-dye labelled M13 is added to the reaction mix. The 10 µL PCR mixture contained 20–50 ng of genomic DNA, 25 µM of each dNTP (Roche Diagnostics), 0.15 µM of each primer, 0.15 µM of labelled M13, 1 µL of 10x PCR buffer (Roche Diagnostics) and 0.25 U of *Taq* DNA polymerase (Roche Diagnostics). Amplifications were performed using a "touch down" PCR procedure consisting of an initial 2 min denaturation step at 94 °C, followed by 16 cycles with 45 s at 94 °C, 45 s at 60 °C with this annealing temperature decreasing by 0.5 °C at each cycle, 30 s at 72 °C, then 35 cycles with 45 s at 94 °C, 45 s at 52 °C, 30 s at 72 °C (25 cycles for IRic04, IRic05 and IRic18) and a final extension step of 10 mins at 72 °C. For genotyping, 0.5 µL of PCR products were pooled with 13 µL of Hi-Di Formamide and 0.25 µL of the GeneScan-500LIZ Size Standard (Applied Biosystems) and analysed on an ABI Prism 3130XL Genetic Analyser (Applied Biosystems). Raw data was sized using the associated GENEMAPPER software V4.0.

Of the 19 loci, we selected nine polymorphic loci that displayed good amplification results. These microsatellite loci were tested on two populations of nymphs from a suburban forest (Forêt de Sénart, Ile-de-France), one collected from five roe deer (N=21) and the other from twenty passerine birds (N=39). We considered these samples as representing potentially independent populations based on previous work that indicated the presence of host-associated races in this tick in some populations (Kempf *et al.* 2011). Data were analysed using GENEPOP 4.0.10 (Raymond and Rousset 1995) and FSTAT 2.9.3.2 (Goudet 1995). All markers were tested for independence using exact probability tests and for Hardy-Weinberg proportions by calculating Weir and Cockerham's (1984) estimator of Wright's F_{IS} for each population. In an attempt to reduce any potential Wahlund effect to a minimum, we also compared Hardy-Weinberg proportions of each population to that from 12 ticks sampled on a single roe deer individual, that is, a tick infrapopulation. Finally, we evaluate how overall F_{IS} estimates changed when our new loci were used in combination with pre-existing markers.

All loci were polymorphic with relatively high genetic diversity (Table 1). One-step mutations were noted for several loci (especially for IRic04 and IRic09). All loci (new and old) were in linkage equilibrium at the 5% threshold except three locus pairs (IRic05 – IRic08, IRic07 – IR27 and IRic08 – IR39). The probability of occurrence of three significant tests out of the 91 possible is less than would be expected by chance at an alpha of 5% (k' = 9, Generalised binomial procedure, MULTI-TEST V1.2; De Meeûs *et al.* 2009). For this reason, and because results of the linkage tests differed between the two studied populations, we consider that all markers represent independent replicates of the tick genome.

Among the nine new markers, we observed large heterozygote deficits for five in the roe deer tick population and six in the bird tick population (Table 2). IRic05, IRic07 and IRic08 showed Hardy-Weinberg proportions in both populations. Deficits were of the same order of magnitude in the tick infrapopulation suggesting that population-level deficits were not due to a Wahlund effect among individual hosts. However, an effect of homogamy within the infrapopulation can not be ruled out. MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2004) suggested the presence of null alleles for several loci: IRic04, IRic08, IRic11, IRic13, IRic17, IRic18 in the roe deer population and, IRic04, IRic07, IRic08, IRic09, IRic11, IRic13, IRic17, IRic18 in the bird population. The pattern used to identify the presence of null alleles at a locus is similar to that expected for a Wahlund effect or homogamy (Van Oosterhout *et al.* 2004), and may therefore account for the variation between the two tick populations.

These results are consistent with previous studies on *Ixodes ricinus* showing heterozygote deficits that were partially explained by technical problems.

TABLE 2: Tests of Hardy-Weinberg proportions for 14 microsatellite loci (nine new markers, IR25, IR27, IR32 and IR39 from Delaye *et al.* 1998, and IRN37 from Røed *et al.* 2006) in two nymphal populations of *I. ricinus* sampled respectively from birds and roe deer and in a tick infrapopulation from a single roe deer host.

Locus	Host	N	*Ho*	*Hs*	F_{is}	*P* value
IRic04	Bird	36	0.389	0.947	0.589	**0.0000***
	Roe deer	20	0.650	0.963	0.325	**0.0000***
	Roe deer infrapopulation	12	0.500	0.977	0.488	**0.0000***
IRic05	Bird	33	0.727	0.813	0.105	0.1002
	Roe deer	20	0.850	0.836	-0.017	0.8174
	Roe deer infrapopulation	12	0.917	0.845	-0.085	0.9493
IRic07	Bird	38	0.684	0.862	0.207	0.0071
	Roe deer	20	0.650	0.795	0.182	0.4144
	Roe deer infrapopulation	11	0.545	0.723	0.245	0.3944
IRic08	Bird	36	0.667	0.856	0.221	0.0176
	Roe deer	21	0.619	0.886	0.301	0.0163
	Roe deer infrapopulation	12	0.500	0.822	0.392	0.0845
IRic09	Bird	39	0.615	0.923	0.334	**0.0000***
	Roe deer	21	0.810	0.907	0.108	0.1134
	Roe deer infrapopulation	12	0.833	0.898	0.072	0.6401
IRic11	Bird	39	0.385	0.891	0.568	**0.0000***
	Roe deer	19	0.263	0.943	0.721	**0.0000***
	Roe deer infrapopulation	10	0.300	0.933	0.679	**0.0000***
IRic13	Bird	33	0.273	0.718	0.620	**0.0000***
	Roe deer	21	0.286	0.815	0.650	**0.0000***
	Roe deer infrapopulation	12	0.250	0.864	0.711	**0.0000***
IRic17	Bird	32	0.063	0.518	0.879	**0.0000***
	Roe deer	19	0.053	0.585	0.910	**0.0000***
	Roe deer infrapopulation	12	0.000	0.621	1.000	**0.0000***
IRic18	Bird	37	0.432	0.922	0.531	**0.0000***
	Roe deer	21	0.381	0.842	0.547	**0.0000***
	Roe deer infrapopulation	12	0.417	0.883	0.528	**0.0002***
IR25	Bird	33	0.455	0.895	0.492	**0.0000***
	Roe deer	18	0.500	0.884	0.434	**0.0000***
	Roe deer infrapopulation	11	0.545	0.864	0.368	0.0087

N: number of genotyped individuals

Ho : observed heterozygosity

Hs : expected heterozygosity

F_{is}: Weir and Cockerham's (1984) estimator

P -value: F_{is} exact probability estimated by the Markov chain method

*: significant test for deviation from Hardy-Weinberg proportions after Bonferroni correction

TABLE 2: Continued.

Locus	Host	N	*Ho*	*Hs*	F_{is}	*P* value
IR27	Bird	38	0.263	0.479	0.451	**0.0000***
	Roe deer	21	0.143	0.665	0.785	**0.0000***
	Roe deer infrapopulation	12	0.167	0.667	0.750	**0.0000***
IR32	Bird	30	0.100	0.730	0.863	**0.0000***
	Roe deer	15	0.200	0.714	0.720	**0.0000***
	Roe deer infrapopulation	9	0.222	0.403	0.448	0.1152
IRN37	Bird	39	0.436	0.857	0.491	**0.0000***
	Roe deer	21	0.571	0.862	0.337	0.0231
	Roe deer infrapopulation	12	0.500	0.883	0.434	0.0232
IR39	Bird	38	0.368	0.867	0.575	**0.0000***
	Roe deer	21	0.571	0.912	0.373	**0.0000***
	Roe deer infrapopulation	12	0.583	0.913	0.361	0.0102

N: number of genotyped individuals

Ho : observed heterozygosity

Hs : expected heterozygosity

F_{is}: Weir and Cockerham's (1984) estimator

P -value: F_{is} exact probability estimated by the Markov chain method

*: significant test for deviation from Hardy-Weinberg proportions after Bonferroni correction

Null alleles therefore seem to be common in this species and will require genome-level information in order to further understand their source. However, despite these technical issues, our new markers slightly improve the precision of previous population genetic estimates (Global F_{IS} estimate across loci and populations for pre-existing markers F_{IS} = 0.549 $\pm$ 0.066, for new markers F_{IS} = 0.418 $\pm$ 0.078, for all markers F_{IS} = 0.464 $\pm$ 0.057), with the addition of one marker that presented no indication of null alleles in either of the examined populations (IRic05). Thus, in tandem with appropriate sampling strategies, these markers should represent useful additional tools for studying the ecology of *I. ricinus* populations and their role as disease vectors.

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