Molecular Identification of Bloodmeal Source in *Ixodes ricinus* Ticks Using 12S rDNA As a Genetic Marker

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ABSTRACT We developed an efficient molecular method for the identification of the bloodmeal sources in the tick *Ixodes ricinus* (L.), the European vector of the agents of Lyme borreliosis and tick-borne encephalitis. A ≈145-bp orthologous fragment of the vertebrate mitochondrial 12S rDNA was used as a molecular marker to discriminate host vertebrate species. The method consists of a single run polymerase chain reaction amplification of the 12S rDNA molecular marker by using nondegenerate primers followed by a reverse line blot hybridization assay by using specific oligonucleotide probes. The palette of probes allowed us to distinguish major groups of host vertebrates (e.g., mammals, small rodents, artiodactyls, birds, lizards) and to identify the bloodmeal sources at the genus or species level. External primers were designed and used to sequence the 12S rDNA molecular marker of a broad range of known or potential host vertebrate species ($n = 60$), including mammal ($n = 28$), bird ($n = 31$), and reptile ($n = 1$) species. The use of this technique coupled with known methods for identification of tick-borne pathogens (e.g., *Borrelia burgdorferi* sensu lato) allowed us to determine the source of infective bloodmeal and to identify reservoir species. The present method was successfully used to identify the source of bloodmeals in all feeding *I. ricinus* ticks and in half of questing field-collected *I. ricinus* ticks. Moreover, the bloodmeal source was identified in 65% of ticks infected with *B. burgdorferi* sensu lato. Further development of this technique may be envisaged for the detection of other vector-borne pathogens and their reservoir hosts.

KEY WORDS bloodmeal, host identification, tick, *Ixodes ricinus*, 12S rDNA

To develop efficient control strategies toward vector-borne zoonoses, a clear understanding of the transmission dynamics of pathogens is needed. This requires an exhaustive identification of reservoir host species of these agents and an assessment on their respective role in habitats of interest. Among tick-borne zoonoses, Lyme borreliosis shows the most frequent occurrence in the Northern Hemisphere. In Europe, the etiological agent of Lyme borreliosis, *Borrelia burgdorferi* sensu lato (s.l.), is mainly vectored by the tick *Ixodes ricinus* (L.), and this pathogen is maintained through cycles involving ticks and vertebrate hosts such as mammals and birds (Gern and Humair 2002). European reservoirs of *B. burgdorferi* s.l. have been largely investigated since the first description of the pathogen in *I. ricinus* (Burgdorfer et al. 1983), and a provisional list of recognized reservoir and nonreservoir species has been drawn up (Gern et al. 1998, Gern and Humair 2002). However, this list is probably not exhaustive, and it may contain some inaccuracies due to the host identification procedure adopted. As a gold standard, reservoir identification implies animal trappings, temporary maintenance in captivity and use of tick xenodiagnosis. This procedure may increase the importance of small rodents as reservoirs, because these animals can be very easily trapped and maintained in captivity compared with birds and larger mammals. Indeed, tick xenodiagnosis has been largely used with small rodents (Aeschlimann et al. 1986; Matuschka et al. 1992; De Boer et al. 1993; Humair et al. 1993, 1999) but less frequently used with medium-sized mammals, birds, and reptiles (Matuschka et al. 1994, 1997; Kahl and Geue 1995; Craine et al. 1997; Gern et al. 1997; Humair and Gern 1998; Humair et al. 1998; Kurtenbach et al. 1998; Dsouli et al. 2006). As an alternative method, the assessment of the prevalence of borrelial infection in host feeding *I. ricinus* larvae or in derived nymphs (compared with the infection prevalence in questing larvae) may give indicative information on the infectivity of a particular vertebrate species for ticks, as shown for some species (Tälleklint and Jaenson 1994, Poupon et al. 2006).

Alternatively, the analysis of bloodmeals in vectors is an elegant and effective approach that was first explored for hematophagous Diptera, in particular, for

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mosquitoes, black flies and tsetse flies, by using immunological techniques (Tempelis 1975, Beier et al. 1988, Hunter and Bayly 1991, Clausen et al. 1998). Then, the use of polymerase chain reaction (PCR) techniques, such as heteroduplex analysis or multiplexed PCR, was introduced (Boakye et al. 1999, Lee et al. 2002, Ngo and Kramer 2003, Kent and Norris 2005). Methods for bloodmeal analysis in ticks have been developed with the use of a PCR amplification targeting the cytochrome *b* gene coupled with sequencing (Tobolewski et al. 1992) or with a reverse line blot assay (RLB) (Kirstein and Gray 1996). The bloodmeal analysis in ticks faces the problem of accessibility of free-living ticks, which have recently fed. After their molt, questing ticks occur on the vegetation, and they may remain there for months, seeking for a host. Consequently, the quality and the quantity of bloodmeal remnants in questing ticks are both very poor. The sensitivity of the technique turned out to be crucial for the identification of bloodmeal source in ticks. Kirstein and Gray (1996) were able to detect host DNA in ticks up to 280 d postengorgement. More recently, Pichon et al. (2003, 2005) developed a similar technique targeting the nuclear 18S rRNA gene to discriminate major groups of vertebrate hosts.

We describe herein an efficient molecular method for the identification of *I. ricinus* bloodmeals by using a variable fragment of the mitochondrial 12S rRNA gene as a genetic marker. This marker allows at the same time to discriminate major groups of vertebrate hosts (e.g., mammals, birds, small rodents, artiodactyls, and lizards) and to identify the bloodmeal source at the genus or species level.

Materials and Methods

Search for an Adequate Molecular Marker. After assessment of various mitochondrial genes, 12S rDNA sequences of various mammal and bird species that are known or potential hosts for *I. ricinus* ticks were retrieved from the GenBank database and aligned using ClustalX multi-alignment software. The interspecific or intergeneric genetic variability of the amplicon, the nondegenerate character of primers, and the small size of the amplicon were the three major criteria in the choice of an adequate molecular marker. Forward and reverse nondegenerate primers (12S-6F and 12S-9R) were designed in conserved regions flanking a variable region (Table 1). The amplicon size was 143–150 bp. External nondegenerate primers (12S-12F and 12S-13R) also were designed for the complete sequencing of the 12S rDNA marker of various vertebrate species (Table 1). Using the external primers, the amplicon was 612–635 bp in size and included the 12S rDNA target sequence.

Collection of Vertebrate Tissues. A large collection of vertebrate tissue samples was established during this study thanks to several collaborators (see Acknowledgments). This collection includes samples from various species of mammals and birds, and one species of reptile, which all belong to the vertebrate fauna of Switzerland and are known or potential hosts for *I. ricinus.* When possible, samples from different individuals coming from different locations in Switzerland were sequenced for each species to assess the intraspecific genetic variability of the 12S rDNA marker.

Samples were taken from dead and frozen animals by using sterile scalpel blades and sterilized forceps. Tissue samples were generally taken from muscles or skin. For some species of small mammals, liver samples kept in alcohol were available. Tissue samples were subjected to DNA extraction, PCR amplification, and sequencing.

Collection of Field-Derived Feeding and Questing Ticks. Feeding ticks were collected from five species of mammals (*Apodemus sylvaticus*, *Clethrionomys glareolus*, *Sciurus vulgaris*, *Capreolus capreolus*, and *Erinaceus europaeus*) and from four species of birds (*Turdus merula*, *Turdus philomelos*, *Sitta europaea*, and *Parus major*). Ticks were identified to species, stage, and sex, and they were kept frozen at −80°C until DNA extraction.

Questing nymph and adult *I. ricinus* ticks were collected by flagging the vegetation in a woodland in Neuchâtel, Switzerland, in spring 2005. Ticks were identified to species, stage, and sex, and they were maintained at relative humidity close to saturation (RH > 95%) and at room temperature until DNA extraction. Questing ticks were analyzed for both host DNA and *Borrelia* identifications.

DNA Extraction. DNA was extracted from vertebrate muscle, skin, or liver tissues by using a DNeasy tissue kit (QIAGEN, Basel, Switzerland) according to the manufacturer's protocol. DNA was eluted in 200 μl of elution buffer (QIAGEN), the DNA concentration was measured with a spectrophotometer, and DNA extracts were stored at −20°C until further use.

Before DNA isolation, ticks were soaked in ethanol 70% and air-dried. DNA was extracted from unfed and feeding ticks by using a protocol described previously (Guy and Stanek 1991, Rijpkema et al. 1995). Briefly, feeding ticks were individually homogenized in 100 μl of 0.7 M ammonium hydroxide by using sterile filter tips or micropestles. Questing field-collected ticks were not homogenized, and they were placed as entire ticks in ammonium hydroxide. Tubes were incubated at 100°C for 15 min. After a quick cooling, tubes were left open and incubated at 100°C for 15 min to evaporate the ammonia. Negative controls (0.7 M ammonium hydroxide without tick) were included during each DNA extraction procedure from ticks. Tick lysates were stored at −20°C until use for PCR amplification.

PCR Amplification. Forward and reverse nondegenerate primers (12S-6F and B-12S-9R) (Table 1) were used to amplify the ≈145-bp 12S rDNA fragment that acts as molecular marker for the discrimination of vertebrates. PCR amplification was performed in a 50-μl reaction volume containing a total of 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 μM of each primers, 1× *Taq* buffer, 1.25 U of *Taq*DNA polymerase (QIAGEN) and ultrafiltrated H_2O. Twenty microliters of tick lysates or 100 ng of vertebrate DNA was used as template.

Table 1. Oligonucleotide sequences of primers and probes used in PCR amplification and RLB assays

Oligonucleotide primer or probe	Nucleotide sequence (5′–3′)	Target organisms
Primers		
12S-6F	CAAACTGGGATTAGATACC	Vertebrates
B-12S-9R	5′biotin-AGAACAGGCTCCTCTAG	Vertebrates
12S-12F	TGCCAGCCACCGCGGTCA	Vertebrates
12S-13R	AGGAGGGTGACGGGCGGT	Vertebrates
Probes		
Mammal	5′amino-AAAACTCAAAGGACTTGGC	Mammals
Small rodent	5′amino-GGCGGTACTTTATATCCAT	Muroidea (Muridae, Cricetidae)
Artiodactyl	5′amino-TATTCGCCAGAGTACTAC	Bovidae, Cervidae, Suidae
Bird	5′amino-TACGAGCACAAACGCTTAA	Birds
Lizard	5′amino-GAGAACTACAAGTGAAAAACT	Lizards
Clethrionomys	5′amino-AAACTTCAACATTTATAAAACAAAAG	*Clethrionomys glareolus*
Apodemus	5′amino-TAAACTTAAATAATTTAATAACAAAACTAT	*Apodemus sylvaticus, A. flavicollis*
M. agrestis/Micromys	5′amino-CTTAGCCCTAAACTTTAATAATT	*Microtus agrestis, Micromys minutus*
M. arvalis	5′amino-AAACCTCAATAATTTAGAAACAAAAA	*Microtus arvalis*
M. minutus	5′amino-AAACTTTAATAATTCCACAACAAAAT	*Micromys minutus*
Mus	5′amino-TGCTTAGCCATAAACCTAAAT	*Mus musculus*
R. norvegicus	5′amino-AACCTTAATAATTAAACCTACAAAAT	*Rattus norvegicus*
R. rattus	5′amino-ACCTTAATAATTACATCTACAAAAAT	*Rattus rattus*
Sciurus	5′amino-AACATAGACACTCAATTAACAAG	*Sciurus vulgaris*
Glis	5′amino-AAACCCTTACTAACGCAAC	*Myoxus glis*
Lepus	5′amino-TTAAACCTAAATAATTTCCTAACAAA	*Lepus europaeus*
Erinaceus	5′amino-GACAGTTACTTAACAAAATTGTA	*Erinaceus europaeus*
Sorex sp.	5′amino-AATACCCGCCAGAGAAC	*Sorex araneus, S. alpinus, S. minutus, S. coronatus*
S. araneus	5′amino-GGTATTTAACCTAACAAAAATAC	*Sorex araneus*
S. minutus	5′amino-ATCTAACAAGAATACCCGC	*Sorex minutus*
Neomys sp.	5′amino-ACAAAATTACTCGCCAGAG	*Neomys anomalus, N. fodiens*
N. anomalus	5′amino-TAAACTCAAGTAATTCACAAACA	*Neomys anomalus*
T. europaea	5′amino-ACCAAGACAATCAAGTTAACA	*Talpa europaea*
Vulpes	5′amino-CTATAACAAAACAATTCGCCA	*Vulpes vulpes*
Meles	5′amino-GATAACTCACAGAACAAAACT	*Meles meles*
M. erminea	5′amino-CATAAATAGTTCTAACAACAAAAC	*Mustela erminea*
M. putorius	5′amino-CTAAACATAAATAATTATCACAACAA	*Mustela putorius*
Capreolus	5′amino-CCTAAACACAAGTAATTAATATAACAA	*Capreolus capreolus*
Sus	5′amino-ACCCAAATAGTTACATAACAAAA	*Sus scrofa*
Homo	5′amino-ACCTCAACAGTTAAATCAACA	*Homo sapiens*
Turdus/Parus	5′amino-TGATGCTCGATATTACCTG	*Turdus merula, T. iliacus, T. philomelos, T. pilaris, Parus major, P. caeruleus*
Erithacus	5′amino-ATCTTGATGCTCCACCTTA	*Erithacus rubecula*
Parus	5′amino-TGAGCGTCCGCCTGA	*Parus major, P. caeruleus*
P. ater	5′amino-TTGATGCTTAACATTACCTGA	*Parus ater*
Fringilla/Pyrrhula	5′amino-TGATGCTTACCCCTACTAA	*Fringilla coelebs, F. montifringilla, Pyrrhula pyrrhula*
Prunella	5′amino-TGATGCTTAACCCTACCTA	*Prunella modularis*
Sitta	5′amino-TATACAACCTAAGCATCCG	*Sitta europaea*
Sylvia	5′amino-GCTCGATCTTACTGGAG	*Sylvia atricapilla*
Troglodytes	5′amino-TGATGCTTTATATAACCCAAG	*Troglodytes troglodytes*
Garrulus	5′amino-TTGACACTCTATGCTACCT	*Garrulus glandarius*

Touch-down PCR conditions were used: initial denaturation step for 3 min at 94°C, first cycle of 20 s at 94°C, 30 s at 60°C, and 30 s at 72°C; the following cycles are identical except for the annealing temperature, which was lowered by 1°C at each cycle until it reached 52°C, then 20 cycles (for vertebrate DNA extracts) or 40 cycles (for tick lysates) were carried out (20 s at 94°C, 30 s at 52°C, and 30 s at 72°C) and a final extension step at 72°C for 7 min. PCR products were stored at 4°C until use for reverse line blotting.

For sequencing, external forward and reverse primers (12S-12 F and 12S-13R) (Table 1) were used to amplify the ≈600-bp 12S rDNA fragment, including the complete molecular marker. The PCR amplification was carried out for 25 cycles (denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 1 min) with initial denaturation step at 94°C for 2 min and a final extension step at 72°C for 10 min. After amplification, the presence of PCR products was checked on a 1% agarose gel. PCR products were stored at −20°C until use for sequencing.

All PCR amplifications were performed in a TGradient thermocycler (Biometra, Goettingen, Germany). For each PCR reaction, negative and positive controls were included. Great care was exercised to prevent contaminations. PCR setup, DNA extraction, sample additions, and PCR amplification and post-PCR analyses were performed in four separate rooms. PCR setup was done in a cabinet with built-in UV lamps. PCR setup and DNA extraction were performed in rooms restricted to this project only. Each area had its own dedicated set of pipettes and sterile filter tips.

Table 2. Vertebrate species 12S rDNA sequences subjected to multi-alignment for search of molecular marker and probe design, and GenBank accession numbers

Species[a]	Common name	Amplicon size (bp)	No. sequenced in this study	Accession nos.[b]
Mammals				
*Apodemus flavicollis**	Yellow-necked mouse	144	2	AJ311127, AJ311164, EF027238, EF027239
*Apodemus sylvaticus**	Wood mouse	144	2	AJ311126, AJ311131, EF027240, EF027241
*Arvicola terrestris**	Ground vole	145	1	EF027242
*Bos taurus**	Cow	145		V00654
*Canis familiaris**	Dog	144		U96639, AY012152, Y08507
*Capra ibex**	Alpine ibex	145		AY846815
*Capreolus capreolus**	Roe deer	145	2	EF027243, EF027244
*Cervus elaphus**	Red deer	145		AF091707
*Clethrionomys glareolus**	Bank vole	144	4	AJ250356, AJ005781, EF027245–EF027248
Crocidura russula	White-toothed shrew	144	3	AY769263, AY769264, EF027249–EF027251
*Eliomys quercinus**	Garden dormouse	143	1	Y16896, EF027252
Equus caballus	Horse	146		X79547
*Erinaceus europaeus**	European hedgehog	143	2	X88898, EF027253, EF027254
*Felis catus**	Cat	146		U20753, AY012149, Y08503
*Homo sapiens**	Human	145		NC_001807
*Lepus europaeus**	European hare	144	2	AJ421471, EF027255, EF027256
Lynx lynx	Eurasian lynx	n.d.		D28891
*Meles meles**	Eurasian badger	145	1	Y08513, EF027257
Micromys minutus	Harvest mouse	143	3	AJ311139, EF027258–EF027260
*Microtus agrestis**	Field vole	144	4	EF027261–EF027264
*Microtus arvalis**	Common vole	144	1	EF027265
*Microtus nivalis***	Snow vole	144		X99464
Mus musculus[c]	Mouse	146	2	X84382, EF027267, EF027268
Muscardinus avellanarius	Hazel dormouse	143	3	X84384, EF027269–EF027271
*Mustela erminea**	Stoat/ermine	145	1	AF068553, EF027272
*Mustela nivalis***	Least weasel	145	1	Y08515, AF068554, EF027273
*Mustela putorius**	European polecat	145	1	Y08516, AF068550, U12844, EF027274
*Myoxus glis**	Fat dormouse	143	1	AJ001562, X84385, EF027266
*Neomys anomalus**	Miller's water shrew	144	2	AF182177, AF182178, AF182173, AF182172, EF027275, EF027276
*Neomys fodiens**	Eurasian water shrew	144	2	AF182174, AF182175, EF027277, EF027278
*Oryctolagus cuniculus**	Rabbit	144		AJ001588
*Ovis aries**	Sheep	144		AF010406
*Rattus norvegicus***	Brown rat	145	1	X14848, EF027279
*Rattus rattus***	Black rat	145		AJ005780
*Sciurus vulgaris**	Red squirrel	144	2	AJ238588, EF027280, EF027281
*Sorex alpinus**	Alpine shrew	145	1	EF027282
*Sorex araneus**	Common shrew	145	4	AY012102, EF027283–EF027286
Sorex coronatus	Millet's shrew	145	3	EF027287–EF027289
Sorex minutus	Pigmy shrew	145	3	EF027290–EF027292
*Sus scrofa**	Wild boar	144	2	AF034253, AJ002189, EF027293–EF027294
*Talpa europaea**	European mole	145	2	Y19192, EF027295, EF027296
*Vulpes vulpes**	Red fox	144		Y08508
Birds				
*Buteo buteo**	Common buzzard	146	2	AF380305, U83719, EF027297, EF027298
Carduelis carduelis	European goldfinch	147	1	EF027299
*Certhia brachydactyla**	Short-toed tree creeper	147	1	EF027300
*Coccothraustes coccothraustes**	Hawfinch	147	1	EF027301
*Corvus corone**	Carrion crow	147		AF386463
*Delichon urbica**	House martin	146	1	EF027302
*Erithacus rubecula**	European robin	147	2	EF027303, EF027304
*Falco tinnunculus**	Common kestrel	148	1	EF027305
*Fringilla coelebs**	Chaffinch	147	3	AF407088, AF447231, EF027306–EF027308
*Fringilla montifringilla**	Brambling	147	1	EF027309
*Gallus gallus**	Chicken	147		AY235571
*Garrulus glandarius**	Eurasian jay	147	1	EF027310
*Parus ater**	Coal tit	147	2	EF027311, EF027312
*Parus caeruleus**	Blue tit	147	1	EF027313
*Parus major**	Great tit	147	2	AY136557, EF027314, EF027315
*Passer domesticus**	House sparrow	147	2	AF407085, AF447245, EF027316, EF027317
*Passer montanus**	Tree sparrow	145		AB042360
*Perdix perdix***	Grey partridge	145	1	AF222590, EF027318
*Phasianus colchicus**	Common pheasant	145	1	U83742, EF027319
Phoenicurus ochruros	Black redstart	147	1	EF027320
*Phylloscopus collybita**	Common chiffchaff	147	1	EF027321
*Phylloscopus trochilus**	Willow warbler	147	2	AF407093, EF027322, EF027323
*Pica pica**	European magpie	147	1	EF027324
Picus viridis	Green woodpecker	150	1	EF027325

(continued)

Table 2. continued

Species[a]	Common name	Amplicon size (bp)	No. sequenced in this study	Accession nos.[b]
*Prunella modularis**	Dunnock/hedge accentor	147	1	AF407081, EF027326
*Pyrrhula pyrrhula**	Eurasian bullfinch	147	2	EF027327, EF027328
*Serinus serinus**	European serin	147		AF447255
*Sitta europaea**	Eurasian nuthatch	147	1	AB042353, EF027329
*Sturnus vulgaris**	Common starling	147	1	AF407091, EF027330
*Sylvia atricapilla**	Blackcap	147	3	EF027331–EF027333
*Troglodytes troglodytes**	Wren	147	1	EF027334
*Turdus iliacus**	Redwing	147	1	EF027335
*Turdus merula**	Blackbird	147	3	EF027336–EF027338
*Turdus philomelos**	Song thrush	147	1	AF484935, EF027339
*Turdus pilaris**	Fieldfare	147	2	EF027340, EF027341
Reptiles				
*Lacerta agilis**	Sand lizard	n.d.[d]		AF149947, AF080298
*Lacerta viridis**	Green lizard	n.d.		AF149958, AF149960–AF149962, AJ001480
Podarcis muralis	Wall lizard	146	1	AF440603, AJ001468–AJ001470, AY190305, EF027342, EF027343

[a] Species marked with an asterisk (*) are known hosts for *I. ricinus* ticks in Switzerland (Aeschlimann 1972, Papadopoulos et al. 2002); species marked with 2 asterisks are known hosts for *I. ricinus* ticks in Europe (Arthur 1963, Matuschka et al. 1997).
[b] Sequences obtained from GenBank database or obtained in this study (accession numbers starting with EF).
[c] Laboratory host species.
[d] n.d., not determined due to incomplete sequence.

Sequencing of Vertebrate 12S rDNA Molecular Marker. The ≈600-bp PCR products were purified with QIAquick PCR purification kit (QIAGEN). Cycle sequencing was performed using the dideoxy chain termination method by using an ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, Rotkreuz, Switzerland). Cycle sequencing parameters were as follows: denaturation at 96°C for 15 s, primer annealing at 53°C for 15 s, and extension at 60°C for 4 min. The cycle sequencing products were purified either by ethanol precipitation or with DyeEx spin columns (QIAGEN) and applied to an ABI Prism 310 genetic analyzer (Applied Biosystems). DNA sequences were checked with Sequence Navigator software (Applied Biosystems). Sequences from the same species were aligned with each other and with respective sequences from the GenBank database when available. The 12S rDNA marker sequences of various vertebrates obtained in this study are available in the GenBank database under respective accession numbers (Table 2).

Sequence Analysis and Probe Design. The 143–150-bp 12S rDNA marker sequences (newly sequenced or retrieved from the GenBank database) of various vertebrate species hosts for *I. ricinus* ticks were aligned using ClustalX multi-alignment software embl.net. Areas of genetic variability between species or between groups of vertebrates were used to design oligonucleotide probes using the Oligo software Primers 3.

Reverse Line Blot Hybridization. The reverse line blotting method for the identification of host DNA in ticks developed herein is based on the RLB technique described by Rijpkema et al. (1995)). Briefly, the probes were covalently linked to an activated Biodyne C membrane (Pall, Dreieich, Germany) by their 5′ amino group. For this purpose, the membrane was placed in a miniblotter system (Immunetics, Cambridge, MA), and line slots were filled with the different probes (100–500 pmol). After a quick incubation (at least 1 min at room temperature), the excess of probes was removed, and the membrane was inactivated in 100 mM NaOH for 10 min (maximum) and washed in 2× SSPE buffer/0.1% SDS (SSPE; Invitrogen, Basel, Switzerland) for 5 min at 60°C. Then, the membrane was placed in the miniblotter, but the position of the membrane was 90° rotated compared with the previous position. The slots were filled with the biotin-labeled heat-denatured PCR products, and the membrane was incubated for 60 min at 42°C. Then, the PCR product solutions were removed, and the membrane was washed twice in 100 ml of 2× SSPE/0.5% SDS for 10 min at 55°C and incubated with streptavidin-peroxidase conjugate (Roche Diagnostics, Rotkreuz, Switzerland) at 42°C for 30 min. After a washing step, the membrane was incubated for 1–2 min. with enhanced chemiluminescence detection liquid (GE Healthcare, Otelfingen, Switzerland) and exposed to X-ray film Hyperfilm (GE Healthcare).

Detection and Identification of *Borrelia* Species in Ticks. For the detection and identification of *B. burgdorferi* s.l. species in ticks, we used the PCR and RLB hybridization method described previously (Alekseev et al. 2001, Burri et al. 2007). Oligonucleotide probes used are described in Poupon et al. (2006).

Statistical Analysis. Statistics were calculated with S-Plus 7.0 for Windows (Insightful Corp., Seattle, WA). The Fisher exact test was used to assess the success of the host DNA identification method between nymphs and adults (statistical significance, $P \leq 0.05$).

Results

Sequencing and Alignment of 12S rDNA Marker of Various Vertebrate Species. Using external primers, the 12S rDNA molecular marker was sequenced for a broad range of vertebrate species belonging to the fauna of Switzerland: 28 species of mammals, 31 spe-

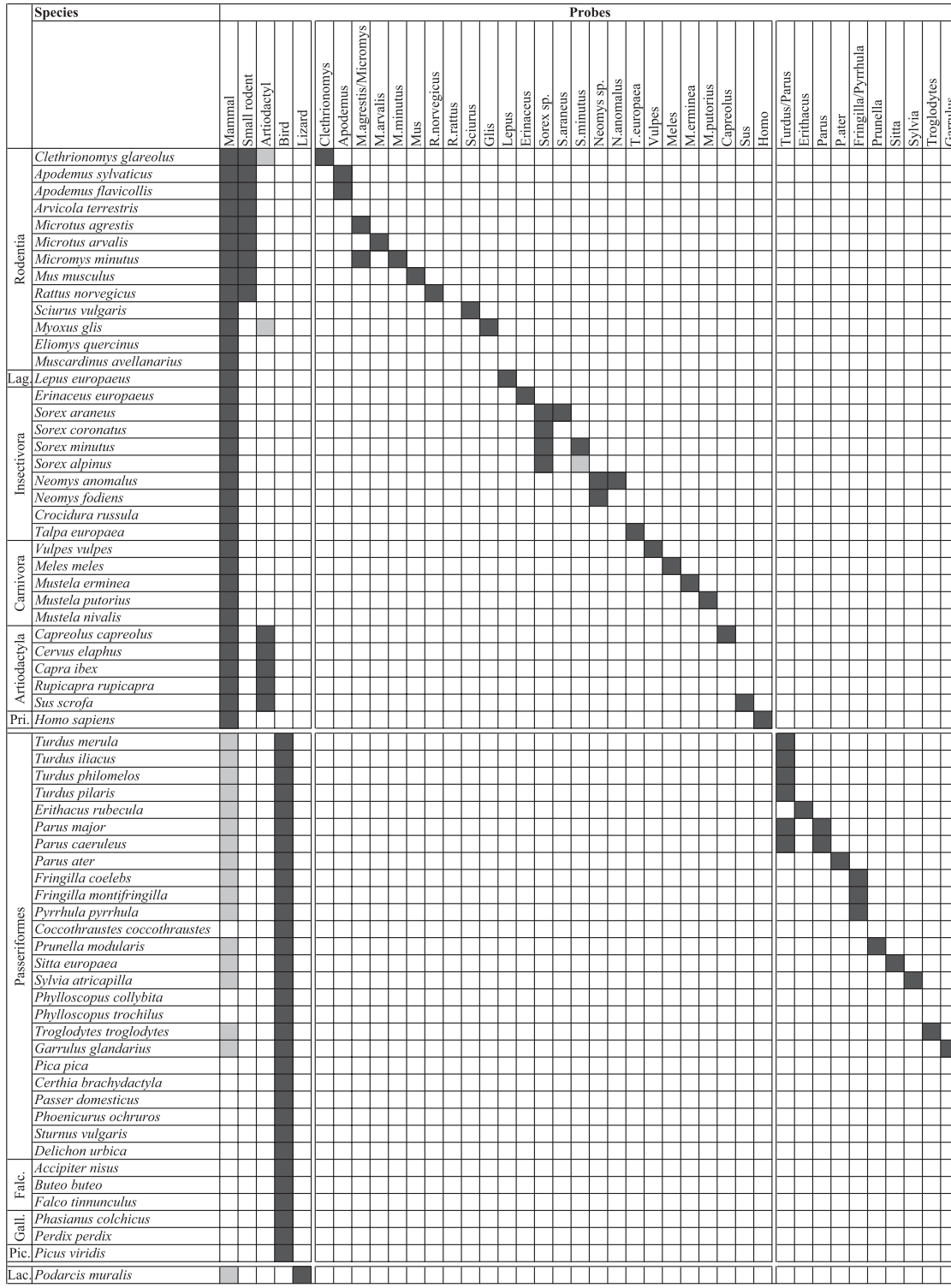

Fig. 1. Schematic representation of the RLB hybridization analysis of oligonucleotide probes with various host DNA. ■, strong hybridization; ▨, slight hybridization may occur. Falc., Falconiformes; Gall., Galliformes; Lac., Lacertidae; Lag. Lagomorpha; Pic. Piciformes; and Pri., Primates.

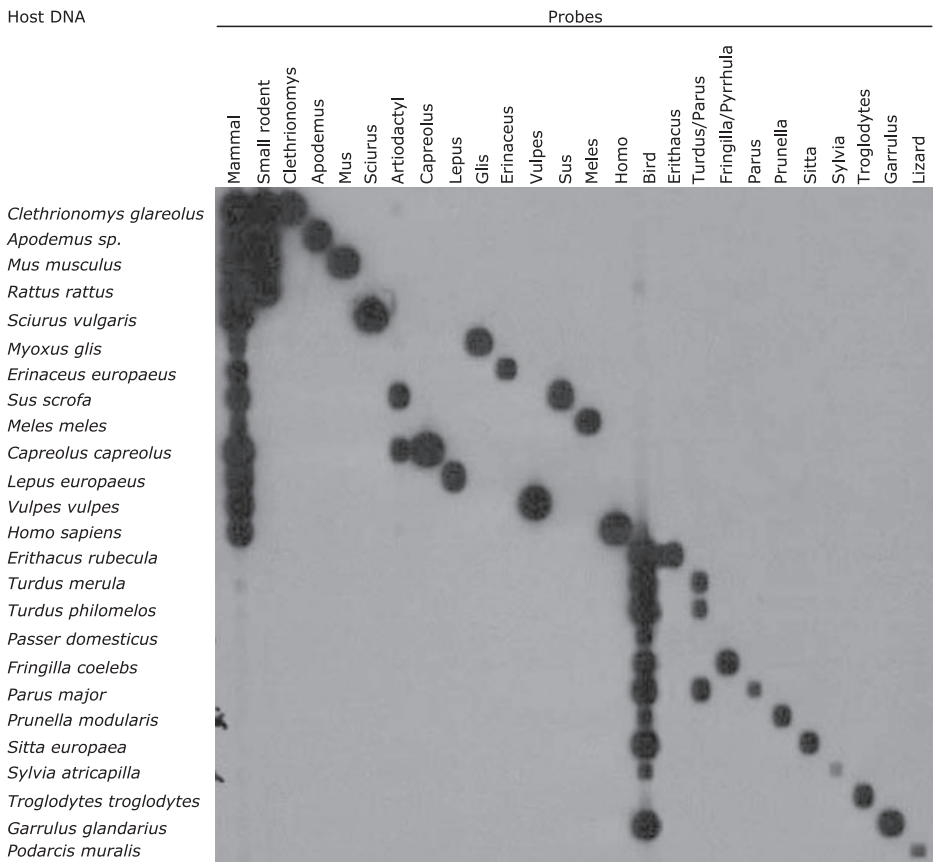

Fig. 2. Reverse line blot assay for the identification of vertebrate host species in host samples by using group- and species/genus-specific probes.

cies of birds, and one species of reptiles (Table 2). Some species are known hosts for *I. ricinus* ticks in Switzerland (Aeschlimann 1972, Papadopoulos et al. 2002, Poupon et al. 2006) and in Europe. If possible, sequences from two or more individuals of each species originating from different sites in Switzerland were obtained and aligned with sequences retrieved from the GenBank database if available. For a few species, only sequences from GenBank database were available. Overall, we aligned 12S rDNA sequences from a large spectrum of vertebrate species including 42 species of mammals, 35 species of birds, and three species of reptiles (Table 2). According to the list of known host species of *I. ricinus* in Switzerland (Aeschlimann 1972, Papadopoulos et al. 2002, Poupon et al. 2006), only three species of mammals (*Martes martes*, *Martes foina*, and *Rupicapra rupicapra*) and 20 species of birds were missing.

The 12S rDNA molecular marker shows very low intraspecies genetic variability. For genetic variability between sequences retrieved from GenBank and those obtained in this study, only sequences derived from vertebrates collected in Switzerland were considered for the consensus sequence.

Design of Specific Probes. The partial 12S rDNA consensus sequences of 42 species of mammals, 35 species of birds and three species of reptiles were multi-aligned to design specific probes. Five probes were designed to discriminate main groups of vertebrate hosts: mammals, birds, small rodents (Muroidea), lizards, and artiodactyls, which include Bovidae, Cervidae, and Suidae (Table 1). Twenty-four and 10 probes, respectively, were designed to identify major mammal and bird *I. ricinus* hosts at the genus or species level (Table 1). The design of species-specific probes seemed to be impossible for the discrimination of some species (e.g., *Apodemus* spp. and *Turdus* spp.).

Identification of Vertebrate DNA by RLB. To assess the specificity of the five group-specific probes and the 34 genus- or species-specific probes (Table 1), identification of vertebrate DNA by RLB was performed using DNA extracted from the aforementioned Swiss fauna tissue collection ($n = 60$) and from one laboratory species (*Mus musculus*) (Fig. 1). Overall, all probes showed a strong hybridization with the respective host DNA (Fig. 1). In a few cases, a weak cross-hybridization occurred, in particular with group-specific probes. The artiodactyl probe showed a weak cross-hybridization with vole DNA (*C. glareolus*) and dormouse DNA (*Myoxus glis*). The mammal probe showed a strong hybridization with all tested

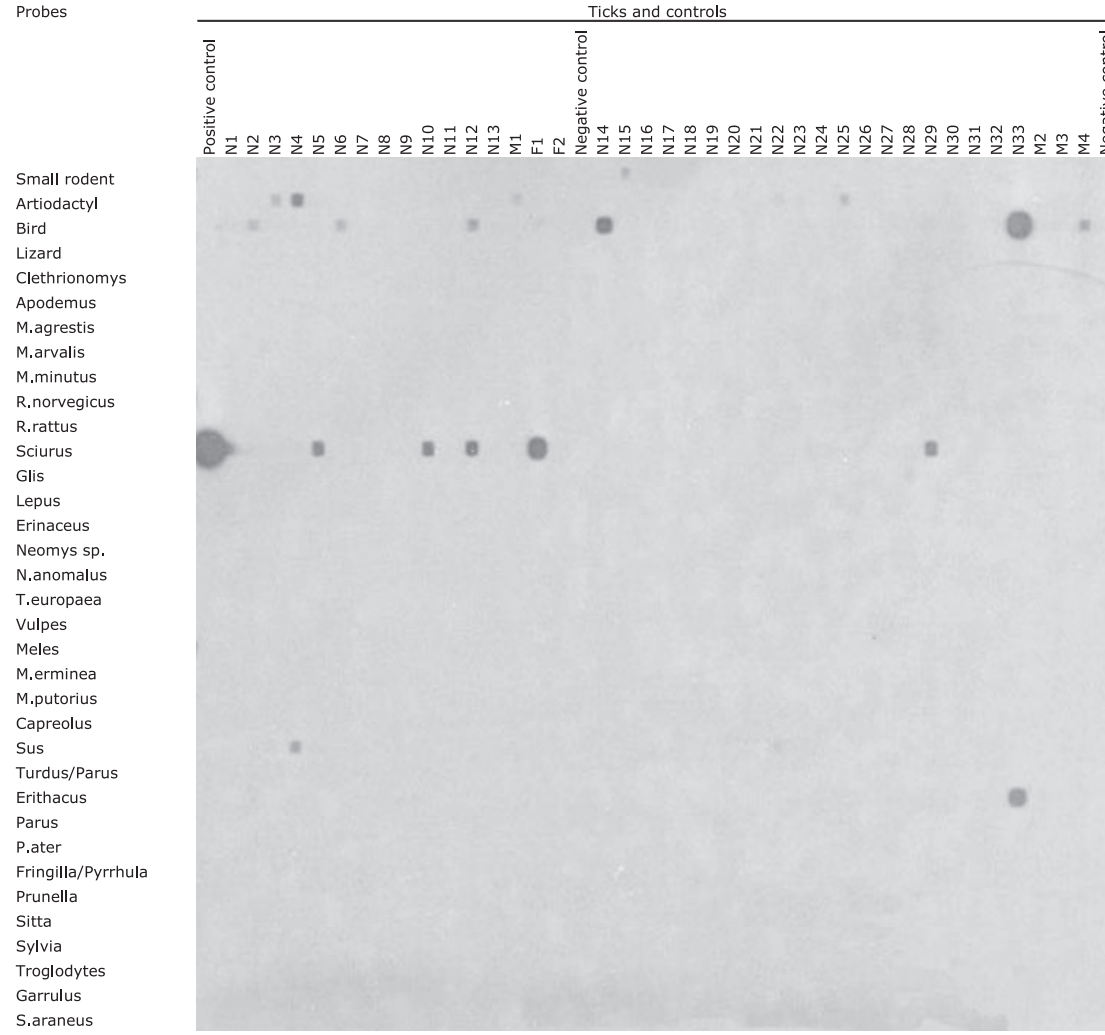

Fig. 3. Reverse line blot assay for the identification of vertebrate host species in questing *I. ricinus* ticks (Neuchâtel, Switzerland) using group- and species/genus- specific probes. N, nymph; F, female; M, male; X and H, different batches of ticks.

mammalian DNA, but turned out to be less specific because it weakly hybridized with DNA from 16 bird species as well as with DNA from a lizard species, *Podarcis muralis*. In contrast, the bird probe was highly specific and allowed to unambiguously discriminate avian hosts from nonavian hosts. The lizard probe was also highly specific. The other 34 probes allowed to clearly identify vertebrate hosts either at the genus level (e.g., the *Apodemus* probe) or at the species level (e.g., the *M. putorius* probe) (Fig. 1). The *Turdus*/*Parus* probe and the *Parus* probe had to be used both to discriminate *Turdus* sp. DNA from *Parus* sp. DNA. In fact, the *Turdus*/*Parus* probe reacted to *Turdus* sp. and *Parus* sp., whereas the *Parus* probe reacted to *Parus* sp. only. Figure 2 shows the RLB hybridization patterns for some host species.

Host Identification in Feeding Ticks. Host identification was performed in nine *I. ricinus* ticks collected while feeding on five mammal and four bird species (*A. sylvaticus* (one larva), *C. glareolus* (one larva), *S. vulgaris* (one nymph), *E. europaeus* (one female), *C. capreolus* (one female), *T. merula* (one nymph), *T. philomelos* (one nymph), *P. major* (one nymph), and *S. europaea* (one nymph). In all nine ticks, host DNA was present and showed a perfect hybridization pattern according to the probe specificity.

Host and *Borrelia* Identifications in Unfed Field-Collected Ticks. To assess the utility of the present host identification method under field conditions, nymphal and adult ticks collected in Neuchâtel were tested for the presence of host and *Borrelia* DNA (Fig. 3). The detection of host DNA in questing ticks required the use of a high number of PCR amplification cycles, and as a result, contamination with human DNA could not be avoided. Therefore, mammal and *Homo* probes for detection of mammalian and human DNA, respectively, were removed from our set of probes.

Table 3. Host DNA identification in field-collected questing *I. ricinus* ticks by RLB assay (Neuchâtel, Switzerland)

Host identification		Nymphs	Adults	Total
Group identification				
Small rodents		1	1	2
Birds		3	4	7
Artiodactyls		5	3	8
Subtotal		9	8	17
Specific or generic identification				
Clethrionomys glareolus	Bank vole		1	1
Sciurus vulgaris	Red squirrel	5	11	16
Capreolus capreolus	Roe deer	1	6	7
Sus scrofa	Wild boar	3		3
Mustela putorius	European polecat		3	3
Mustela erminea	Ermine		1	1
Erithacus rubecula	Robin	1		1
Turdus sp.			1	1
Subtotal		10	23	33
Multiple identification				
Artiodactyl/bird/European mole (*T. europaea*)		1		1
Bird/red squirrel (*S. vulgaris*)		1		1
Bird/wild boar (*S. scrofa*)			1	1
Subtotal		2	1	3
Total		21	32	53

In total, 55 nymphal, 28 male, and 26 female *I. ricinus* ticks were tested for host and *Borrelia* identifications. Host DNA could be detected in half of questing *I. ricinus* ticks (53/109; 48.6%). Detection was significantly higher in adults (32/54; 59.3%) than in nymphs (21/55; 38.2%) ($P = 0.035$; Fisher exact test) and slightly varied between sexes (15/28 males; 53.6% and 17/26 females; 65.4%). For one third of the questing ticks with a bloodmeal identified (17/53; 32.1%), host identification was possible only at the group level (small rodents [Muroidea], birds, or artiodactyls) (Table 3). For virtually two thirds of the *I. ricinus* ticks with identified host DNA (33/53; 62.3%), host identification could be achieved at the species or genus level (Table 3). In three questing *I. ricinus* ticks, the presence of a mixture of DNA from two or three different hosts was observed (Table 3). Red squirrel (*S. vulgaris*) followed by roe deer (*C. capreolus*) were the species from which DNA was most frequently detected in questing ticks (Table 3). DNA from artiodactyls ($n = 20$) (including eight artiodactyls, seven *C. capreolus*, three *S. scrofa* and two multiple identifications), and rodents ($n = 20$) (including two Muroidea, 16 *S. vulgaris*, one *C. glareolus*, and one multiple identification) were most frequently detected followed by birds ($n = 12$) (including seven birds, one *E. rubecula*, one *Turdus* sp., and three multiple identifications) and carnivores ($n = 4$) (including three *M. putorius* and one *M. erminea*).

Borrelia infection was detected in 23/109 (21.1%) questing *I. ricinus* ticks. In 15 of these 23 infected ticks (65.2%), identification of the blood source was also possible (Table 4). In these ticks, single infection with *B. burgdorferi* sensu stricto ($n = 5$), *Borrelia afzelii* ($n = 2$), *Borrelia garinii* ($n = 1$), *Borrelia valaisiana* ($n = 5$), and untypeable *Borrelia* ($n = 2$) was observed (Table 4). No multiple infections were detected. *B. burgdorferi* ss and *B. afzelii* infections were observed with squirrel DNA, and *B. valaisiana* was observed with bird DNA. In ticks containing blood remnants from artiodactyls, DNA from *B. afzelii*, *B. burgdorferi* s.s., and *B. valaisiana* was detected (Table 4).

Discussion

This report describes the development of an efficient new technique for the identification of bloodmeal sources in the tick *I. ricinus*, the European vector of the agents of Lyme borreliosis and tick-borne encephalitis. The method consists of a single-run PCR amplification of the 12S rDNA molecular marker using a single set of nondegenerate primers followed by RLB hybridization assay by using specific probes. This method allowed identifying the bloodmeal source in half of questing *I. ricinus* ticks collected from vegetation (49%). This sensitivity was similar to those obtained in previous studies using 18S rDNA as target (Pichon et al. 2003, 2005; Estrada-Peña et al. 2005). The identification to the genus or species level occurred for two thirds of ticks with bloodmeal identification. This represents a more precise identification compared with previous studies that identified host

Table 4. Bloodmeal source identification in *B. burgdorferi* s.l.-infected questing *I. ricinus* ticks (Neuchâtel, Switzerland)

Host identification	No. infected *I. ricinus* ticks[a]				
	Bb s.s.	*Ba*	*Bg*	*Bv*	*Borrelia* sp.
Red squirrel	2N, 1M[b]	1N			1N
European polecat	1F				
Small rodents					1F
Birds				2N, 1M	
Artiodactyls	1N	1N		1N	
Bird/wild boar				1F	
Artiodactyl/bird/mole			1N		
Total	5	2	1	5	2

[a] *Ba*, *B. afzelii*; *Bb* s.s., *B. burgdorferi* sensu stricto; *Bg*, *B. garinii*; *Bv*, *B. valaisiana*; and *Borrelia* sp., untypeable *Borrelia*.

[b] N, nymph; M, male; and F, female.

groups rather than host species (Pichon et al. 2003, 2005).

That questing ticks were collected at the beginning of the tick activity season (spring) suggests that the previous bloodmeal occurred during the previous tick activity season and that the method is sensitive enough to detect and identify the source of bloodmeals, which are several months old. A better sensitivity might be expected with the summer/autumnal tick cohort, as observed by Pichon et al. (2005). The identification of DNA from two or three different hosts in a single tick suggests that interrupted feeding may occur in *I. ricinus.* Possibly, this may contribute to the transfer of tick-borne pathogens. However, such transmission probably rarely occurs.

Coupled with the RLB method for the detection and identification of *B. burgdorferi* s.l. (Alekseev et al. 2001, Poupon et al. 2006, Burri et al. 2007), the host identification technique could confirm the existence of associations between hosts and *Borrelia* genospecies, notably between red squirrels, *B. burgdorferi* s.s., and *B. afzelii* (Humair and Gern 1998) as well as between birds, *B. garinii,* and *B. valaisiana* (Humair et al. 1998). Currently, our sample sizes are too small to confirm previous observations. The presence of *B. afzelii*, *B. valaisiana*, and *B. burgdorferi* s.s. in ticks in which artiodactyl blood remnants were detected was more surprising, because these hosts are generally considered zooprophylactic (Gern and Humair 2002). One explanation may be that these ticks have been infected through cofeeding transmission of the pathogens from infected to uninfected ticks feeding on uninfected artiodactyls (Hu et al. 2003). The overall prevalence of *Borrelia* infection in ticks (21.1%) in this study was similar to prevalences observed in other tick populations in Switzerland (Jouda et al. 2003, 2004a,b; Burri et al. 2007).

The target gene for the identification of bloodmeal sources is the 12S rRNA gene, which encodes for the small subunit ribosomal RNA in mitochondria. Mitochondrial DNA (mtDNA) presents peculiar features, such as lack of recombination, maternal inheritance, and presence of orthologous genes, and it has been used extensively in phylogenetic and evolutionary studies. In addition, mtDNA occurs in high copy numbers in a eukaryote cell; therefore, is a molecule of choice when analyzing small quantity of degraded biological material, as in the case of bloodmeal remnants in tick midguts. The 12S rRNA gene was investigated because it is described with some tRNAs as the most conserved regions of the mitochondrial genome (Saccone et al. 1999). Various authors have used the cytochrome *b* gene as a molecular marker to identify bloodmeals in Diptera (Boakye et al. 1999, Lee et al. 2002, Ngo and Kramer 2003) and in ticks (Tobolewski et al. 1992, Kirstein and Gray 1996). A preliminary analysis (unpublished) demonstrated that the cytochrome *b* gene is subject to a high inter- and intraspecific genetic variability, which could have lead to a problematic probe and primer design for the large spectrum of *I. ricinus* hosts. However, the nuclear 18S rRNA gene, used in other studies (Pichon et al. 2003, 2005; Estrada-Peña et al. 2005), is too much conserved to identify the genus or species origin of tick bloodmeals.

The small size of the molecular marker is crucial for the host identification in questing ticks. In the case of degraded DNA, as occurs in questing ticks, an inverse correlation exists between the efficiency of PCR amplification and the size of the amplicon (Kirstein and Gray 1996). The nondegenerate character of primers avoids mispriming and enhances the specificity of DNA amplification reaction. High interspecies or intergenus genetic variability and a low intraspecies polymorphism of the molecular marker allow a precise identification of the hosts that ticks have been feeding on.

The palette of oligonucleotide probes used in the RLB allowed at the same time to distinguish major groups of host vertebrates and to identify bloodmeal sources at the genus or species level. Forty oligonucleotide probes were designed to identify groups, genera or species that may serve as natural or laboratory hosts for *I. ricinus.* The analysis of various vertebrate DNA samples from our tissue library demonstrated that the specificity of the method was very good and allowed a correct identification of a large range of host species. However, in some cases, the precise identification at the species level was not possible. For mammals, for example, the *Apodemus* probe did not permit to distinguish *A. flavicollis* from *A. sylvaticus,* and the *M. agrestis/Micromys* probe did not discriminate *M. agrestis* and *M. minutus.* For some avian hosts, the specificity was even lower as in the case of the *Turdus/Parus* probe that reacts to four *Turdus* species and two *Parus* species. The concurrent use of the *Parus* probe is necessary to distinguish *Turdus* sp. from *Parus* sp.

The sensitivity of the technique becomes crucial when analyzing host-seeking *I. ricinus* ticks collected from the vegetation. Host blood remnants in field-collected tick midguts come from the previous instar, may be several months old and are present in small quantity and in a degraded state. To enhance the technique's sensitivity, the number of PCR amplification cycles was increased. The use of nested PCR was avoided because of the high risk of contaminations with products from the first PCR run. Using the 40-cycle PCR procedure, human contamination was very frequent. Therefore, the mammal and the *Homo* probes were discarded when analyzing field-collected ticks. In addition, to avoid any other contamination, extremely strict conditions were applied to the method. For example, PCR setup and DNA extraction were performed in rooms dedicated to this use and restricted to this project.

In conclusion, the present technique based on PCR amplification of a 12S rDNA molecular marker and RLB hybridization assays provides a rapid and accurate method for determining the source of bloodmeals in *I. ricinus* ticks collected in the field. This method could be used to identify new host species of arthropod vectors. Coupled with methods for identification of vector-borne pathogens, this technique should allow researchers to determine the source of infective

bloodmeal, identify reservoir hosts at the species level, and assess the importance of a host species in a habitat. Further development of this technique may be envisaged for other arthropod vectors.

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