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Short Communication

Diagnostic DNA amplification from individual tick eggs, larvae and nymphs

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ABSTRACT

The species of single tick eggs, larvae and nymphs was determined by PCR amplification and characterization of the hypervariable, second transcribed spacer (ITS2) of the multicopy ribosomal RNA gene (rDNA). Engorgement of larvae and nymphs did not preclude species identification. The method is generally applicable for ixodid and argasid ticks and can be used for epidemiological studies requiring the identification of individuals from pre-adult stages.

Key words: ticks, species identification, DNA amplification, ribosomal DNA.

It is difficult, most often even impossible, to determine the species of tick nymphs, larvae and especially eggs by their morphology. The application of molecular genetic techniques, however, offers an alternative approach: DNA-based methods have already been employed to identify the species of adult ticks by single-strand conformation polymorphism (SSCP) analysis (Hiss *et al.*, 1994) and DNA sequencing (Wesson *et al.*, 1993; Black and Piesman, 1994; McLain *et al.*, 1995; Zahler *et al.*, 1995). Even more desirable than for adult ticks would be the application of molecular techniques to the species identification of eggs and in particular larvae and nymphs. Until now, the only successful attempt has been on single nymphs and on egg masses (Wesson *et al.*, 1993), but not with individual eggs or larvae.

In this report, using *Dermacentor reticulatus* as a model, we describe a highly sensitive and specific method to determine genetically the species of individual eggs, larvae and nymphs. The method is applicable to both unfed and engorged larvae and nymphs.

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DNA isolation

Individual eggs as well as larvae and nymphs, both unfed and engorged on rabbits, of *D. reticulatus* were placed in 1.5 ml microcentrifuge tubes and coarsely crushed with a disposable inoculation needle. The material was thoroughly mixed with 100 μl TET buffer (50 mM Tris–HCl, pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.4% Triton X-100) and 2 μl (20 mg ml^{-1}) proteinase K (United States Biochemical Corp., Cleveland) by stirring and shaking on a vortex mixer. After incubation at 50°C for 2 h, the DNA was extracted by shaking with 100 μl PCI (phenol : chloroform : isoamyl alcohol 25 : 24 : 1) which had been saturated with 10 mM Tris–HCl, pH 8.0, containing 1 mM EDTA (Sigma, St Louis). After centrifugation, the aqueous phase was extracted twice with equal volumes of chloroform. Identical set-ups without tick material were processed simultaneously as negative controls.

DNA amplification and characterization

Ten microlitres of each of the DNA solutions were used for polymerase chain reaction (PCR) amplifications of the second internal transcribed spacer (ITS2) of the ribosomal RNA gene (rDNA) with primers RIB-3 (CGG GAT CCT TC(A,G) CTC GCC G(C,T)T ACT) and RIB-4 (CCA TCG ATG TGA A(C,T)T GCA GGA CA) as described before (Zahler *et al.*, 1995). The use of more than 10 μl repeatedly prevented PCR products, presumably due to the presence of polymerase inhibitors. PCR amplifications were performed employing a 'hot start' technique in which the DNA solution, 28 μl water, 6 μl 50 mM $MgCl_2$, 2 μl DMSO and 1 μl each of both primers (50 μM each) were overlain with two drops of mineral oil (Sigma, St Louis) and denatured at 96°C for 2 min. After cooling to 90°C, a mixture of 5 μl buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl), 0.5 μl dNTP-mix (25 mM each; United States Biochemical Corp., Cleveland) and 0.5 μl (5 u μl^{-1}) *Taq* DNA polymerase (Amersham Buchler, Braunschweig, Germany) was added. Thirty-five cycles were performed with denaturation at 92°C for 60 s, annealing at 64°C for 60 s and extension at 72°C for 90 s. PCR products were visualized in a 1.3% agarose gel containing 0.2 μg ml^{-1} ethidium bromide (Sigma, St Louis). For restriction fragment length polymorphism (RFLP) analyses, the DNA was excised from the agarose gel, purified by silica gel adsorption (Geneclean; Bio 101, La Jolla) and eluted in 20 μl water. Ten microlitres each were digested with 1 μl of either *Pst*I (20 u μl^{-1}) or *Xho*I (12 u μl^{-1}) after the addition of 1.2 μl of the appropriate 10X buffer and separated on a 1.3% agarose gel as above. For DNA sequencing, 20 μl of the PCR reaction was run on an agarose gel, the DNA purified by silica gel adsorption as described above and then eluted in 7 μl water. After the addition of 1 μl (5 μM) sequencing primer (RIB-4: Zahler *et al.*, 1995), boiling for 5 min and snap-cooling on ethanol/dry ice (Seetharam and Dicker, 1991) the DNA was directly sequenced using a Sequenase 2.0 kit (United States Biochemical Corp., Cleveland).

The application of the described DNA extraction and amplification method resulted in PCR products of 1.2 kb from all tick stages examined; unfed and engorged larvae and nymphs and even from eggs as young as 24 h after deposition (Fig. 1). Digestion of the PCR products with *Pst*I resulted in visible bands of 0.9 and 0.3 kb and digestion with *Xho*I gave products of 0.7 and 0.5 kb (data not shown). Direct sequencing of 0.2 kb with primer RIB-4 resulted in sequences identical to that of *D. reticulatus* (Zahler *et al.*, 1995).

PCR amplifications of serial DNA dilutions of single *D. reticulatus* eggs, incubated at 20°C and 95% relative humidity for 12–14 days after deposit and then frozen at − 20°C, showed a detection limit of 1/100 egg (Fig. 2).

PCR products generated with primers RIB-3 and RIB-4 have also been obtained with DNA of other genera of ixodid and argasid ticks (*Ixodes*, *Rhipicephalus*, *Haemaphysalis*, *Hyalomma* and *Persicargas*; Fig. 3). The authenticity of these products was determined by direct sequencing of the flanking regions which confirmed the presence of the 3′ end of the 5.8S rRNA gene on one side of the product and the presence of the 5′ start of the 28S rRNA gene on the other side.

The DNA extraction and amplification method presented in this report constitutes a prerequisite suitable for the identification of the species of individual nymphs, larvae and eggs. Engorgement of larvae and nymphs and the consequent presence of host DNA did not prevent the amplification of the tick target sequence.

Beyond the example of *D. reticulatus* described here, two generally applicable DNA sequence analysis methods can be used to identify other tick genera and species as well. First, if the determined ITS2 sequence is already known, as is the

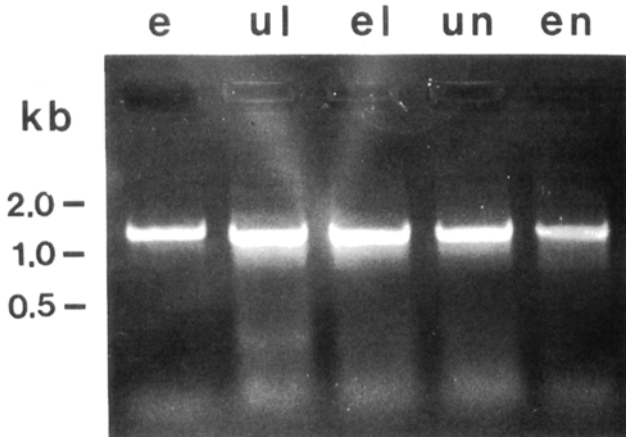

Fig. 1. PCR products from a single *D. reticulatus* egg (e), unfed larva (ul), engorged larva (el), unfed nymph (un) and engorged nymph (en).

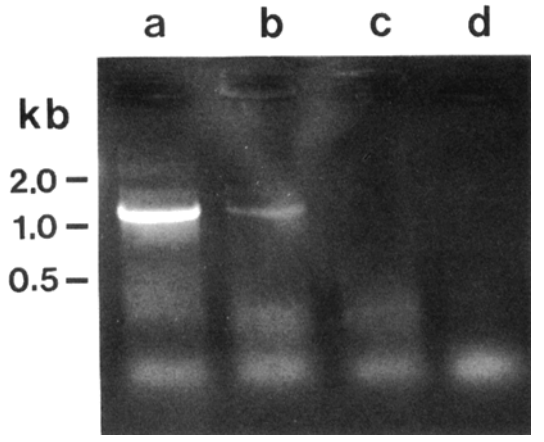

Fig. 2. PCR products from serial dilutions of DNA extracted from a single *D. reticulatus* egg, corresponding to (a) 1/10, (b) 1/100, (c) 1/1000 and (d) 1/10 000 of the total egg DNA.

case to date for *Ixodes scapularis*, *Ixodes pacificus* (Wesson *et al.*, 1993), *D. reticulatus*, *Dermacentor marginatus*, *Dermacentor andersoni* and *Dermacentor variabilis* (Zahler *et al.*, 1995), the species can immediately be identified. Alternatively, if a hitherto unknown sequence is obtained, it may be matched against sequences of different genera in a dendrographic plot. For another target sequence, a 0.46 kb fragment of the mitochondrial 16S rDNA, sequences of nine ixodid and four argasid genera are already available and have been used to construct a phylogenetic tree (Black and Piesman, 1994).

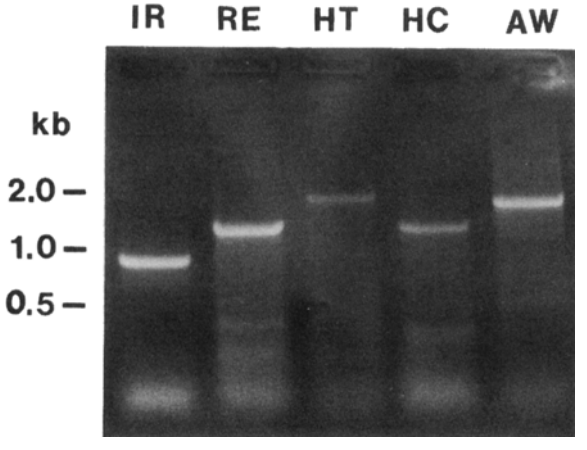

Fig. 3. PCR products from individual adult *Ixodes ricinus* (IR), *Rhipicephalus evertsi evertsi* (RE), *Hyalomma truncatum* (HT), *Haemaphysalis concinna* (HC) and *Argas walkerae* (AW) ticks.

However, the discriminatory power of coding target sequences such as the 16S rDNA is less than that of non-coding, hypervariable genomic regions, for example the ITS2 spacer used in this report, because the latter are less affected by evolutionary constraints for conservation. Therefore, we favour the ITS2 as an amplification target, not least because the ITS spacer regions have already been shown to allow the differentiation of genotypes even below the species level (McLain *et al.*, 1995; Zahler *et al.*, 1995). Depending on the problem to be investigated, the high degree of polymorphisms of the rDNA spacers might even allow the substitution of cumbersome sequencing methods by more simple RFLP analyses for the characterization of the PCR product. The *Pst*I- and *Xho*I-generated RFLP patterns obtained here were identical to both *D. reticulatus* and *D. marginatus*, but not to *D. andersoni*, *D. variabilis* or the other previously examined ixodid or argasid genera (see below). But if the problem under consideration requires a higher degree of specificity, the option to sequence the PCR product is still available.

The internal transcribed spacers ITS1 and ITS2 offer the additional advantage of being flanked by conserved, coding sections of the rDNA gene, for which a single PCR primer pair can be used to amplify the intervening, much more variable spacer region of a wide range of, possibly all, tick genera. We have already obtained PCR products from other ixodid genera (*Ixodes*, *Rhipicephalus*, *Haemaphysalis* and *Hyalomma*) and from argasid ticks (*Persicargas*) with the same PCR primers which have been used in this report (Fig. 3).

Besides the high specificity due to the large number of polymorphisms, a further advantage of rDNA spacers over other hypervariable genomic regions is the characteristic feature of rDNA genes of being present in several hundred copies in each cell (Hillis and Dixon, 1991). This leads to a corresponding increase in the sensitivity of the DNA amplification.

We consider the technique described in this report to be particularly suitable in epidemiological studies, for example for the unequivocal identification of tick larvae and nymphs on different host animals, with regard to questions of host specificities under field conditions as well as with respect to tick-borne pathogens at the host–vector interface.

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