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Genetic differences in internal transcribed spacer 1 between *Dermanyssus gallinae* from wild birds and domestic chickens

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Abstract. We investigated the presence of the poultry red mite or the chicken mite, *Dermanyssus gallinae* De Geer, Acari: Dermanyssidae, in wild bird populations in four different geographical regions of Sweden. The mites identified as *D. gallinae* were compared genetically with *D. gallinae* from egg-producing poultry farms in the same regions. The small subunit (SSU) gene, the 5.8S ribosomal RNA (rRNA) gene and the two internal transcribed spacers (ITS) of the rRNA genes were used in the genetic analysis. All *D. gallinae* mites had identical SSU rRNA, 5.8S rRNA and ITS2 sequences independent of their origin. By contrast, we identified significant differences in the ITS1 sequences. Based on the differences in the ITS1 sequences, the mites could be divided into two genotypes, of wild and domesticated origin, with no variation within the groups. These results imply that wild bird populations are of low importance, if any, as natural reservoirs of *D. gallinae* in these four geographical regions of Sweden.

Key words. *Dermanyssus gallinae*, chicken, ectoparasite, genetic analysis, ITS1, reservoir host, ribosomal RNA, SSU.

Introduction

The blood-feeding mite *Dermanyssus gallinae* De Geer, also known as the poultry red mite or chicken mite, has worldwide distribution and is an economically important parasitic pest of domestic chickens (Chauve, 1998). Affected birds show decreased production, irritation and, in severe infection, anaemia leading to death. The mite spends most of its life aggregated in cracks and crevices in the poultry house, where mating, reproduction and development take place. The parasite only visits its host for bloodmeals about every third day (Kirkwood, 1963), and may, under optimal conditions in poultry operations, complete the feeding–oviposition cycle within 1 week, resulting in the rapid establishment of dense populations of mites.

Dermanyssus gallinae has been suggested as a potential vector of pathogens causing fowl spirochaetosis, chicken pox, encephalitis, fowl cholera and Newcastle disease (Zeman *et al.*, 1982; Chauve, 1998). *Erysipelothrix rhusiopathiae*, a bacteria causing septicaemia in hens, has been isolated from mites originating from poultry facilities with outbreaks of erysipelas (Chirico *et al.*, 2003). The zoonotic potential of these pathogens should also be considered as additional concerns associated with the parasite, as *D. gallinae*, in the absence of its definitive host, occasionally attacks dogs, cats, rodents, horses and humans (Brockis, 1980), causing dermatitis and intense pruritus. There are also limited options to control the parasite in production facilities with active acaricides because of food safety regulations. Consequently, the focus of control involves treating facilities between flock cycles, and avoiding the re-introduction of mites into the production sites. Thus, the transmission route of *D. gallinae* into the poultry facilities is not fully understood (J. Strömberg and J. Chirico, unpublished data, 2003).

A wide range of Mesostigmata parasitic mite species may be found in wild bird populations, and wild birds have been suggested to be potential carriers of the parasite or reservoir hosts. Determining the species identity of such mites with keys based on morphological features is challenging because it is time-consuming and laborious. Moreover, reliable identification may

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be impossible in closely related species or varieties of the same species. However, molecular data have proven to be highly discriminatory tools for resolving relationships between species; for instance, much of modern taxonomy is based on analysis of ribosomal RNA (rRNA) genes. In many applications, the internal transcribed spacers (ITS1 and ITS2) of rRNA repeats are powerful markers for detecting genetic variation between closely related species (Berrilli *et al.*, 2002).

The aim of this study was to investigate the potential of some wild bird populations to act as natural reservoirs of *D. gallinae*. We hypothesized that *D. gallinae* from wild birds and domestic chickens would be genetically identical. If the mites were genetically identical, wild birds could transmit *D. gallinae* into poultry facilities. *Dermanyssus gallinae* from egg-producing poultry facilities and from wild birds collected in different geographical regions of Sweden were studied, both morphologically and with molecular tools. The small subunit (SSU) rRNA, 5.8S rRNA and the ITS1 and ITS2 of the rRNA repeats were used for the molecular study.

Materials and methods

Collection of mites

Egg-producing poultry facilities and wild bird nests were investigated regarding mites in four different geographical regions of south-central Sweden (Öland, Skåne, Västergötland and Uppland). Mites from six layer facilities, consisting of aviary systems as well as furnished cages, were recovered using 7×10-cm semi-transparent corrugated plastic traps (Nordenfors *et al.*, 2001). Traps were placed in the nests for 7 days, after which they were put into plastic bags and sent to the laboratory. Engorged female mites were selected and placed in wells of a round-bottomed ELISA plate, and stored at room temperature for 8–10 days to allow larvae to hatch. Both larvae and protonymphs were collected and used in the molecular study.

Nests from wild birds were collected from experimental nesting boxes utilized by the Universities of Uppsala and Lund for other research purposes. In total, 78 wild bird nests from the same geographical regions, except Västergötland, were collected and examined for mites. Nests were individually placed in plastic bags, marked with location and bird species, and transported to the laboratory. Mites were recovered by placing the nest in double plastic bags according to the method of Bram (1978). Briefly, the inner bag was opened and the outer bag closed, allowing those live mites emerging into the outer bag to be collected. Dead and live mites still in the nest were recovered by flotation (Bram, 1978). The nests were pressed to the bottom of a bucket with a wire net, and water was added. After 2 h, floating mites were collected with a small strainer. Samples of recovered mites determined as *D. gallinae* were sent to Dr L. Lundqvist, University of Lund, for verification. Verified specimens of *D. gallinae* were then investigated in the molecular study.

DNA preparation

DNA was prepared from each individual mite using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems Inc., Foster City, CA, U.S.A.). The mite was placed in an Eppendorf tube and 30 μL PrepMan™ was added. The preparation was homogenized for 2×30 s directly in the tube with a mini-pestle, and thereafter boiled for 10 min and cooled on ice for 2 min. After cooling the tube was centrifuged for 3 min at 17 000 **g**, and the supernatant containing the DNA template was removed and stored at 4 °C.

PCR amplification and sequencing

Universal primers targeting the start, the central part and the end of the rRNA SSU gene were used to amplify the SSU region of the mite DNA. The forward primer was NSF4 (5′-CTG GTT GAT [C,T]CT GCC AGT-3′) (Wuyts *et al.*, 2004). We used two different reverse primers: NSR951 (5′-TTG G[C,T][A,G] AAT GCT TTC GC-3′) and NSR1787 (5′-C[C,T]G CAG GTT CAC CTA C[A,G]G-3′) (Wuyts *et al.*, 2004). To amplify the ITS1 and ITS2 regions we used universal primers situated at the end of the rRNA SSU gene and at the start of the rRNA large subunit (LSU) gene. The forward primer was NSF1624 (5′-TTT GYA CAC ACC GCC CGT CG-3′) (Wuyts *et al.*, 2004) and the reverse primer was RIB-3 (5′-CGG GAT CCT TC[A,G] CTC GCC G[C,T]T ACT-3′) (Zahler *et al.*, 1999). The polymerase chain reaction (PCR) mixture (50 μL) contained 5 μL GeneAmp® 10 × PCR buffer (100 mM Tris-HCl [pH 8.3]), 500 mM KCl, 15 mM $MgCl_2$, 0.01% [w/v] gelatine; Applied Biosystems), 1 U AmpliTaq DNA polymerase (Applied Biosystems), 200 μM of each deoxynucleotide (GE Healthcare, Uppsala, Sweden), dH_2O, 20 pmol of each primer and 1 μL DNA template. For the PCR reaction a PTC 200 machine (MJ Research, Inc., Waltham, MA, U.S.A.) was used. After 2 min denaturation at 95 °C the DNA was amplified for 40 cycles with the following programme: 15 s of denaturation at 95 °C, 30 s annealing at 55 °C and 2 min of elongation at 72 °C. The PCR reaction ended with an elongation for 4 min at 72 °C. The PCR product was purified from gel using GENECLEAN® II KIT (Qbiogene, Inc., Carlsbad, CA, U.S.A.), or from solution using JetQuick (SaveenWerner, Malmo, Sweden) eluted with 10 μL dH_2O.

DNA was sequenced using ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence reaction mixture of 20 μL contained 2 μL BigDye® solution, 3 μL BigDye® 5 × sequencing buffer, dH_20, 5 pmol primer and 1 μL purified PCR product. The primers were as described above or by Wuyts *et al.* (2004). After the sequence reaction, unincorporated terminators were removed by ethanol precipitation. The sequencing reactions were separated on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were analysed using the Vector NTI program, Suite 9 (Informax Inc., Oxford, U.K.).

Results

Collection of mites

Mites were recovered from all six egg-producing poultry farms from the four different geographical regions investigated.

The nests analysed were inhabited by nine bird species: pied fly-catcher (*Ficedula hypoleuca*); collared fly-catcher (*Ficedula albicollis*); spotted fly-catcher (*Muscicapa striata*); starling (*Sturnus vulgaris*); tree sparrow (*Passer montanus*); blue tit (*Parus caeruleus*); swallow (*Hirundo rustica*); great tit (*Parus major*), and wryneck (*Jynx torquilla*). Mites were found in 45 of the nests, but only 16 contained *D. gallinae*. We also found *Hypoaspis* sp., *Ixodes* spp., *Parasitus* sp., *Pergamasus* sp. and an Ascidae. The *D. gallinae* mites were recovered from the nests of: *F. albicollis* (2), *M. striata* (1), *F. hypoleuca* (5), *P. major* (1) and *S. vulgaris* (7). The mites from the *H. rustica* nest were not possible to identify because of their poor condition. We found no *D. gallinae* in any of the 10 nests of *P. montanus*.

Molecular analysis

Firstly, the SSU rRNA gene was analysed from 19 different mites from wild and domesticated birds. All of these sequences were identical. To increase the resolution, we then analysed the ITS1 and ITS2 of the rRNA repeats. We successfully amplified the ITS1-5.8S-ITS2 fragment from 10 of 20 *D. gallinae* mites from wild birds *F. albicollis* (6), *M. striata* (1), *F. hypoleuca* (2) and *S. vulgaris* (1). We also amplified and analysed 23 individual mites from the six different layer facilities.

The region covering the ITS1-5.8S-ITS2 was 457 bp irrespective of the origin of the mites. All amplified sequences from wildbird *D. gallinae* mites were identical, as were those of all mites from domestic chickens. However, the ITS1 sequences differed between the two groups of mites. The 160-bp 5.8S rRNA gene sequences were identical in all mites, as were the 90-bp ITS2 sequences. In the ITS1 comparison there were 10 fixed differences between the two groups. The differences clustered in an 80-bp region of the central part of the 207-bp ITS1. A model of the secondary ITS1 structure including the potential stem-loops (based on that of Morrison, 2006) is shown in Fig. 1. The positions where the two groups differ are highlighted.

Discussion

To our knowledge this is the first study to demonstrate genetic differences between *D. gallinae* from wild and domestic bird populations. Since the end of the 1970s, rRNA genes have been the most extensively used genes for evolutionary studies (Wuyts *et al.*, 2004). The combined structures of rapidly evolving regions and evolutionarily conserved sequences have made it possible to resolve important issues in phylogenetics at several taxonomic levels. By contrast, ITS1 and ITS2 of the rRNA repeats have been used as standard tools to understand closer relationships (Schultz *et al.*, 2005), such as among species belonging to the same genus. Within this study all investigated mites shared the same SSU rRNA sequence, independent of both geographic and host origin. By contrast, all mites originating from wild birds had one ITS1 genotype and all mites from domestic chickens had a second genotype. A secondary structure analysis of ITS1 between the two types of mites strengthens the evidence for separation of the mites into two groups (Fig. 1). The nucleotide differences are not all randomly distributed, but appear as compensatory base changes in the putative paired stems of the RNA transcripts. The importance of compensatory base changes in ITS as a molecular classifier was tested recently by Muller *et al.* (2007), who concluded that the presence of compensatory mutations between samples implies the presence of independent species.

Sampling mites from wild bird nests was a limiting factor of this study, for various reasons. Most importantly, we used nests from experimental nesting boxes, conveniently located for mating and ethological studies, but not always close to poultry facilities. Nevertheless, as the genetic sequences of these mites were identical, independent of bird species and geographical location, we assume that *D. gallinae* infesting other wild bird species building nests adjacent to or on poultry houses will be of the same ITS1 genotype.

Additionally, there were technical problems in recovering DNA from dead mites. Dead and dry mites were more resistant to mechanical rupture than live mites, which led to difficulties in recovering DNA, which might account for PCR failures. Alternatively, the DNA might have been degraded before our isolation of DNA. Contamination of host DNA may also account for some of the PCR failures. To minimize this problem, we strove to use larvae and protonymphs hatched in the laboratory. This was possible from all the egg-producing flocks, but no newly fed females were recovered from the nest material.

Our results suggest that the role of wild birds as a reservoir for introducing *D. gallinae* into commercial poultry production

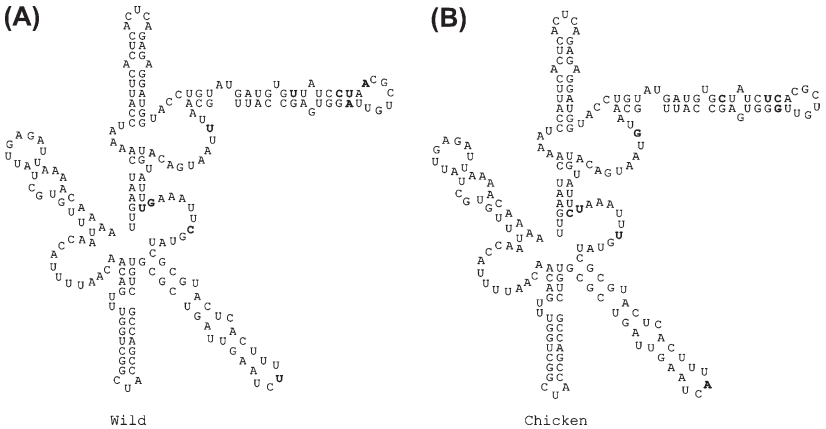

Fig. 1. Stemloop structures of the ITS1 region of the rRNA, of *Dermanyssus gallinae* from (A) wild birds and (B) chickens. Nucleotide differences are in bold.

systems is probably of low importance, at least in Sweden. However, our results cannot rule out the possibility that *D. gallinae* from poultry can infest wild birds. The transmission routes are more likely to be affected by the management of the poultry production system (e.g. by transport of live birds and eggs). In a recent Swedish investigation, some poultry farmers admitted that they find wild bird nests within their facilities (Engström *et al.,* 2007). This may cause sanitation problems in terms of faecal contamination and potential infectious agents related to such contamination, such as *Salmonella* spp. (Boqvist *et al.*, 2003), but it is probably not a major transmission route for *D. gallinae*.

In conclusion, *D. gallinae* is divided into at least two genotypes, one infecting egg-producing poultry flocks and one infecting wild birds. This result suggests that wild birds may play only a minor role, if any, as a reservoir of *D. gallinae*. If wild birds are infected with a different genotype of *D. gallinae* than chickens, the risk of transmission of vector-borne infectious agents from wild life into poultry facilities is probably also low.

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