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Population genetics of *Parascaris equorum* based on DNA fingerprinting

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ABSTRACT

The large roundworm of horses, *Parascaris equorum* is considered ubiquitous in breeding operations, and is regarded as a most important helminth pathogen of foals. Over the past decade, this parasite has been reported increasingly resistant to anthelmintic drugs worldwide. This paper reports analysis of the population genetic structure of *P. equorum*. Adult parasites (n = 194) collected from Sweden, Norway, Iceland, Germany, Brazil and the USA were investigated by amplified restriction fragment length polymorphism (AFLP) analysis. The genetic variation was low (Hj = 0.12–0.4), for the global population of worms. This was accompanied by a weak degree of population structure (Fst = 0.2), low gene flow (Nm = 1.0) and low mutation rate (4 Nμ = 0.07). Thus, the low genetic diversity is probably a result of a low mutation rate in DNA, although the gene flow (due to global movement of horses) is large enough to allow the spread of novel mutations. Surprisingly, isolates from Icelandic horses were not found to be different from other isolates, in spite of the fact that these have been isolated for thousands of years. The study indicates that the global *P. equorum* population is essentially homogenous, and continents do not appear to be strong barriers for the population structure of this species. Consequently, the potential spread of rare anthelmintic resistance genes may be rapid in a homogenous population.

1. Introduction

The large roundworm of horses, *Parascaris equorum,* is the most pathogenic parasite of foals and yearlings worldwide. Infection may cause nasal discharges, coughing and impaired growth, and large burdens may even lead to death caused by obstruction or penetration of the small intestine (Boyle and Houston, 2006; Cribb et al., 2006).

Anthelmintic resistance (AR) has become a major problem in veterinary medicine and constitutes a threat to animal welfare and productivity (Kaplan, 2004). Failure of macrocyclic lactone (ML) treatment to reduce the shedding of *P. equorum* eggs has been reported in several countries, such as the Netherlands (Boersema et al., 2002), Canada (Hearn and Peregrine, 2003), the United Kingdom (Stoneham and Coles, 2006), the United States (Craig et al., 2007), Denmark (Schougaard and Nielsen, 2007), Germany (von Samson-Himmelstjerna et al., 2007), Brazil (Molento et al., 2008), Sweden (Lindgren et al., 2008), Italy (Veronesi et al., 2009) and Finland (Näreaho et al., 2011). In ML-resistant populations of *P. equorum*, treatment with pyrantel pamoate, fenbendazole and oxibendazole has been successful (Slocombe et al., 2007; Lyons et al., 2008; Schougaard and Nielsen, 2007; Hearn and Peregrine, 2003). One study has reported apparent lack of pyrantel efficacy against *P. equorum* (see Lyons et al., 2008), while there are no published studies reporting benzimidazole (BZ) resistance in this parasite. However, BZ resistance might be developing, as the use of this drug class in foals is likely to increase due to the reduced efficacy of MLs. Little is still known about how anthelmintic resistance arises, and how genes conferring resistance are spread among parasite populations.

Molecular techniques have provided valuable tools to conduct detailed population genetic investigations of almost all organisms. For parasitic nematodes of veterinary interests it has been shown that gene flow and diversity are determined by the life history of both the parasite and its host (Anderson et al., 1998). For example, in the closely related large roundworm of pigs, *Ascaris suum,* host dispersal effectively determines and controls the patterns of gene flow (Nadler, 1986). Thus, the potential spread of AR may be rapid due to extensive transport of infected hosts across large geographical distances. While this is still only a preliminary conceptual idea for the spread of AR, it has been shown that large nationwide transport of domestic pigs has led to a homogenisation of the gene pool of *A. suum* in Denmark (Nejsum et al., 2005).

Accordingly, an investigation of the population genetic structure and diversity of *P. equorum* can be used to predict the pattern of spread of this parasite, and also to explore whether there is

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evidence for distinct isolates of *P. equorum* representing local subpopulations, or whether the members can rather be regarded as a single panmictic population. This knowledge is essential for understanding whether there is an imminent risk for the selection and spread of drug-resistance genes in response to an excessive and indiscriminate use of anthelmintics as practiced in many equine establishments. Clearly, the rate of spread of any genes conferring anthelmintic resistance will be directly correlated with the degree of gene flow among hosts and farms (Anderson et al., 1998; Hawdon et al., 2001; Nejsum et al., 2005).

In Sweden, transportation of horses between farms is readily apparent. Many horse farms routinely accommodate mares and foals, especially during the breeding and grazing seasons. Although *P. equorum* lives for less than a year in its host, foals become infected on pasture by parasite eggs deposited from the previous generation of foals on the same farm (Lindgren et al., 2008). Moreover, these eggs are reported to be very resistant to both climatic changes and disinfectants and, apparently, they can remain viable in the soil for up to 10 years (Urquhart et al., 1996). Thus, *P. equorum* possesses numerous potential pathways for gene flow between different host generations.

The aim of this study was to investigate the population structure of *P. equorum* among young horses in Sweden and to compare it with other isolates both from Europe and other continents using the DNA fingerprinting technique amplified fragment length polymorphism (AFLP). We have addressed the question as to what extent geographical locations have influenced the population structure of *P. equorum*. AFLP is commonly used in population genetic studies (Bensch and Åkesson, 2005; Bonin et al., 2007; Meudt and Clarke, 2007) and one of the major advantages of the AFLP technique are the detection of a large number of polymorphism and the generation of a unique genetic pattern for each individual (Vos et al., 1995). Another advantage is that it is efficient to use on worms with no previous knowledge of the DNA sequence (Höglund et al., 2004; Nejsum et al., 2005). Identification of nematodes analysed in the AFLP was also performed with universal primers targeting the conserved genes flanking the spacers of the internal transcribed spacers 2 (ITS-2) of the ribosomal rDNA gene array.

2. Materials and methods

2.1. Parasite material

In Table 1 sample code of parasite isolates, geographical origin, collection method and breed of horses are given. The *Parascaris* isolates from Sweden and Norway were sampled from faeces that were collected 1–2 days after treatment with fenbendazole. Expelled worms (a minimum of 10 adult worms from each farm) were collected in a plastic bag by the horse manager and immediately submitted to the laboratory at the Section for Parasitology at the Swedish University of Agricultural Sciences (SLU) in Uppsala. The isolates from Brazil and Iceland were collected from the intestine at slaughter. One isolate from the US (U1) was obtained from a well-characterised, closed research horse herd, maintained at the University of Kentucky without anthelmintic intervention since 1979 (Lyons et al., 1997), and parasites maintained here are therefore ML-naïve. The second US isolate (U2) was collected during routine necropsy performed at the Veterinary Diagnostic Laboratory at University of Kentucky. All worms collected outside Sweden and Norway were preserved in ethanol (70–90%) and sent by airmail to the laboratory at the Section for Parasitology at the Swedish University of Agricultural Sciences (SLU) in Uppsala. Upon arrival all worms were stored in −70 °C until DNA extraction was performed.

2.2. DNA extraction

DNA was extracted from individual worms using nucleospin tissue (Macherey–Nagel, Düren, Germany) according to the manufacturer's recommendation. RNase A (400 μg/sample) was used to obtain RNA-free samples. The purified DNA was stored at 4 °C until further analysis.

2.3. AFLP

A total of 194 worms were used for AFLP analysis (Table 1). As an internal control replicate analyses (5) were performed for one of the isolates from farm S3 (Heby). The AFLP procedure was performed as described by Applied Biosystems (ABI, Carlsbad, California, USA) in their Plant Mapping Protocol and as described by Höglund et al. (2004). All reagents were supplied in the AFLP™ Plant Mapping Kit except the restriction enzymes and the T4 DNA ligase, which were from New England Biolabs (Ipswich, Massachusetts, USA).

For all worms, the maximum allowed volume (5.5 μL), of extracted DNA was used. The DNA from each worm was digested with *Mse*I and *Eco*RI (ABI, Carlsbad, California, USA), and adaptors for *Mse*I and *Eco*RI restriction products were ligated in the same

Table 1
Parascaris isolate code, geographical origin, collection method and breed of horses.

Code	Continent	Geographical origin	Collection method	Number of worms	Breed of horses
S1	Europe	Sweden, Boden	Expelled by fenbendazole, faecal sample	10	Standardbred
S2	Europe	Sweden, Borlänge	Expelled by fenbendazole, faecal sample	10	Standardbred
S3	Europe	Sweden, Heby	Expelled by fenbendazole, faecal sample	10	Standardbred
S4	Europe	Sweden, BrO	Expelled by fenbendazole, faecal sample	10	Standardbred
S5	Europe	Sweden, Ekerö	Expelled by fenbendazole, faecal sample	10	Standardbred
S6	Europe	Sweden, Tystberga	Expelled by fenbendazole, faecal sample	10	Standardbred
S7	Europe	Sweden, Västervik	Expelled by fenbendazole, faecal sample	10	Standardbred
S8	Europe	Sweden, Västervik	Expelled by fenbendazole, faecal sample	10	Standardbred
S9	Europe	Sweden, Landskrona	Expelled by fenbendazole, faecal sample	10	Standardbred
S10	Europe	Sweden, Svenljunga	Expelled by fenbendazole, faecal sample	10	Standardbred
S11	Europe	Sweden, Sjöbo	Expelled by fenbendazole, faecal sample	10	Standardbred
G	Europe	Germany	Collected at an abattoir	10	Standardbred
I1	Europe	Iceland, Hvolsvöllur	Collected at an abattoir	9	Icelandic horse
I2	Europe	Iceland, Hella	Collected at an abattoir	9	Icelandic horse
I3	Europe	Iceland, Hvolsvöllur	Collected at an abattoir	9	Icelandic horse
N1	Europe	Norway, Vestfold	Expelled by fenbendazole, faecal sample	11	Standardbred
N2	Europe	Norway	Expelled by fenbendazole, faecal sample	10	Standardbred
B	S. America	Brazil	Collected at an abattoir	6	Not known
U1	N. America	US, Kentucky	Collected during necropsy	10	Mixed light breed
U2	N. America	US, Kentucky	Collected during necropsy	10	Thoroughbred