Applicability of molecular markers to determine parasitic infection origins in the animal trade: a case study from Sarcoptes mites in wildebeest

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Abstract The development of non-manipulative molecular tools to determine the origin of parasite infections in the animal trade (if infected before their export or import) is of great interest worldwide for both the animal trade industry and for animal welfare. Molecular tools have a wide range of applications, including forensic identification, wildlife preservation and conservation, veterinary public health protection, and food safety. Nonetheless, genetic markers were not reported to detect the source of infection in the animal trade. In this study we tested the applicability of molecular tools to detect the origin of Sarcoptes mite infection of wildebeest imported by the United Arab Emirate (UAE) from Tanzania. Using one multiplex of seven microsatellite markers and control samples from UAE, Kenya and Italy, we demonstrated the usefulness of the multiplex STR-typing as a molecular tool of pivotal interest to help commercialist, authorities, and conservationists, to identify the geographical origin of parasitic infections.

Keywords Sarcoptes scabiei · Genetic structure · Microsatellite markers · Forensic parasitology · Infection source · Tanzania · UAE · Kenya · Italy

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Introduction

With the recent advances in genetics, the use of molecular DNA in forensic science has added a new dimension to wildlife management and conservation [1], food safety [2], and veterinary public health [3]. Additionally, molecular data is occasionally the only source of information available for conservation officials throughout their investigations [4].

Trading with pathogen pre-infected animals is illegal [5], and when it occurs, it is usually by chance when importing live animals [6]. Infected animals may go undetected because of the incubation period of some pathogens, like scabies, which can be weeks or even months [7], and because there is a clear lack of accurate diagnostic tests for many animal species [8]. Hence, genetic molecular forensic science tools could have a potential applicability to detect possible parasitic infection origins in the animal trade.

The development of accurate methods for determining the origin of parasitic infection in traded animals is of vital interest to protect both consumers from fraud [9], and the

country of destination from receiving a new parasite variety/species, which may have important negative consequences [10]. In this study we test the efficiency of one multiplex of microsatellite markers to detect the origin of Sarcoptes mite infections in traded animals.

While the question as to whether Sarcoptes scabiei may be split into different species or subspecies, or whether they are in fact monospecific, is still the subject of ongoing debate, Sarcoptes mite continues to affect humans and a wide range of mammalian hosts worldwide [11]. Introduction of a single case of scabies in areas of high animal densities can result in an epidemic [12], which ultimately can have devastating consequences in wild and domestic animals [13].

The life cycle of Sarcoptes is about 2 weeks; however the pathogenesis and concordant clinical symptoms of mange, depending on the immune status of the respective host, could be detectable months after the first contact between the host and the infected animals [14]. Hence it is difficult to determine if the traded animal was infected before or after the exportation/importation from one locality to another.

Case report

Six wild caught wildebeest (Connochaetes taurinus) calves were imported from Arusha National Park (Tanzania) to Fujeirah (United Arab Emirates) in December 2010 (Fig. 1). According to the owner (who obviously ordered healthy animals), all animals already showed different levels of skin lesions on arrival, mainly on the head and neck. The owner complained that the imported animals had been infected before leaving Tanzania, and that these animals had not been in contact with other animals after arriving in UAE. The worst infected animal, a 50 kg female, died and was sent for necropsy to the Central Veterinary Research Laboratory in Dubai (United Arab Emirates). The carcass showed generalized skin alterations caused by Sarcoptes scabiei. The remaining five wildebeests were treated with ivermectin (Ivomec) at a dosage of 200 mg/kg b.w. twice during a 2 week period. The condition of the animals improved after treatment.

Specimen collection and DNA extraction

Nine mites were collected from the skin crust of the infected wildebeest imported by United Arab Emirates from Tanzania, using postponed isolation and direct isolation (with aqueous potassium hydroxide digestion) techniques [15]. Five mites were collected from three

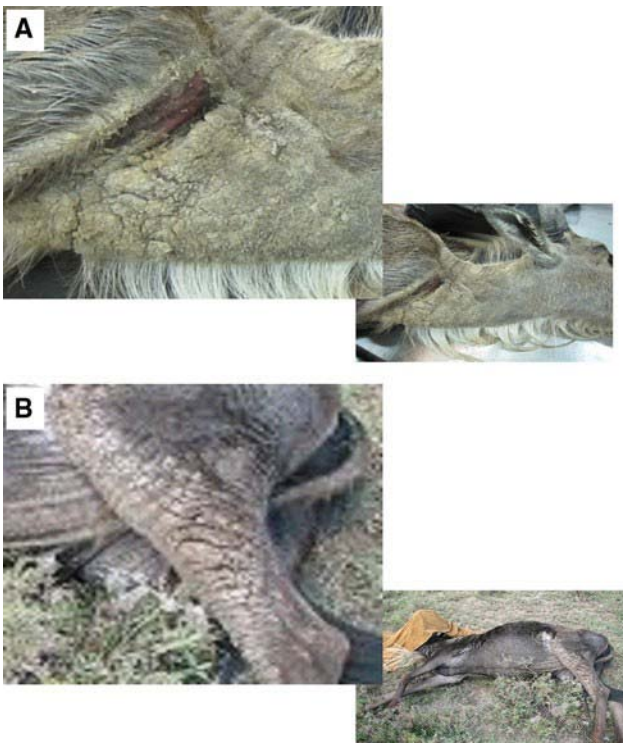

Fig. 1 Photographs of mangy wildebeest, showing alopecia and encrustation in a an exported animal from Tanzania to the UAE, and b a positive control from Masai Mara, Kenya

wildebeest from Masai Mara (Kenya) as a positive control, one mite was collected from a domestic rabbit (Oryctolagus cuniculus) from Nakhlee, Dubai (United Arab Emirates), and another fifteen mites were collected from nine chamois (Rupicapra rupicapra) from the Eastern Italian Alps (Italy) as negative controls. All mites were identified as S. scabiei on the basis of known morphological characteristics [16]. The DNA of individual Sarcoptes mites was extracted using the HotSHOT Plus ThermalSHOCK technique [17].

Fluorescent-based polymerase chain reaction analysis of microsatellite DNA

As described by Alasaad et al. [18], seven specific Sarcoptes mite microsatellites (Sarms33, 35–38, 40 and 45) were amplified in one 7 multiplex PCR. One primer from each set 5 was labelled with 6-FAM, VIC, NED or PET® fluorescent dye tag (Applied Biosystems, Foster City, CA, USA). Each 15 l PCR mixture consisted of 3 l of the single mite DNA, together with the PCR mixture containing all primer pairs (range: from 0.04 to 0.1 M per primer), 200 M of each dNTP, 1.5 l of 10 PCR buffer

(200 mM KCl and 100 mM Tris–HCl, pH 8.0), 1.5 mM $MgCl_2$ and 0.15 ▮l (0.5 U/reaction) HotStar Taq (QIAGEN, Milano, Italy). The PCR conditions (2720 thermal cycler, Applied Biosystems, Foster City, CA, USA) were as follows: 15 min at 95°C (initial denaturation), followed by 37 cycles of three steps of 30 s at 94°C (denaturation), 45 s at 55°C (annealing) and 1.5 min at 72°C (extension), before a final elongation of 7 min at 72°C. Fluorescent PCR amplification products were analyzed, using formamide with 500 Liz Size Standard (Applied Biosystems, Foster City, CA, USA), by ABI PRISM 310 Genetic Analyzer with pop4. Allele calls and editing were performed using GeneMapper v. 4.0 software (Applied Biosystems, Foster City, CA, USA). To track and minimize the amount of errors associated with genotyping, the genetic data were collected twice.

Molecular analyses

Expected (H_E) and observed (H_O) heterozygosity, linkage disequilibria (LD), and Hardy–Weinberg equilibrium (HWE) tests were calculated in GENEPOP (v.3.4; [19]). Deviations from HWE and tests for LD were evaluated using Fisher's exact test and sequential Bonferroni corrections. Possible genotyping mistakes (scoring error due to stuttering, large allele dropout) were estimated using MICROCHECKER [20]. Null-alleles were estimated using ML-NULLFREQ [21].

The analysis of relationships between mites was carried out by a Bayesian assignment test of the software STRUCTURE (v.2.3.3; [22]). Burn-in and run lengths of Markov chains were 100,000 and 500,000, respectively. We ran 20 independent runs for each K (for K = 1–10). Selection of K was determined using two methods (1) by plotting the negative log likelihoods [(ln P(D)] versus K [22], and (2) using the ΔK method described in Evanno et al. [23]. Finally, each of the inferred clusters was associated with the component populations of its mites.

Discussion

We used seven microsatellite markers on purified DNA extraction samples from 30 Sarcoptes mites originating from (1) imported wildebeest from Arusha (Tanzania) to Fujeirah (UAE), (2) positive control wildebeest from Masai Mara (Kenya) (considered as positive controls since wildebeest are seasonal migratory species [24], and they move frequently between Kenya and Tanzania, and are expected to be genetically similar to those from Tanzania), (3) a rabbit negative control from Dubai (United Arab Emirate), and (4) chamois negative controls from the Eastern Italian Alps (Italy). As with other highly divergent taxa, with Sarcoptes scabiei few loci and low sample sizes are sufficient to find strong population differentiation between host species [25, 26].

Twenty-one alleles were detected from the seven microsatellites loci. The allele count for each of the seven loci ranged from two (Sarms33, Sarms36 and Sarms37) to five (Sarms45). Thirteen private alleles were detected: six alleles in the Sarcoptes mite from chamois (Italy), six alleles in rabbit mites (UAE) and one allele from wildebeest mites from Kenya, while no private alleles were detected in mites from wildebeests from Tanzania. The high number of private alleles in all studied mite populations indicates high genetic divergence and reduced gene flow between mite populations.

There was no evidence of deviation from HWE, LD (P ▮ 0.05), scoring errors due to stuttering, large allele dropout, or null alleles for all microsatellite loci examined.

The results of the Bayesian clustering analysis of multilocus genotypes in Structure showed that the log likelihood (ln [P(D/K)] for the likely number of populations K was 3, the same K value was obtained when applying Evanno et al. [23] criteria.

All cluster assignments were consistent with the population of origin. Sarcoptes mites from the two wildebeest populations (imported from Tanzania to the UAE and the positive control from Kenya) formed one cluster that was highly supported, with 99.5% probability of belonging to the same cluster. Mites from the rabbit (negative control from the UAE) and chamois (negative control from the Eastern Italian Alps) formed two, well formed, clusters, supported by 99.3 and 99.4% respectively (Fig. 2). These results support the proposition that the imported wildebeest were infected before leaving Tanzania, and that they had not been infected after arriving in the UAE. We obtained the same results when we applied K = 4 (the number of Sarcoptes mite populations in this study), which demonstrates the complete population divergence into distinct clusters, and illustrates the high levels of isolation and subsequent differentiation (Fig. 2).

Our study revealed the usefulness of molecular markers as forensic tools to detect the origin of parasitic infections in the animal trade which may have further economic and legal implications. Moreover, the detection of the increasing invasion of diseases into new areas as emerging and re-emerging pathogens, and hence the application of such tools in animal conservation, should be considered. We encourage parasitologists to develop a standardised set of molecular protocols to detect the origin of animal infections and their pathogens. Such molecular tools will

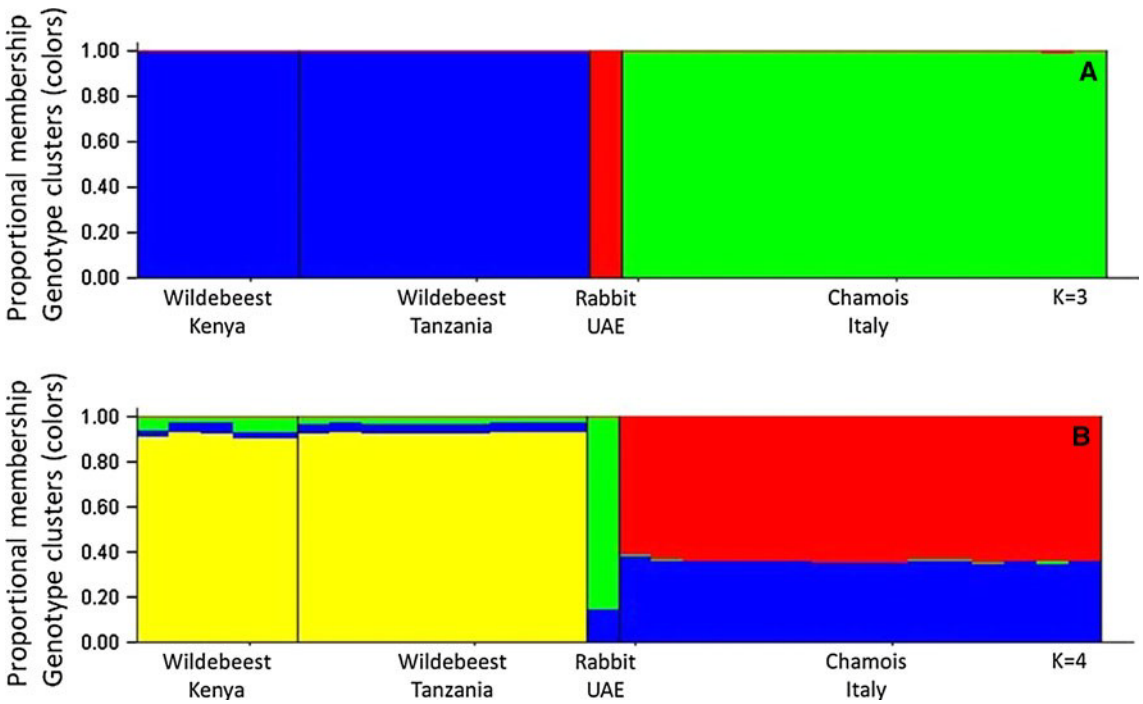

Fig. 2 Bar plotting of the proportion of individual variation of 30 Sarcoptes mite from wildebeest (from Tanzania and Kenya), rabbit (from United Arab Emirates), and chamois (Italy) assigned to genetic clusters in STRUCTURE, when three populations (a: K = 3) and four populations (b: K = 4) are assumed in the dataset

be of great benefit to guarantee welfare within the animal trade, law enforcement officials and conservationists.

Key points

1. Molecular techniques have a wide range of applications, including forensic identification, wildlife preservation and conservation, veterinary public health protection and food safety.
2. Trading with infected animals is illegal, unless, the infection goes undetected because of the long incubation period of the pathogen.
3. In this study we show the applicability of molecular markers as useful tools to be used in forensics to detect the origin of parasitic infections in the animal trade.
4. The use of molecular tools in "forensic parasitology" to detect the origin of parasitic infections is nevertheless limited without a vast sample collection, such as that proposed by the Sarcoptes-World Molecular Network; the Sarcoptes-Specimens World Bank [4].

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