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Using the genetics of *Echinococcus multilocularis* to trace the history of expansion from an endemic area

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ABSTRACT

Alveolar echinococcosis, caused by the cestode *Echinococcus multilocularis*, is the most serious parasitic disease for humans in Europe, with a sylvatic life cycle generally between small rodents and red foxes. General expansion of the range of *E. multilocularis* has been observed across Europe over the last 15 years. In France, a westward spread of the known endemic areas of the parasite was described recently. For genotyping, the microsatellite EmsB was used to trace expansion in five French areas. A total of 22 EmsB profiles were identified, with five similar to those previously described in other parts of Europe. An imbalance of genetic diversity was observed between the five areas which also revealed their interconnection with the presence of common profiles, notably the two main profiles both present in all regions except one in the North. These two findings are similar to those described at the European level, highlighting transmission of the parasite by a mainland–island system. A spatio-temporal scenario of the expansion of *E. multilocularis* can be proposed with spread from the French historical focus in eastern France to the Lorraine, the Champagne-Ardenne and finally the North, while simultaneously another expansion has occurred from the historical focus into the West. The colonization by the parasite into the West and North areas from the historical focus was probably due to the migration of foxes several decades ago. Recent detection of the parasite in new endemic "départements" may be due to more active research rather than a recent spread of the parasite. Regarding the numerous data obtained by the different EmsB analyses, principally across Europe, centralization of all the profiles described in a public databank appears necessary in order to obtain a precise understanding of transmission of the parasite from one country to another.

1. Introduction

Alveolar echinococcosis, caused by the cestode *Echinococcus multilocularis*, is the most serious parasitic disease for humans in Europe (Romig, 2009). The end result of oral ingestion of the tapeworm eggs is to cause chains of small interconnected cysts almost exclusively in the liver, with tumor-like, infiltrative, destructive growth (Eckert and Deplazes, 2001; Jenkins et al., 2005). Humans are considered to be dead-end hosts in the sylvatic life cycle of the parasite, which is based on a prey–predator relationship. In Europe, red foxes (*Vulpes vulpes*) and small rodents (*Microtus arvalis* and *Arvicola terrestris*) are the main definitive and intermediate hosts, respectively (Giraudoux et al., 2002). General expansion of the distribution range of *E. multilocularis* has been observed across Europe over the last fifteen years (Eckert et al., 2000; Romig, 2009; Romig et al., 2006). The south-central endemic foci described before the 1980s are now assumed to have spread to most parts of Europe, with the exception of the British Isles and the Mediterranean area (Romig, 2009). Although Fennoscandia was assumed to be free of *E. multilocularis*, cases in foxes were recently reported in Sweden and Norway (Svalbard) (Fuglei et al., 2008; Osterman-Lind et al., 2011). In Europe, the eastern and central parts of France were considered as the endemic border, mainly due to absence of data in other parts of the country. Recently, a westward spread of the known endemic areas of the parasite has been described by fox analysis with identification of *E. multilocularis* in 35 of the 42 "départements" (French administrative units) investigated, including 25 "départements" where the parasite had not previously been described (Combes et al., 2012). The western endemic border for *E. multilocularis* in Europe is therefore known to be located several hundred kilometers further west from the historical descriptions (Eckert et al., 2000; Romig et al., 2006).

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The tandemly repeated multilocus microsatellite EmsB has proved its usefulness for exploration of the genetic diversity of *E. multilocularis*, with discriminating power higher than 10 single-locus microsatellites combined (Bart et al., 2006; Knapp et al., 2007). Using this tool, the spatial and temporal spread of *E. multilocularis* in Europe from nine sub-regions (corresponding to 571 worms from 123 foxes) was characterized by the presence of 32 profiles. A mainland–island system of transmission was described with spread ruled by founder events across historical and peripheral areas (Knapp et al., 2009a). In the same way, an autochthonous focus was described in northern Italy based on the analysis of 17 worms in which four unique profiles were identified compared to those from others parts of Europe (Casulli et al., 2009). Hungary was considered as a peripheral area of the European focus due to the analysis of 81 worms and with regard to the low genetic diversity observed (Casulli et al., 2010), while in Denmark the hypothesis of an introduction from neighboring countries could not be documented since the isolate described did not closely cluster with any other European isolates (Enemark et al., 2013). In France, the usefulness of the microsatellite EmsB marker to assess genetic polymorphism was confirmed at a local scale in the Ardennes "département" (Knapp et al., 2008), but the *E. multilocularis* genetic diversity of all the known endemic areas of France was not explored. The aims of this study were to describe the genetic diversity of *E. multilocularis* in the historically endemic parts of France as well as in the newly described endemic areas, and consequently propose a spatio-temporal scenario for the westward spread of the known endemic areas of *E. multilocularis* recently described in France (Combes et al., 2012).

2. Materials and methods

2.1. Collection of adult E. multilocularis worms

The worms sampled for the present study came from fox intestines collected within the framework of a large-scale survey of fox infection by *E. multilocularis* in France (Combes et al., 2012). The Ille-et-Vilaine "département", recently found to be an endemic area, was added to the sampling area (Combes et al., 2013). Analyses of intestines were performed by the departmental veterinary laboratories involved in the study using the Segmental Sedimentation and Counting Technique (Umhang et al., 2011). On positive animals, worms were isolated and maintained in 70% (v/v) ethanol before transfer to the National Reference Laboratory (NRL) for confirmation of the parasite species. At the NRL, a maximum of 5 *E. multilocularis* worms per fox were used to perform the EmsB analyses. The final panel was composed of 383 worms from 128 foxes collected between 2007 and 2012 in 15 "départements" corresponding to five different geographical areas of France (Table 1). The geographical position of each fox and estimation of *E. multilocularis* prevalence in foxes were determined as reported by Combes et al. (2012) (Fig. 1).

2.2. DNA extraction and fragment size analysis

The worms were individually subjected to DNA extraction with the help of the Nucleospin Tissue kit (Macherey–Nagel, Germany) then stored at −20 °C until use. A fluorescent PCR assay was carried out as previously described (Knapp et al., 2009b). Briefly, the reaction was performed in a 25 µl reaction mixture, containing 200 µM of each dNTP, 0.4 µM of fluorescent forward primer EmsB A, 0.7 µM of classical reverse primer EmsB C, and 0.5 U of Platinum *Taq* DNA Polymerase enzyme (Life Technologies, Foster City, CA), with the addition of Platinum 1 × PCR Buffer (Life Technologies, Foster City CA, USA). The amplification reaction was performed in a Veriti thermal cycler (Life Technologies, Foster City, CA), under the following conditions: a pre-amplification step of 94 °C for 2 min, followed by 45 cycles with a denaturing step at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, with a final elongation at 72 °C for 45 min. Capillary electrophoresis of PCR products was performed on a sequencer machine (ABI Prism 310; Life Technologies, Foster City, CA). The size and height of each peak of the EmsB profiles were determined with the use of GeneMapper 4.1.

2.3. Genotyping, richness and diversity analysis

Unlike classical microsatellite profiles, the EmsB profile is composed of several peaks or alleles between 209 and 241 bp due to its multilocus nature in the parasite DNA (Bart et al., 2006). The characterization of each EmsB profile was performed as previously described (Knapp et al., 2007). The hierarchical clustering analysis was done using the Euclidean distance and the average link clustering method (UPGMA) (Legendre and Legendre, 1998). The uncertainty of clusters was evaluated by a multiscale bootstrap resampling ($B = 1000$) and given by approximately unbiased *P*-values (AU), according to Shimodaira (Shimodaira, 2002, 2004). Clustering analyses were performed using the R statistical software (R Development Core Team, 2005) and pvclust library (Suzuki and Shimodaira, 2006). The genetic threshold of 0.08 was used to determine the genotyping status of each sample while two *E. granulosus* sensu stricto (G1) were used as an outgroup (Knapp et al., 2007; Umhang et al., 2013). The EmsB profiles obtained from this French collection were compared with the 32 profiles obtained from the European collection (Knapp et al., 2009a) in order to identify identical profiles.

Species accumulation curves, plotting the cumulative number of profiles as a function of sampling effort (i.e. number of worms analyzed), were built for each area (Magurran, 2004). The curve reaches an asymptote when the sampling is sufficient to catch the actual number of genetic profiles in the area. If an asymptote was not reached, the first order Jackknife estimator of species richness, and its standard error, were computed to estimate the total number of profiles in the area (Magurran, 2004). Genetic diversity (α diversity) was computed in each area using the reciprocal Simpson index (1/D). Since sampling effort differed between areas, a

Table 1
Number of worms and foxes sampled for each of the five areas and the estimated prevalences of *E. multilocularis* in foxes.

"Départements" investigated	AREAS				
	Historical focus	Lorraine	Champagne-Ardennes	North	West
	Jura (39), Savoie (73), Doubs (25), Ain (1)	Moselle (57), Meurthe-et-Moselle (54)	Aube (10), Ardennes (8), Marne (51)	Nord (59), Somme (80), Oise (60)	Calvados (14), Ille-et-Vilaine (35), Manche (50)
No of worms	185	79	45	23	51
No of foxes	59	27	14	12	16
Prevalence in foxes in % (CI95%)	36.3% [31.7–41.2%]	42.8% [35.9–50.0%]	22.2% [17.8–27.3%]	11.8% [8.5–16.2%]	11.5% [8.2–15.9%]