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Population genetics, invasion pathways and public health risks of the raccoon and its roundworm *Baylisascaris procyonis* in northwestern Europe

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Abstract

The geographic range of the zoonotic raccoon roundworm (*Baylisascaris procyonis)* is expanding together with the range of its host, the raccoon (*Procyon lotor*). This creates a new public health risk in parts of Europe where this parasite was previously absent. In the Netherlands, a raccoon population is becoming established and incidental findings of *B. procyonis* have been reported. To assess the risk to public health, the prevalence of *B. procyonis* was determined in the province of Limburg, where currently the largest Dutch raccoon population is present, as well as in the adjoining region of southern Belgium. Furthermore, genetic methods were employed to assess invasion pathways of both the raccoon and *B. procyonis* to aid in the development of control measures.

Macroscopic analysis of intestinal content and testing of faecal samples were performed to detect *B. procyonis* adults and eggs. The population genetics of both *B. procyonis* and its raccoon host were analysed using samples from central and northwestern Europe.

B. procyonis was found in 14/23 (61%, 95% CI: 41%–78%) raccoons from Limburg, but was not detected in 50 Belgian raccoons. Genetic analyses showed that the majority of the Dutch raccoons and their roundworms were introduced through ex-captive individuals.

As long as free-living raccoon populations originate from captivity, population control methods may be pursued. However, natural dispersal from the border regions will complicate prolonged population control. To reduce the public health risk posed by *B. procyonis*, public education to increase awareness and adapt behaviour towards raccoons is key.

KEYWORDS
Baylisascaris procyonis, epidemiology, phylogenetic analyses, public health, raccoons, zoonotic infectious diseases

1 | INTRODUCTION

The raccoon roundworm (*Baylisascaris procyonis*) is a gastrointestinal nematode parasite of the raccoon (*Procyon lotor*). As the definitive host, infected raccoons can excrete millions of *B. procyonis* eggs via their droppings, that, under suitable moisture conditions, can remain infective in the environment for years (Page et al., 2011). In the definitive host, *B. procyonis* infections are usually asymptomatic. In non-primary hosts; however, the nematodes larvae hatch after ingestion and begin an aggressive, extra-intestinal migration (referred to as larva migrans), which can result in the host's death after larvae grow and migrate within the central nervous system (Sorvillo et al., 2002). The parasite is considered to be highly non-specific: over 130 vertebrate species have been reported to exhibit clinical symptoms of infection with the parasite (Page, 2013).

Humans are also susceptible to *B. procyonis* infection as accidental hosts. The precise symptoms of the ensuing baylisascariasis depend on the extra-intestinal migration of the ingested larvae and include visceral, ocular and neural larva migrans syndrome. The latter is of particular concern as there is no known effective treatment and published reports of neural larva migrans cases frequently mention fatal outcome or neurological impairment (Gavin et al., 2002; Wise et al., 2005). Infections usually occur in infants, which are especially at risk because of the faecal–oral transmission route (Gavin et al., 2002). Also at risk are people exhibiting pica or geophagia syndromes, those with (occupational) contact with raccoons, as well as inhabitants of housing with raccoon activity nearby (Conraths, 1996; Sorvillo et al., 2002; Wise et al., 2005).

The raccoon and the raccoon roundworm *B. procyonis* are native to North America. However, both have expanded their global distribution through export of raccoons. In the raccoon populations that are establishing themselves in Europe, the prevalence of *B. procyonis* varies. The parasite is particularly widespread in free-living raccoons in central Germany, where a median prevalence of 43.6% per administrative district ('Landkreis') has been reported (Heddergott et al., 2020). Although the reported number of human baylisascariasis in Europe is limited thus far to one non-fatal case (Küchle et al., 1993) and four seropositive persons (out of a group of 13) (Conraths, 1996) from Germany, infected raccoons often live in urban areas in close proximity to humans (Gey, 1998; Rentería-Solís et al., 2018). Consequently, the World Health Organisation has classified baylisascariasis as a zoonosis 'with current and potentially increasing impact' in Europe (Anonymous, 2004). From a public health perspective, the identification of *B. procyonis* risk areas is thus necessary, as, aside from prevention, early recognition and rapid treatment can sometimes prevent severe outcomes (Dunbar et al., 2019).

In the Netherlands, after decades of infrequent raccoon observations that likely concerned escaped or released pet animals, as well as an occasional immigrant from the neighbouring raccoon populations in Germany or Belgium (Delbroek & Janssen, 2018; Lammertsma et al., 2008), a population of free-living raccoons in Limburg has reached a sufficiently high density to allow reproduction (Delbroek & Janssen, 2018). *B. procyonis* has been detected sporadically in the Netherlands: in 2007 in a raccoon of unknown origin; in 2014 in two road-killed raccoons near the Dutch-German border in the east-central Netherlands (Dutch Wildlife Health Centre (DWHC), 2014) and in 2016 in a road-killed raccoon in the southern province of Limburg (Maas et al., 2018). However, it is not clear whether the parasite is established in the Dutch raccoons in general and in Limburg in particular or whether these occurrences were incidental and limited to these specific animals. In the adjoining region of southern Belgium, raccoons are established (Salgado, 2018) but it is unclear whether *B. procyonis* is present in this population and whether this raccoon population may have served as a source for the Limburg population.

Therefore, the first aim of this study was to estimate the prevalence of *B. procyonis* in Dutch and Belgian raccoon populations. Furthermore, for effective eradication and/or management actions, it is important to identify the invasion pathways of the parasite (and its host). Thus, the population genetic structures of the raccoon and its *B. procyonis* parasite in northwestern Europe were analysed to ascertain whether host and parasite originated from captivity or whether they entered the Netherlands by natural dispersal, or whether the parasite may have spread into a nematode-free raccoon population.

2 | METHODS

2.1 | Sample collection and preparation-field study

Between September 2019 and March 2020, 23 raccoons were captured as part of a relocation project to control the population in the Dutch province of Limburg. Age was estimated based on size, weight, eruption of permanent teeth and tooth wear. EDTA blood samples were collected for genetic analysis and stored at −20°C. After collection of a first faecal sample, all animals were subjected to an anthelmintic treatment with 1 mg/kg ivermectin (Ivomec 1%, Boehringer-Ingelheim; Alkmaar, the Netherlands). The two following days, faeces were checked for the presence of worms, which were collected, together with a second faecal sample. Excreted worms were determined morphologically and stored in 70% ethanol while faecal samples were processed using faecal centrifugal filtration and flotation (FFF; for details see Appendix) to detect *B. procyonis* eggs. In one case, faecal samples were tested for the presence of nematode eggs using a formol-ether sedimentation (FES) method (Allen & Ridley, 1970).

Between 2012 and 2015, 50 hunted and road-killed raccoons from Wallonia, the southern Belgian region adjoining Limburg, were tested. Presence of *B. procyonis* was investigated by macroscopic analysis of intestinal content (eggs or worms) and faecal samples were analysed to detect eggs using zinc chloride flotation (for details see Appendix).

Raccoons were considered positive when *B. procyonis* worms were excreted upon anthelmintic treatment or detected in the intestines or when *B. procyonis* eggs were detected in the faecal samples. Except in the case of the eggs obtained using the FES method, the species identity of the eggs was confirmed using genetic methods (see below).

2.2 | Sample collection – population genetic analysis

In addition to the 23 raccoons from Limburg, one raccoon trapped in 2019 in the eastern Dutch province of Drenthe was subjected to the same treatment and included in the population genetic analysis. Furthermore, from six road-killed raccoons collected between 2011 and 2016 in various locations in the Netherlands muscle tissue samples and their roundworms (if applicable) were included (Table 1). Between 2006 and 2016, 103 raccoon muscle tissue samples from Wallonia and 28 from northeastern France were opportunistically collected from hunted and road-killed raccoons to be included in our population genetic reference dataset (see below).

2.3 | Molecular laboratory work

DNA was extracted from *B. procyonis* adult worms using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), from *B. procyonis* eggs using the QIAamp mini stool kit (Qiagen, Hilden, Germany) and from raccoon blood using the Sherlock AX kit (A&A Biotechnology, Gdynia, Poland). In each case, we followed the manufacturer's instructions. DNA was extracted from the muscle tissue using an ammonium-acetate-based precipitation method (Miller et al., 1988).

The species identity of the recovered nematodes and the nematode eggs were confirmed based on the mitochondrial cytochrome oxidase 1 (CO1) gene (Franssen et al., 2013). Seventeen microsatellite loci were used to generate a genetic profile for each raccoon (consisting of a minimum of 15 genotyped loci) (Osten-Sacken et al., 2018). Working on the Germany/Luxembourg raccoon reference dataset also used here (see below), Fischer et al. (2015) obtained no evidence for systematic deviations from Hardy–Weinberg and linkage equilibria among these 17 loci. *B. procyonis* samples were genotyped using 13 microsatellite loci that were shown to be inherited in a Mendelian fashion (Osten-Sacken et al., 2018) and only genetic profiles genotyped at a minimum of 11 of 13 loci were considered in the statistical analysis.

2.4 | Statistical and genetic analyses

The Wilson score interval was used to calculate the 95% confidence intervals of the proportion of infected animals in the Limburg raccoon population (Newcombe, 1998). For the genetic analysis of the raccoons, the 154 genetic profiles (from Belgium, France and the Netherlands) generated in this study were added to a reference dataset consisting of 390 genetic profiles from northern Belgium, Luxembourg and Germany that had been genotyped in the same laboratory using the same protocols (Fischer et al., 2015). Based on this reference dataset, five distinct raccoon populations were previously inferred to be present in Saxony (eastern Germany), in Brandenburg and surrounding areas (northeastern Germany), around the Harz low mountains (Central Germany), in Hesse and its neighbouring regions (Central Germany), as well as in western Germany and Luxembourg (Fischer et al., 2015; Heddergott et al., 2020; see also Figure 1). For the genetic analysis of *B. procyonis*, the 15 genetic profiles generated here were added to a reference dataset (Osten-Sacken et al., 2018) consisting of 217 genetic profiles from Central Germany (Figure 2a). So far, *B. procyonis* has been reported to occur only in the Harz and Hesse raccoon populations (Heddergott et al., 2020). The genetic structure of the parasite mirrors that of its host insofar as *B. procyonis* in (neighbouring regions of) Hesse and around the Harz form two distinct genetic populations (Osten-Sacken et al., 2018). *B. procyonis* has not been found yet in Luxembourg (Heddergott et al., 2020) or northeastern France (Umhang et al., 2020), so that no *B. procyonis* samples from these countries could be included in the genetic analysis.

For both datasets, the number of genetic clusters (K) was inferred using STRUCTURE v. 2.3.4 (Pritchard et al., 2000), performing 10 independent runs of $K = 1–12$ (raccoon) or $K = 1–10$ (roundworm) with 10^6 Markov chain Monte Carlo (MCMC) iterations after a 10^5-iteration burn-in length, based on the admixture and correlated-allele-frequency models. ALPHA, the Dirichlet parameter for the degree of admixture, was allowed to vary between clusters. The choice of the most likely number of clusters was based on the ten log-likelihood values estimated for each K and their convergence. After accounting for label switching and confirming the lack of multimodality,

TABLE 1 Characteristics of the Dutch raccoon and *B. procyonis* samples. Positive raccoons had excreted worms, unless indicated otherwise. Negative status was based on no excretion of worms, a negative result with faecal centrifugal filtration and flotation (FFF) and a negative PCR, unless indicated otherwise. The 'inferred STRUCTURE cluster' columns indicate the cluster to which a raccoon or its *B. procyonis* parasite(s) were assigned to. Except in two cases, we analysed one parasite per raccoon. For more information on the geographic spread of the 'BeNeGe', 'Hesse' and 'Limburg' raccoon clusters, please refer to Figures 1 and 2 for the distribution of the genetic clusters of the parasite

Raccoon ID	Age class	Gender	Year	Municipality (province)	*B. procyonis* status	Inferred STRUCTURE cluster	
						Raccoon	*B. procyonis*
D280	Unknown	Unknown	2011	Nijkerk (G)	Not tested	Hesse	NA
3141021018	Subadult	F	2014	Doetinchem (G)	+[†]	Hesse	2× Hesse, 1× Limburg
3141208029	Unknown	M	2014	Doetinchem (G)	+[†]	Hesse	3× Hesse
3151029005	Adult	M	2015	Nederweert (L)	–[†]	BeNeGe	Not present
3160215001	Adult	M	2016	Boxmeer (NB)	–[†]	BeNeGe	Not present
3161031032	Adult	Unknown	2016	Stein (L)	+[†]	Limburg	Limburg
Yas	Subadult	F	2019	Roerdalen (L)	+	Limburg	Limburg
Xela	Adult	M	2019	Sittard–Geleen (L)	+	Limburg	Limburg
Sittard	Subadult	M	2019	Beekdaelen (L)	+	Limburg	Limburg
Ahmik	Subadult	M	2019	Beekdaelen (L)	+	Limburg	Limburg
Paiute	Adult	F	2019	Beekdaelen (L)	+	Limburg	Limburg
Tigua	Adult	F	2019	Beekdaelen (L)	–	Hesse	Not present
Tadi	Adult	F	2019	Venlo (L)	–	Hesse	Not present
Weeko	Adult	F	2019	Beekdaelen (L)	–	Limburg	Not present
Payat	Adult	F	2019	Emmen (D)	–	BeNeGe	Not present
Guyapi	Adult	F	2019	Beekdaelen (L)	+	Limburg	NA
Stein	Adult	M	2019	Beekdaelen (L)	+[‡]	Hesse	NA
Schin	Adult	F	2019	Beekdaelen (L)	+	NA	NA
Dell	Adult	M	2019	Beekdaelen (L)	–	NA	Not present
Hachi	Adult	F	2019	Sittard–Geleen (L)	–	NA	Not present
Sihu	Adult	M	2020	Sittard–Geleen (L)	+	Limburg	Limburg
Tuketu	Adult	M	2020	Beekdaelen (L)	+	Hesse	Limburg
Walapai	Adult	M	2020	Beekdaelen (L)	–	Limburg	Not present
Geleen	Adult	F	2020	Beekdaelen (L)	–	Limburg	Not present
Alawa	Adult	F	2020	Sittard–Geleen (L)	+[§]	Limburg	NA
Atohi	Subadult	F	2020	Sittard–Geleen (L)	+[§]	Limburg	NA
Schinveld	Adult	F	2020	Beekdaelen (L)	–[¶]	NA	Not present
Susteren	Adult	F	2020	Echt–Susteren (L)	–	NA	Not present
Laatste	Adult	M	2020	Echt–Susteren (L)	+	NA	NA
Tipais	Adult	M	2020	Beekdaelen (L)	+	NA	NA

[†]Macroscopic examination, followed by molecular confirmation when worms were identified.
[‡]Positive status based on FES.
[§]Positive status based on FFF, PCR and sequencing. Eggs were used for the STRUCTURE analysis.
[¶]No worm excretion, negative FFF, PCR positive, but sequencing negative. NA = either no tissue or blood sample was collected, or it was not possible to generate a microsatellite profile for the animal in question.
Abbreviations of provinces: G = Gelderland, L = Limburg, NB = North-Brabant, D = Drenthe.

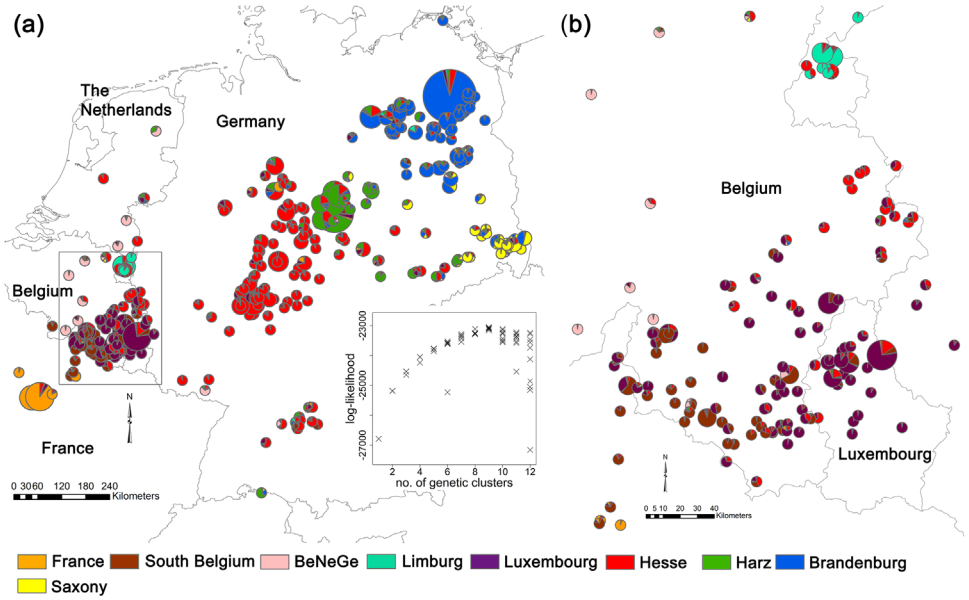

FIGURE 1 Results of the analysis of the population genetic structure of the raccoon in its northern European distribution. (a) Spatial distribution of the genetic clusters inferred by program STRUCTURE for $K = 9$. Inset: Plot of the number of STRUCTURE clusters tested against their estimated log-likelihood. (b) Focus on the clustering results from the region indicated by a black square in (a). Different colours represent different genetic populations. Pie charts represent the genetic populations of origin of the individuals and their size is indicative of the number of samples included.

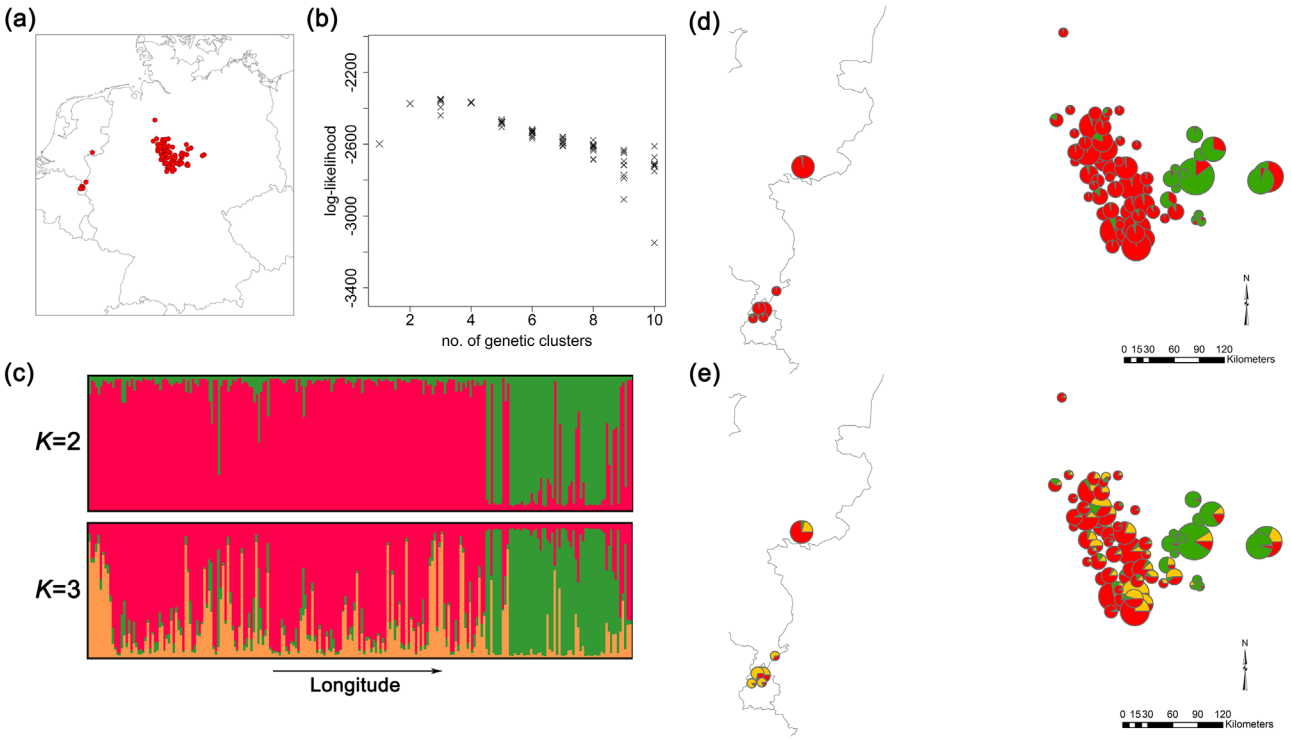

FIGURE 2 Results of the analysis of the population genetic structure of *B. procyonis*. (a) Geographic origin of Dutch and German reference samples. (b) Plot of the number of STRUCTURE clusters tested against their estimated log-likelihood. (c) Bar plots of the proportion of membership of each individual (represented by a vertical line) to the different clusters inferred by STRUCTURE for $K = 2$ and $K = 3$ genetic clusters. (d) Spatial distribution of the genetic clusters inferred by program STRUCTURE for $K = 2$ genetic populations. (e) Spatial distribution of the genetic clusters inferred by program STRUCTURE for $K = 3$ genetic populations. Different colours represent different genetic populations. Pie charts represent the genetic populations of origin of the individuals and their size is indicative of the number of samples included.

the proportion of membership of each individual was averaged over replicate runs for a specific value of *K*. For subsequent analyses, animals were modally assigned to the STRUCTURE cluster for which the highest proportion of membership had been estimated. The program GENETIX v.4.05.2 (Belkhir et al., 2004) was used to perform a factorial correspondence analyses (FCA) to visualize the genetic distance between animals. Individuals whose FC scores were more than six standard deviations away from the mean score of one of the first two eigenvectors were defined as outliers. The program SPAGEDI 1.5 (Hardy & Vekemans, 2002) was employed to estimate the degree of genetic differentiation between the genetic clusters based on F_{ST} values (Weir & Cockerham, 1984) with 10,000 permutations of individual genetic profiles between populations. Unbiased expected heterozygosity (uH_e) was estimated (Nei, 1978) using GENETIX v.4.05.2 and the allelic richness (A_R) using the program FSTAT v.2.9.3.2 (Goudet, 1995).

3 | RESULTS

3.1 | Prevalence of *B. procyonis*

Of the 23 raccoons that were captured within Limburg (Netherlands), 14 tested positive for *B. procyonis* (61%, 95% CI: 41%–78%). Of these, excretion of adult *B. procyonis* worms was recorded for 11 raccoons. Two raccoons were confirmed to be positive by the FFF and sequencing, and the last one raccoon by the FES (Table 1). A 405-base-pair-long fragment of the CO1 gene was generated from seven worms from different raccoons, as well as from the two FFF samples. All nine sequences were completely identical (GenBank acc. no.: MW465179) and matched the (48-bp shorter) *B. procyonis* haplotype HT1 previously reported from *B. procyonis* from Germany (GenBank: MF680533). Positive raccoons were obtained from 4/5 municipalities, and in the municipality of Sittard–Geleen 4/5 raccoons tested positive. No significant association of infection with *B. procyonis* with gender (Fisher's exact test $p = .20$) or age (Fisher's exact test $p = .13$) was detected.

None of the 50 raccoons from Wallonia (Belgium) was found positive for *B. procyonis* (Appendix Table A1 and Figure A1).

3.2 | Genetic analysis raccoons

The highest STRUCTURE log-likelihood values were obtained for $K = 9$ (Figure 1a). Thirteen out of 17 raccoons in Limburg, including one older sample, were inferred to belong to a distinct genetic population (referred to as the 'Limburg' population hereafter). The clustering program also identified the four main German populations (Brandenburg, Harz, Hesse, Saxony) and showed that the Luxembourg cluster extended in a northwestern direction into Belgium (Figure 1). STRUCTURE also inferred the presence of distinct genetic populations in northeastern France and southern Belgium as well as a small cluster consisting of animals sampled in northern Belgium, in the Netherlands and in a few dispersed localities in Germany (referred to as the 'BeNeGe' cluster hereafter; Figure 1). The majority of the animals

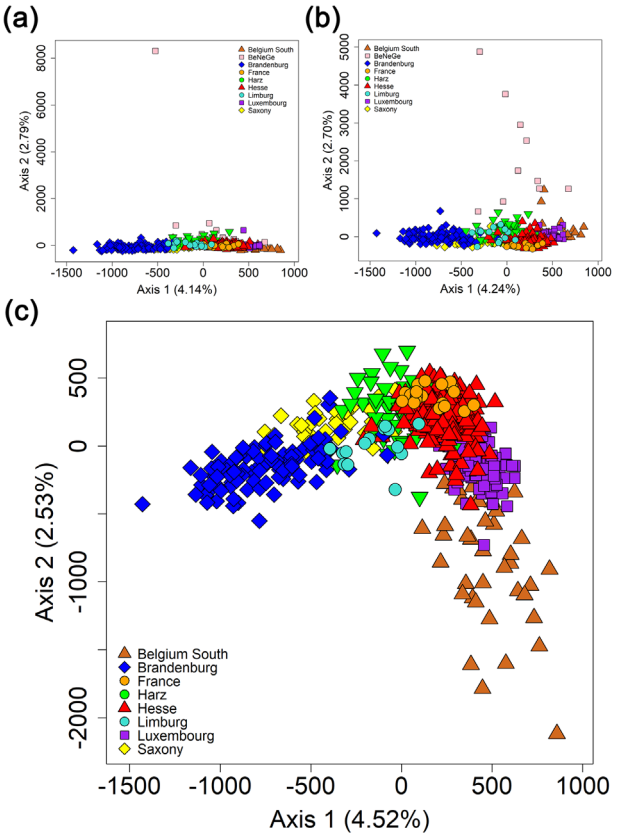

FIGURE 3 Factorial correspondence analysis of microsatellite-based genetic profiles of the raccoons (a) in the complete dataset, (b) in a dataset excluding one BeNeGe outlier, (c) in a dataset excluding all all animals from the BeNeGe cluster. The percentage of the total variation explained by each of the three axes is indicated

from southeastern Belgium and some animals from Luxembourg and the Netherlands were assigned to the German Hesse population (Figure 1b; Table 1).

When considering the FCA of the whole dataset, one animal assigned to the BeNeGe cluster was a genetic outlier (Figure 3a). After removing this animal and recalculating the eigenvectors, four animals from the BeNeGe cluster were statistical outliers (Figure 3b). Because the remaining BeNeGe animals were also slightly distinct from the main clusters, eigenvectors were recalculated with the dataset without BeNeGe animals to better focus on the differentiation between the main clusters. This new FCA (Figure 3c) confirmed the genetic distinctness of all but one of the STRUCTURE-inferred clusters (the extent of the France cluster completely overlapped with the Hesse cluster). The Limburg cluster was distinct, but a little dispersed and located at the intersection of the Brandenburg, Harz, Hesse and Saxony clusters (Figure 1c). All the F_{ST} estimates between the nine STRUCTURE clusters were statistically significant ($p < .001$) and varied between 0.054 and 0.306, with an average of $F_{ST} = .130$ (Appendix Table A2). The F_{ST} values of all pairwise comparisons involving the Limburg cluster varied between 0.098 (Limburg–Hesse) and 0.306 (Limburg–France). The

France cluster was strongly differentiated from all other partitions: the seven pairwise comparisons with the highest F_{ST} values all included the France cluster and the remaining comparison (France–Hesse) had $F_{ST} = .127$, indicating that the France cluster is distinct even from the Hesse cluster.

There was a statistically significant difference in the median of the uH_e and A_R (calculation based on 10 diploid individuals) between the different clusters (uH_e: Kruskal–Wallis $\chi^2 = 28.07$, df = 8, $p < .001$; A_R: Kruskal–Wallis $\chi^2 = 38.08$, df = 8, $p < .001$). The France cluster had a reduced ($uH_e = 0.36$; $A_R = 2.6$) and the BeNeGe cluster a relatively high genetic diversity ($uH_e = 0.72$; $A_R = 5.8$). The genetic diversity of the Limburg cluster ($uH_e = 0.57$; $A_R = 4.0$) fell within the range of the estimates for the other clusters ($0.57 \leq uH_e \leq 0.66$; $3.6 \leq A_R \leq 4.8$; Appendix Table A3).

3.3 | Genetic analysis *B. procyonis*

The STRUCTURE log-likelihood values started to plateau at $K = 2$ (Figure 2b). The two inferred *B. procyonis* clusters were geographically coherent and consisted of an eastern ('Harz') and a western ('Hesse') cluster, with the worms from Limburg raccoons assigned to the latter (Figure 2c–d). However, ignoring two runs that converged at lower values, the highest log-likelihood values were obtained for $K = 3$ (Figure 2e). At $K = 3$, STRUCTURE in essence inferred again the presence of a Harz and Hesse cluster, but additionally, the worms from raccoons from southern Limburg together with those from 27 German raccoons formed a third cluster. While this third cluster lacked a certain degree of geographic coherence, an FCA (Figure 4a–c) confirmed – after iterative removal of two outliers (German animals, one of which assigned to the Limburg cluster) – the genetic distinctness of the worms from the raccoons in the Limburg cluster. According to F_{ST} estimates, all three *B. procyonis* clusters were significantly differentiated (Harz–Hesse: $F_{ST} = .283$, $p < .001$; Harz–Limburg: $F_{ST} = .332$, $p < .001$; Hesse–Limburg: $F_{ST} = .162$, $p < .001$). While genetic diversity estimates ($uH_e = 0.12$; $A_R = 2.0$) for the Harz cluster were lower than the corresponding values for Hesse ($uH_e = 0.27$; $A_R = 2.2$) and Limburg ($uH_e = 0.22$; $A_R = 2.2$), these differences were not statistically significant (uH_e: Kruskal–Wallis $\chi^2 = 2.808$, df = 2, $p = .246$; A_R: Kruskal–Wallis $\chi^2 = 0.525$, df = 2, $p = .769$; the A_R estimations were based on 29 diploid individuals).

Of the 10 genotyped Dutch raccoons from which 1–3 *B. procyonis* roundworms were also genotyped, 7 were assigned to the Limburg and 3 to the Hesse STRUCTURE cluster (Table 1). While the roundworms were generally assigned to the cluster corresponding to the one of their raccoon host, there were two exceptions: a raccoon from Beekdaelen (Limburg) assigned to the Hesse cluster was parasitized by a roundworm assigned to the Limburg cluster, and a raccoon from Doetinchem (eastern Netherlands) assigned to the Hesse cluster harboured two roundworms assigned to the Hesse cluster but also one roundworm assigned to the Limburg roundworm cluster (Table 1, Figures 1 and 2). No Dutch raccoon assigned to the BeNeGe cluster tested positive for the presence of *B. procyonis*.

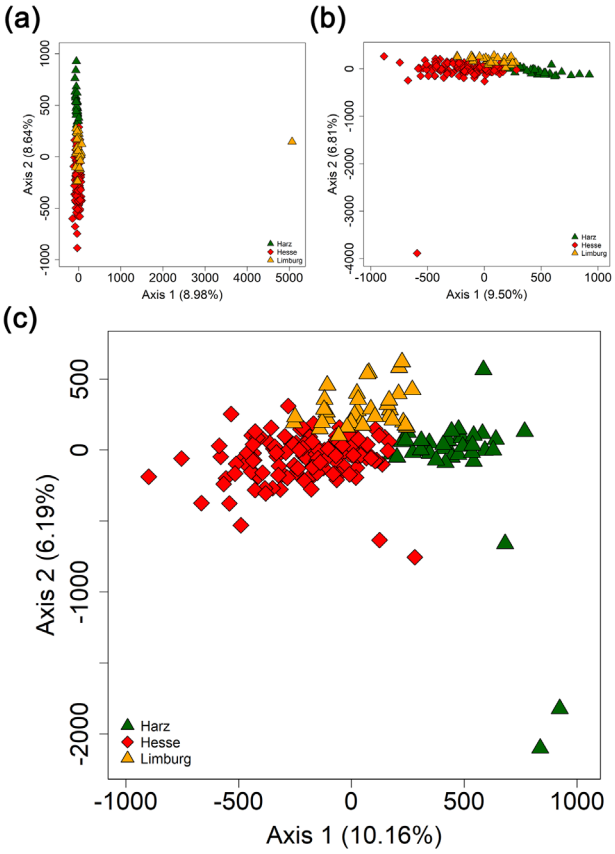

FIGURE 4 Factorial correspondence analysis of microsatellite-based genetic profiles of *B. procyonis* (a) in the complete dataset and (b–c) in a dataset excluding two outliers. The percentage of the total variation explained by each of the three axes is indicated

4 | DISCUSSION

The present study confirms that the roundworm *B. procyonis* is established in raccoons in the province of Limburg in the south of the Netherlands, while providing no evidence so far for the parasite in Belgium. Based on a sample size of 23 raccoons, a prevalence of 61% (95% C.I.: 41-78%) was observed in Limburg. This is high; for comparison, out of the 69 administrative districts in Germany where the parasite was present and 23 or more raccoons had been sampled, only 3 districts had prevalence estimates of ≥61% (M. Heddergott, unpublished data). These *B. procyonis* infected raccoons occurred in 4/5 of the investigated municipalities in Limburg. Given the high infection rate found in this sample of Limburg raccoons and considering that *B. procyonis* eggs can remain infective in the environment for years, there is a risk of severe environmental contamination and an ensuing public health risk if the raccoon population is able to conclusively establish itself in Limburg. However, to further substantiate this finding, a larger number of raccoons needs to be tested from more areas, to account for potential hotspots or seasonal variation in *B. procyonis* infection rates (Page et al., 2005).

The study results clearly show that raccoons in Limburg form a distinct genetic population differentiated from the raccoons in the

surrounding areas. In contrast, the Dutch raccoons sampled outside of Limburg were assigned either to the Hesse cluster, and thus probably have a German raccoon origin, or to the BeNeGe cluster. The BeNeGe cluster is geographically widespread, consists of animals that are genetically distinct from the main clusters and has a high genetic diversity, suggesting that its constituent animals largely originated from separate introduction events. Thus, with a few exceptions, Dutch feral raccoons seem to originate from captivity rather than from free-living raccoon populations in bordering countries that are spreading into the Netherlands. Raccoon introductions originating from pet animals have been reported in several European countries and appear to be the major introduction pathway nowadays (Salgado, 2018). Also, in contrast to raccoons from northeastern France, for example, the genetic diversity of the Limburg raccoons appears to be relatively high and similar to estimates obtained for the larger surrounding populations. The Limburg population was thus either founded by a larger number of individuals or from animals with a different genetic background. The latter appears perhaps more likely, given the dispersion of the cluster in the FCA. In Belgium, four different raccoon clusters were distinguished (Luxembourg, Hesse, southern Belgium and BeNeGe). The continuity of the Luxembourg cluster in Luxembourg and Belgium supports natural movement of raccoons across the Belgium–Luxembourg border.

The analysis of the genetic structure of *B. procyonis* produced results that were less clear. STRUCTURE confirmed the presence of the two *B. procyonis* genetic populations around Hesse and the Harz mountains, respectively (Osten-Sacken et al., 2018), and also provided indications for all the nematodes in southern Limburg forming a third cluster. However, a further 27 German nematodes were also assigned to this third population. Further analyses tended to support the presence of three partitions as they were shown to be to be clearly differentiated. The lack of clear geographic separation could be artificial and result from employing a relatively small number of microsatellite loci that also lacked variability (Manel et al., 2002). Taking into consideration that all southern Limburg *B. procyonis* were assigned to the third cluster and that the Limburg raccoons formed a separate population, we tentatively suggest that the roundworms were introduced from captivity alongside (some) of their raccoon hosts.

When both raccoon and *B. procyonis* DNA were available, they were generally assigned to the corresponding cluster, with two exceptions. From two raccoons found in 2014 near the German border in the eastern Netherlands, six *B. procyonis* worms were collected. Five of these nematodes were assigned to the roundworm Hesse cluster. As the raccoons were also assigned to the Hesse cluster, the results provide support for spread of the parasite into the Netherlands via infected hosts. It remains unclear if these two raccoons were human-mediated import cases or specimens that naturally crossed the border, as there is no information on raccoons and roundworms in the direct border area in Germany. In the case of one of these raccoons, STRUCTURE assigned one of its roundworms to the Limburg cluster and the other two to the Hesse cluster. This result could be an artefact resulting from the uncertainty associated with the STRUCTURE assignments (see above), rather than the raccoon genuinely having been infected with *B. procyonis* from two different genetic populations. Alternatively, the raccoon could have picked up a dual infection in captivity if several raccoons from different origins had been kept in one pen. The second exception was a raccoon from the Hesse cluster infected with a roundworm assigned to the Limburg cluster. In this case, the infection could have been picked up either in captivity or after arrival in the Limburg environment.

Collectively, these results provide evidence for an independent introduction of raccoons in the Netherlands, likely as a result of released or escaped captive raccoons. Between 1995 and 2008, raccoons were frequently observed in urban areas in the Netherlands, away from natural corridors such as rivulets, suggesting that many of these concerned released pet raccoons (Lammertsma et al., 2008). This is supported by the finding of a microchip in one of the raccoons from this study. Furthermore, our results suggest that, rather than spreading into a nematode-free population, the roundworm was introduced alongside the ex-captive raccoons. As long as natural dispersion across borders seems to be rather limited, eradication measures could be relatively effective. Currently, both lethal control methods and capture-and-relocation are used to reduce raccoon population densities in the Netherlands, following the EU regulation on Invasive Alien Species (European Parliament, 2014). However, despite the possibility of controlling the parasite and its host in the short term, the risk that *B. procyonis* will become established in the Netherlands in the future remains high as natural immigration will likely gain in importance.

We did not detect *B. procyonis* in the sampled raccoons from Wallonia, confirming results by Heddergott et al. (2020) who did not detect the roundworm in raccoons from Luxembourg, that belonged to the same 'Luxembourg' genetic cluster. However, our STRUCTURE analysis showed that the raccoons in southeastern Belgium (i.e. the area immediately to the south of the Netherlands) partly originated from the German Hesse population. It is thus surprising that no *B. procyonis* adults were detected in this region (Appendix Table A1 & Figure A1) and further monitoring efforts in Belgium should probably be centred on the eastern side of the country.

The exponential growth of the raccoon population in Germany will probably lead to (increased) natural dispersal into the Netherlands and Belgium during the next decades (Fischer et al., 2016), limiting the effect of population control measures. Therefore, to reduce the public health risk posed by *B. procyonis* in the long term, it is important to reduce anthropogenic food sources that support high densities of raccoons, to already start to raise awareness among people that have occupational contact with raccoons and to invest in public education to avoid the contact with raccoons and raccoon latrines (Gavin et al., 2002).

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. For the study in Limburg, faeces samples were used that were already collected as part of the capture and relocation program of the Province of Limburg. Furthermore, dead raccoons that had been hunted or road-killed were tested. The Belgian raccoons concerned dead raccoons that had been hunted or road-killed.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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SUPPORTING INFORMATION

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