



Raccoons foster the spread of freshwater and terrestrial microorganisms—Mammals as a source of microbial eDNA

Wojciech Solarz | Kamil Najberek | Elżbieta Wilk-Woźniak | Aleksandra Biedrzycka

Institute of Nature Conservation, Polish Academy of Sciences, Kraków, Poland

Correspondence

Aleksandra Biedrzycka, Institute of Nature Conservation, Polish Academy of Sciences, al. Adama Mickiewicza 33, 31-120 Kraków, Poland.

Email: biedrzycka@iop.krakow.pl

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Abstract

Aim: The aim of the study was to test the role of raccoon (*Procyon lotor*), an invasive alien species, in the spread of microorganisms. We tested whether the spread of microorganisms can be detected by sampling microbial DNA sourced from the raccoon body, thus facilitating biodiversity research.

Location: Warta Mouth National Park, western Poland.

Methods: We used the V4 hyper-variable region of the 18S ribosomal RNA gene and Illumina MiSeq amplicon sequencing to identify microorganisms present on the body surface of raccoons.

Results: Out of 170 DNA samples, we obtained 15 PCR products that contained the target sequences of freshwater or terrestrial microorganisms. We found that raccoons carry and spread chlorophytes, alveolates, amoeboids and fungi on their body surface. We identified 16 different microbial organisms. The sequences of four organisms, *Micronuclearia podoventralis* (amoeboid), *Parachloroidium lobatum* and *Jaagichlorella roystonensis* (chlorophyta), and *Mortierella polygonia* (fungi), exhibited 100% identity to the best GenBank hit and were thus identified to the species level. The two chlorophyte species, *Parachloroidium lobatum* and *Jaagichlorella roystonensis*, are particularly noteworthy, as they were first described recently, in 2013 and 2019, respectively, and knowledge about their global distribution is very scarce.

Main conclusions: We demonstrated that raccoons may effectively spread terrestrial and aquatic microorganisms. By utilizing this novel source of microbial DNA, we also showed that mammals may be effective living samplers. This perspective is worth exploring, as in some cases it may efficiently reduce the burden required in traditional sampling and provide valuable insights into local biodiversity and distributions of species.

KEYWORDS

18S rRNA, biological invasions, chlorophytes, eDNA, expansion, microorganisms, *Procyon lotor*, raccoon, transport, vectors

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1 | INTRODUCTION

The dispersal of organisms by animals is a well-known phenomenon, and dispersal units include whole organisms or their parts, such as pollen, spores or seeds. The endo- and epizoochory of diaspores have received a particularly high amount of attention (Albert et al., 2015; Illuz, 2010). While for some endozoochorous diaspores, the suite of vectors may be limited to a narrow group of organisms, epizoochorous diaspores can be transported, as stowaways or contaminants (Hulme et al., 2008), by many types of casual vectors. However, even though they are not strictly dependent on specific vectors, epizoochorous diaspores usually have evolved properties, such as hooks, barbs or adhesive mucus that facilitate attachment to different organisms that increase the chance of successful transport (Howe & Smallwood, 1982). Even more casual links involve situations in which a stowaway dispersal unit is accidentally taken on-board the vector, with no direct relevance to the fitness of the transported organism. Such long-distance accidental hiking is usually associated with highly mobile vectors, such as birds (Green, 2016; Hessen, Jensen, & Walseng, 2019; Reynolds, Miranda, & Cumming, 2015; Solarz, Najberek, Pocięcha, & Wilk-Woźniak, 2017). In the case of vector organisms that have lower mobility, such as small- and medium-sized mammals, the methods by which they disperse other organisms can have considerably different dynamics and spatial patterns. Although the role of mammals in this respect has been less studied than the role of birds, the extent of the accidental short-distance transport of stowaway dispersal units attached to mammal skin, fur or hooves is likely to be great (Baltzinger, Karimi, & Shukla, 2019; Liehrmann et al., 2018; Vanschoenwinkel et al., 2011). However, over short time-scales, the contribution of such short-distance movements to the general biogeography of transported species is small, as the global distribution of these species has long been governed by processes operating at larger temporal and spatial scales, such as climate shifts related to glaciation.

The significance of mammal vectors as direct drivers of rapid large-scale changes in diversity and community composition might have increased as a result of human-mediated introductions of alien species into new areas. It is well known that diaspores of newly introduced alien species pre-adapted for endo- or epizoochory are spread by local mammals (Bartuszevige & Bryan, 2008; Chuong et al., 2016; Eschtruth & Battles, 2009). It can therefore be concluded that mammal vectors may also accidentally carry novel alien stowaways even if the stowaways are not specifically adapted for transport. Even if single hiking events are only over short distances, they may be critical for the establishment of the alien stowaway in a new area. Consequently, the combined effects of such step-by-step movements may result in the spread of an alien organism, thus leading to rapid biogeographical changes at large spatial scales. Even less studied is a scenario in which the introduced species itself is a mammal that becomes a novel accidental vector for local stowaways other than pre-adapted diaspores. While expanding its range in the new area, the alien mammal may carry stowaway organisms beyond the limits of their former range. Taking into account the rate of spread of some invading mammals, even single and short-distance

transport events of stowaways may rapidly alter their large-scale biogeography. In this respect, alien vectors differ from the native ones, in which rapid range expansion is rare.

The aim of this study was to assess the role of the North American raccoon (*Procyon lotor*) as a casual dispersal vector for microorganisms and as a source of microbial DNA. After its primary introduction in Germany in the 1930s and a lag phase when the population persisted at small numbers, the expansion of this species accelerated in the 1980s (Lutz, 1984). Recently, in Germany, the raccoon population has been estimated to be approximately 1,000,000, and it has been predicted that the total European population will double every 3–5 years (Jernelöv, 2017). Currently, the range of the raccoon population in Europe has extended hundreds of kilometres to the west, east and south of the invasion core (Salgado, 2018). A number of risks posed by raccoons for nature conservation and human health have been identified, including the transmission of the co-introduced parasitic nematode *Baylisascaris procyonis* (Bartoszewicz, Okarma, Zalewski, & Szczęśna, 2008; Beltrán-Beck, García, & Gortázar, 2012; García et al., 2012; Kornacka, Cybulska, Popiołek, Kuśmierk, & Moskwa, 2018; Popiołek et al., 2011).

The role of large mammals as casual dispersal vectors of microorganisms, until recently, has received little attention from scientists. For instance, it was demonstrated that at the local scale, small freshwater organisms (e.g. Rotifera) are translocated with mud by the wild boar *Sus scrofa* (Vanschoenwinkel et al., 2008), in fur of the nutria *Myocastor coypus* (Waterkeyn, Pineau, Grillas, & Brendonck, 2010) and on the skin of the African elephant (*Loxodonta africana*; Vanschoenwinkel et al., 2011).

In our study, we used methods developed for environmental DNA (eDNA) coupled with high-throughput sequencing. Following Taberlet, Coissac, Hajibabaei, and Rieseberg (2012), we refer to eDNA as all genetic material that is obtained directly from the environment, including DNA from whole organisms (i.e. prokaryotes and microscopic eukaryotes) and cellular material (i.e. blood, mucous, tissue and faeces), or DNA that is released from the cytoplasm as free nucleic acids. The method analyses genetic material that is not collected through methods targeted at specific organisms but that is extracted from bulk environmental samples without any obvious signs of the biological source material (Hopkins & Freckleton, 2002). Using massive parallel sequencing to assess the diversity of environmental samples enables the simultaneous detection of many species. This provides an opportunity to find organisms whose presence was not previously suspected (e.g. newly introduced alien species, species new to science or species not known to occur in a particular country and region) or species that are difficult to identify due to their high phenotypic plasticity (Shubert, Wilk-Woźniak, & Ligęza, 2014) or a lack of taxonomic expertise (Hopkins & Freckleton, 2002).

2 | METHODS

We collected 170 samples from raccoons between 2007 and 2012. The ear fragments were taken in Poland from roadkill or

from individuals live-trapped in the “Warta Mouth” National Park (52°34'N, 14°43'E). This area is dominated by wetlands, scrub willow, marshes, meadows and pastures.

DNA was extracted from dried ear fragments that were stored in dry envelopes at -20°C prior to DNA extraction. We used the NucleoSpin Tissue Kit (Macherey-Nagel) according to the manufacturer's protocol. To detect the widest possible range of freshwater microorganisms that could be present on the raccoons, we used highly conservative PCR primers to amplify the V4 hyper-variable region of the 18S ribosomal RNA gene, the fragment that has proven to be an optimal marker for the assessment of eukaryotic diversity in different microbial ecosystems (Hugerth et al., 2014). The amplification was conducted using a semi-nested PCR protocol. The first amplification was carried out using the primers RLB-F2 (5'-GACACAGG GAGGTAGTGACAAG-3') and RLB-R2 (5'-CTAAGAA TTTCACCTCTGACAGT-3') (Zanet et al., 2014). Products (1 µl) of the first PCR step were used as a template for the second amplification, which used RLB-FINT (5'-GACAAGAAATAACAATACRGGGC-3') as an internal forward primer with RLB-R2 (Zanet et al., 2014). Amplification was performed using HotStar Master Mix (Qiagen) in a final volume of 25 µl 20 pM of each primer and 100 ng of the DNA template. The amplification included a 5-min denaturation step followed by 25 cycles for the first PCR and 40 cycles for the second PCR of 30 s at 95°C, 45 s (at 50°C in the first PCR and 55°C in the second PCR) and 1.5 min at 72°C followed by a final extension at 72°C for 10 min. To enable Illumina sequencing of the obtained products and to distinguish between sequences amplified from different raccoon individuals, the sequencing primers used in the second PCR step (RLB-FINT and RLB-R2) were followed by a unique 6-bp barcode and Illumina-specific primers. We included one negative control per 16 samples, and 10 samples were run as duplicates to control for sequencing errors. As we did not expect any specific organisms to be found, no positive control was used. Then, we followed the procedure described by Biedrzycka, Sebastian, Migalska, Westerdahl, and Radwan (2017). Paired-end sequencing runs were performed on an Illumina MiSeq machine with the MiSeq Reagent Kit v3 for 600 cycles (Illumina, Inc.). Read merging, filtering, quality control, the preliminary control of the length, coverage and frequency of the most abundant variants, chimaera identification and final sequence calling were performed using the AmpliSAS pipeline. We adopted a minimum per sample frequency threshold of 2% and a minimum sequence depth of 30 (Sebastian, Herdegen, Migalska, & Radwan, 2016). The AmpliSAS pipeline applies a similar strategy for variant calling as software commonly used in microbiome studies, in which potential artefactual variants are clustered to suspected parental sequences using Shannon entropy (Eren et al., 2013) or similar clustering methods (Amir et al., 2017; Callahan et al., 2016).

The obtained sequences were compared to the available GenBank database for species identification. Due to the low number of detected sequences, we did not perform any clustering into molecular operational taxonomic units (MOTUs) based on the identity level. The sequences were annotated by BLASTn.

Nevertheless, the DNA marker (V4 hyper-variable region of 18S rRNA gene) used for species identification is suitable for assessing the diversity of microbial eukaryotic communities and acted as a phylogenetical discriminant of microbial organisms (Hugerth et al., 2014). Additionally, we used a semi-nested PCR approach that increased the chances of amplifying organisms with a low abundance in the extracted DNA, as well as degenerated primers in second PCR, increasing the chances of amplifying a wide range of different species. Nevertheless, our results may be biased towards the identification of certain species, while others might have been underrepresented or not identified at all.

3 | RESULTS

Out of 170 analysed samples, we obtained 15 PCR products of expected size. The V4 region can present variations in size of over 100 bp (Wuyts et al., 2000), and the amplified fragment ranged from 415 to 232 bp. No PCR products were obtained from the negative controls, and the concordance between duplicated samples was 100% after the application of all steps in the genotyping pipeline. Upon sequencing, 5,845,214 raw reads were produced. After merging sequence reads and filtering by quality and length, 131,471 reads were retained (mean of 1,750 reads/sample). Between one and four different sequences per PCR product representing different microorganisms were identified. In total, 16 different sequences were confirmed after the genotyping steps were performed. The sequences detected in our data set, along with probable species designations and GenBank assignment scores, are presented in Table 1. The per cent identity to the best GenBank hit ranged from 100% to 91%. The identified sequences were deposited in GenBank under accession numbers MN103981-MN103997. We found several groups of microorganisms, including alveolates, amoeboids and fungi (including *Penicillium* sp.), with 4 organisms identified to the species level: *Micronuclearia podoventralis* (amoeboid), *Parachloroidium lobatum* and *Jaagichlorella roystonensis* (chlorophyta), and *Mortierella polygonia* (fungi).

4 | DISCUSSION

Using a method based on eDNA metabarcoding, we provide evidence of raccoon acting as an accidental dispersal vector of both terrestrial and freshwater externally attached microorganisms. Moreover, this is the first time the processes of passive dispersal have been studied using genetic identification methods.

The concept and terminology of environmental DNA were first applied in microbiological studies in which both extracellular and intracellular eDNA were detected in sediment samples (Ogram, Saylor, & Barkay, 1987). A classic definition from Taberlet et al. (2012) uses the term eDNA for environmental samples that may include DNA from whole organisms or their cellular material or

TABLE 1 List of the microorganisms identified with a GenBank database comparison using a BLASTn search

Identified reference source	GenBank reference sequence acc. no.	Identity % to the reference sequence	BLAST e-value	Sequence length	No. of amplicons
<i>Micronuclearia podoventralis</i> (amoeboids)	AY268038.1	100	0.00	415	2
<i>Parachloroidium lobatum</i> (chlorophyta)	HF586461.1	100	0.00	403	8
<i>Parachloroidium lobatum</i> (chlorophyta)	HF586461.1	98.76	0.00	403	2
<i>Jaagichlorella roystonensis</i> (chlorophyta)	MH780941.1	100	0.00	402	3
<i>Penicillium</i> sp./Eurotiales/fungus (fungi)	NG062803.1	100	0.00	402	2
<i>Mortierella polygonia</i> (fungi)	HQ667463.1	100	0.00	401	2
Uncultured Glomeromycota clone (fungi)	KJ740955.1	99.33	9,00E-67	236	2
<i>Colpodella</i> sp. (alveolates)	KT600661.1	99.31	1,00E-64	232	2
<i>Colpodella</i> sp. (alveolates)	KT600661.1	93.87	1,00E-157	375	2
Uncultured <i>Colpodellidae</i> isolate (alveolates)	KC486331.1	90.93	1,00E-150	402	2
Uncultured freshwater alveolate clone	FJ765407.1	99.29	2,00E-62	234	2
Uncultured alveolate clone	KP213246.1	97.17	2,00E-94	234	2
Uncultured alveolate clone	KP213246.1	93.27	3,00E-79	232	2
Uncultured alveolate clone	KP213246.1	92.68	4,00E-78	233	2
Uncultured dinoflagellate clone	HQ259046.1	96.77	0.00	401	2
Uncultured eukaryote/alveolate	LN582271.1	91.55	3,00E-75	392	2

DNA released from the cytoplasm as free nucleic acids. Currently, the concept of eDNA is more frequently associated with analyses of extracellular (or extraorganismal) microbial DNA that is shed into the environment by the target species, for example, in the form of faeces, urine or hair (Ibáñez de Aldecoa, Zafra, & González-Pastor, 2017; Levy-Booth et al., 2007). However, the array of eDNA sources has been widening, recently encompassing flowers (Thomsen & Sigsgaard, 2019), blood-feeding sand flies and mosquitoes (Kocher et al., 2017) and leeches (Schnell et al., 2018). Analysed samples may contain different combinations of extra- and intracellular DNA.

The medium from which we collected our samples has been neglected as a source of eDNA (but see Vanschoenwinkel et al., 2008; Waterkeyn et al., 2010). We sampled a large and mobile mammal species that moves through different habitat types and serves as a vector that accidentally transports stowaway aquatic microorganisms. Unlike transported organisms that are strictly associated with their vectors, such as parasites or pathogens, the microorganisms we detected were accidentally attached to the raccoon's hair and "contaminated" the specific environment. It is very likely that the total eDNA in our samples contained both microbial eDNA that originated from the genomes of full microscopic organisms (i.e. intracellular or intraorganismal eDNA) and the eDNA that those microorganisms had shed in the environment, for example, following their death (i.e. extracellular or extraorganismal eDNA). However, we were not able to discriminate between intra- and extracellular eDNA in our samples, and we argue that in the case of the microorganisms that we detected, this discrimination is not crucial for the results of biodiversity research. It can be assumed that any sample that contains DNA from microscopic organisms is likely to contain propagules that

are capable of growth and reproduction in areas to which they are transported.

Although preliminary, the results of our analyses provide support for the usefulness of unconventional methods of genetic material collection for biodiversity and biogeography studies, particularly in the detection of organisms that are otherwise difficult to sample or to identify. With the exception of *Penicillium* sp., which could have potentially grown on the samples after their collection, the present knowledge on the phylogeny and distribution of the species that we identified is incomplete; therefore, the new records of these species outside of their previously known ranges provide valuable information. The records of two of the chlorophytes that we identified, *Parachloroidium lobatum* Neustupa & Škaloud and *Jaagichlorella roystonensis*, are especially noteworthy (S. Ma, V.A.R. Huss, X. Sun & J. Zhang). The first species was described only in 2013 as a novel genus of coccoid green algae from subaerial corticolous biofilms from Slovenia (Neustupa, Němcová, Veselá, Steinová, & Škaloud, 2013), and our sequence shows perfect agreement with the V4 fragment of the 18S rRNA gene obtained for this species. *J. roystonensis* was described even more recently, in 2019, from the bark of the royal palm in Haikou, Hainan province of China, as a terrestrial species (Darienka & Pröschold, 2019). Here, the 18S rRNA gene was used as a diagnostic fragment. Despite this perfect match, we are aware that the resolution of the gene fragment we used does not always allow the designation of organisms at the species level. On the other hand, the high level of polymorphism represented by the V4 region, coupled with its length, makes it a perfect marker for the identification of eukaryotic microorganisms from unknown environmental samples by means of next-generation sequencing. The records from

Poland provide the primary distribution data for these two species of chlorophytes.

In addition to providing a broad overview of a region's biodiversity, the use of mammals as living eDNA samplers is worth exploring because in some cases, this source of genetic material may efficiently reduce the burden of traditional sampling. Such samples may be by-products of programmes not specifically focused on biodiversity research, such as invasive alien species eradication campaigns or regular hunting. Moreover, this perspective can be an additional asset to studies in which the main objective is to collect genomic DNA of the "sampler" species itself. Although the method may not be immediately straightforward, the use of the same samples to extend the scope of analyses to the stowaway organisms accidentally transported by the target species can provide valuable data on the distribution of the stowaways. Genetic material, if well-preserved, is very stable, and due to the development of parallel massive sequencing and rapid progress in the reduction of its costs, eDNA-inspired methods may allow the effective re-assessment of museum samples collected in the distant past (Barnes & Turner, 2016). The results of such analyses could be particularly valuable for increasing knowledge on the biodiversity of understudied areas, in which the collection of new data is difficult or expensive.

Although the body of knowledge on community richness, diversity and colonization at different temporal and spatial-temporal scales is extensive, proper assessments on the impacts of living vectors that translocate other organisms are still rarely included in studies on biogeography. This particularly refers to vectors other than birds, although even the role of this group has not been properly identified (Hessen et al., 2019). Irrespective of the vector taxonomy, the consequences of the casual movements of stowaway dispersal units that are not directly related to the fitness of the transported organisms receive less attention than, for example, the dispersal of diaspores. This also pertains to ungulates, which represent one of the best studied groups of mammal vectors (Baltzinger et al., 2019; Liehrmann et al., 2018).

To our knowledge, casual associations between carnivorous mammals and the stowaways that they accidentally carry have not been previously described. In this respect, our example for the raccoon is particularly interesting because of the fast and ongoing spread of this species in Europe. Combined with the history of the invasion of racoons (Jernelöv, 2017; Salgado, 2018), our preliminary results suggest that as a novel, abundant and quickly spreading element in local ecosystems, racoons may act as a new vector contributing to the expansion of stowaway aquatic and terrestrial microorganisms. While the role of vectors operating at large spatial scales is usually attributed to birds (Figuerola & Green, 2002; Green & Figuerola, 2005), our results suggest that short-distance dispersal events by racoons could, over the decades, push the range of the stowaway organisms they carry by hundreds of kilometres, thus altering the distribution of organisms not only at the local but also at the continental scale. The impact of the expansion of alien mammals as dispersal vectors may therefore exceed the geographic scale that is usually attributed to this group of animals (Baltzinger et al.,

2019; Pellerin, Picard, Saïd, Baubet, & Baltzinger, 2016). Moreover, projections from environmental niche modelling show the availability of unoccupied extensive areas suitable for racoons, including Russia and the Middle East, while predictions for the next several decades indicate that due to climate change, new areas will become suitable for racoons further to the north (Loupe, Leroy, Herrel, & Veron, 2019). These results emphasize the potential of racoons to influence large-scale biogeography in the context of not only their associated pathogens and parasites but also the stowaways racoons accidentally carry.

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DATA AVAILABILITY STATEMENT

The DNA sequences obtained in this study are deposited in GenBank under accession numbers MN103981-MN103997. The files containing the results of sequence identification using the AmpliSAS pipeline will be deposited in the Dryad Digital Repository service before the manuscript publication.

ORCID

Wojciech Solarz  <https://orcid.org/0000-0002-9459-2144>

Kamil Najberk  <https://orcid.org/0000-0003-0280-0186>

Elżbieta Wilk-Woźniak  <https://orcid.org/0000-0002-4929-6733>

Aleksandra Biedrzycka  <https://orcid.org/0000-0001-5096-2767>

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BIOSKETCHES

The research team interests focus on different aspects of biological diversity and species distribution, including causes of biological invasions, their effects and methods to mitigate them. We use molecular methods to reveal genetic variability of natural populations of micro- and macro-organisms, population spread and migration patterns focusing on the role of genetic diversity in the invasion success.

Author contributions: W.S., K.N., E.W-W. and A.B conceived the idea and designed the study. A.B. performed laboratory experiment and analysed the data. W.S. wrote the manuscript. All authors discussed the results and reviewed the manuscript.

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