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Preliminary results on the molecular study of fish-eating by 'trawling *Myotis*' bat species in Europe

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Abstract

Piscivory in vespertilionid bat species in the genus *Myotis* and trawling as typical element of behaviour in the hunting flight above the water surface are widely acknowledged. However, among the European bat fauna, only long-fingered bats (*Myotis capaccinii*) are known to consume fish. In Daubenton's bat (*Myotis daubentonii*) as well as the pond bat (*Myotis dasycneme*), both also members of the guild of European 'trawling *Myotis*' species, piscivory is possible, but has not yet been sufficiently recorded by morphological analyses of prey in faecal pellets. Previous experimental studies have shown that fish remains are difficult to detect in faecal pellets of *M. daubentonii;* thus, piscivory may not always be reliably detected by morphological identification of prey remains. We analysed the DNA of faecal samples of all three trawling *Myotis* species from different regions of Europe by PCR amplification and Sanger sequencing of the molecular markers 16S RNA and the DNA barcode region of the cytochrome oxidase subunit I (COI) for detection of possible fish DNA and taxonomic identification of species. In on 30% of the samples we were able to amplify fish-DNA based on COI in faeces of *M. capaccinii* and *M. daubentonii* from Bulgaria and in 8% using the 16S RNA locus from *M. dasycneme* faeces from Germany. Based on technical problems of our molecular approach, our data do not reveal a definitive and reliable molecular record of diet-derived fish DNA in the faecal pellets of the investigated trawling *Myotis* species nor proof for piscivory in *M. dasycneme* and *M. daubentonii*. Our results may be a stimulation and draft for other researchers to test the detection of diet-derived fish DNA in trawling *Myotis* with improved molecular genetics approaches.

Key words

Feeding ecology, hunting behaviour, *Myotis*, piscivory, trawling bat species.

Introduction

Three species of the guild of 'trawling *Myotis*' species are known from Europe: the long-fingered bat (*Myotis capaccinii*), Daubenton's bat (*Myotis daubentonii*) and the pond bat (*Myotis dasycneme*). Through several experiments it was documented that all three mentioned European species use unique foraging techniques: during hunting flight above the water surface, they use their feet and tail membrane to catch insects, such as arthropods, which float on, or fly above the water (Kalko & Schnitzler, 1989; Siemers *et al*., 2001a; Siemers & Schnitzler, 2004). Additionally, all three have relatively large feet in proportion to their body dimensions. They share this morphological feature with other trawling bats, among others with *N. leporinus*. Recent studies on the feeding ecology of *M. daubentonii* and *M. dasycneme* in Europe, which were based on morphological and molecular analyses of the bat guano, show that both species feed on insects and, to a much lesser degree on spiders, but no signs of fish remains could be detected in the guano of the two species (Ciechanowski & Zapart, 2012; Krüger *et al*., 2012,

2014; Nissen *et al*., 2013). In contrast to these two species, the long-fingered bat *M. capaccinii*, distributed in Mediterranean regions of Europe is known to, in addition to insectivory, also exhibit piscivory (Aihartza *et al*., 2003; Levin *et al*., 2006; Biscardi *et al*., 2007). Recent work has also provided insight into how *M. capaccinii* discern prey and adjust their movements during trawling when attacking fish (Aizpurua *et al*., 2015).

The similar hunting behaviour of trawling *Myotis* species and their tight connection to aquatic ecosystems led to the assumption that piscivory could be possible also among Daubenton's bat and the pond bat in Central and northern Europe. As shown by experiments (Siemers *et al*., 2001b), Daubenton's bat is able to catch small fishes under laboratory conditions, where it grasped fishes with its feet. The fish was taken directly from the claw into the mouth and was eaten (Siemers *et al*., 2001b). We know from the experiments of Siemers *et al*. (2001a) that after a specimen of *M. daubentonii* had consumed a lot of insects and approximately 30 small fish during a four week period, only insect remains were found in the guano, but only two fish scales and one bone remained. Because the remains of the small fish are so difficult to identify in faeces, probably because of demineralisation in the stomach, the late Björn Siemers concluded that piscivory will be very difficult to assess quantitatively in the field for the European trawling *Myotis* (Siemers *et al*., 2001a). In contrast to that, Brosset & Delmare (1966) claimed to have found fish remains in the guano of *M. daubentonii* in France several decades ago.

An alternative to morphological analyses of faeces for dietary studies uses DNA remains from prey items in the faeces (e. g. Pompanon *et al*., 2012; Krüger *et al*., 2014). This method allows the detection of dietary elements that are difficult to identify by morphological features. Here, we used a molecular genetic approach to guano samples of *M. dasycneme, M. capaccinii* and *M. daubentonii* in order to detect fish DNA and ultimately test our hypothesis that *M. daubentonii* and *M. dasycneme* also prey on fish based on the observed behaviour of piscivory under laboratory conditions (Siemers *et al*., 2001b).

Material and Methods

Fieldwork and sample selection

During May-August in 2008 and 2009, *M. capaccinii*, *M. daubentonii* and *M. dasycneme* were caught at different localities using mist nets placed over the water surface (supplementary table S1). Bat individuals were kept separately in soft cotton bags for a maximum of one hour and released after taking measurements and collecting faecal samples (cf. Krüger *et al*., 2012). These samples contain "individual samples" in *M. capaccinii* (7 – 11 pellets of one bat individual) and "mixed samples" from *M. daubentonii* (14 – 15 pellets of two individuals) from Bulgaria and "mixed samples" from *M. dasycneme* (2 pellets of two individuals) from northern Germany (Supplementary Information Tab. S1). From northern Germany we used also samples from nursing colonies of *M. dasycneme* (generally ten faecal pellets per sample) from two different localities in 2009 and an unknown number of contributing animals. Samples were either stored dried and frozen, or in 70 % ethanol at room temperature (further information s. supplementary information Tab. S1).

Laboratory procedures

We handled every sample series (ID 1 – 8, 9 – 10, 11 – 20, 21 – 34 and 36 – 46; supplementary information Tab. S1) in dedicated cleanroom facilities at the University of York, where no previous work was performed on either bats or fish (former laboratory of M. Hofreiter, University of York). After processing each sample series, we cleaned the bench and all surrounding laboratory equipment and irradiated the whole cleanroom with UV light for at least 6 hours to avoid possible cross-contamination between the subsequent sample series. We extracted DNA from pellet samples using the QIAamp DNA Stool Mini Kit (Qiagen, UK) according to the manufacturer's instructions (QIAamp DNA stool handbook, 2th edition, April 2010). In order to detect possible contamination during DNA isolation, we included a blank sample (ID 35).

We chose to analyse the faecal samples for the presence of two different molecular markers: the relatively conserved 16S RNA and the DNA barcode region of cytochrome oxidase subunit I (COI), which usually allows for higher taxonomic resolution (Vences *et al*., 2005). Primers were designed based on available DNA sequences of the16S and COI regions of different common European fish species (*Rutilus rutilus, Scardinius erythrophthalmus, Abramis brama, Perca fluviatilis, Cyprinus carpio, Alburnus alburnus, Tinca tinca, Carassius carassius, Esox lucius, Gasterosteus aculeatus, Thymallus thymallus,* and *Salmo trutta*). For primer design we chose the common fish found in Europe, but they also covered a wide taxonomic range, from salmonids to cyprinids and percids. From our experience, primers designed on a wide taxonomic range of species should amplify other species not used for primer design. Even though, we still may miss some fish DNA target if they are not in the species we used for primer design and they have mutations in the priming sites. Screening for suitable mini-barcode regions (ca. 150 bps) with conserved flanking primer-binding sites was performed manually from the resulting multiple sequence alignment. Where necessary, degenerate sites were included in the primer sequence to increase amplification universality.

As positive control for the designed primers, we analysed DNA isolates of common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) (see supplementary information Tab. S2). Preparation of the PCRs was carried out inside laminar flow hoods in the ancient DNA

Tab. 1. Primer sequences used in the PCR.

name	primer sequence (5´→ 3`)	target fragment size
Fw. COI_F1	TGCCTCAGCCGGRATAGT (18)	112 bps
Rv. COI_R1	AGAATTGGYATTACTATAAARAA (23)	
Fw. 16S_F1	ACAAGACGAGAAGACCCTWTG (21)	ca. 78 bps
Rv. 16S_R1	GTGGTCGCCCCAACCRAA (18)	

cleanroom, which was irradiated with UV light at least 6 hours before and after each working step.

For each PCR, a final reaction volume of 20 µl was prepared containing: 13.3 µl of HPLC water, 0.1 U of AmpliTaq Gold (Thermofisher Scientific), 2.0 µl of AmpliTaq Gold buffer, 1.7 µl $MgCl_2$, 0.2 µl BSA, 0.2 µl dNTPs (mix), and forward and reverse primers (1.0 µl each, 10 pmol/µl). We ran two sets of PCR, one with 0.5 µl and one with 5 µl of DNA template, reducing HPLC water accordingly. A negative control containing all PCR reagents except template DNA was always included. Each PCR was run with the following temperature profile: 95°C for 9 min, followed by 60 cycles 95°C for 15 sec., 53°C for 20 sec., 72°C for 30 sec. and a final extension of 72°C for 10 min.

Validation of the designed mini-barcodes

Apart from the *in silico* design and evaluation of the new primers using the multiple sequence alignment, four common freshwater fishes were selected as *in vitro* test in order to evaluate the performance of the designed primers and validate the approach: common carp, rainbow trout, roach and gudgeon, all native to Europe except for rainbow trout, which is a popular game fish and stocked throughout Europe (Savini *et al*., 2010). DNA aliquots of the four species were obtained from colleagues and used as template for PCRs with the same settings as outlined above. PCR products of all reactions showing a clear band of expected product size on an agarose gel (supplementary information Tab. S2) were then Sanger sequenced in both directions using the same primers as used for the PCRs. Data processing and sequence assembly was done with the software Geneious Pro (Biomatters, 2013) and sequences manually screened for unexpected indels or stop codons. The obtained consensus DNA sequences were either submitted to the Barcode of Life Database Systems (BOLD, Ratnasingham & Hebert, 2007) ID engine (COI fragments), or to the NCBI GenBank (16S fragments) using the megablast function (Zhang *et al*., 2000) for reverse identification via sequence comparisons.

Results and Discussion

Sequencing results

Altogether, 23 samples (IDs) out of 45 showed successful PCR amplification based on visible products on agarose gels with either the newly designed COI or 16S mini-barcode primers or both (Tab. 2). Of the 23 amplicons with clear gel bands, 22 were successfully Sanger sequenced in one or both directions (Tab. 2). The majority of the obtained DNA sequences (16 out of 22) did not match any fish species, but was assigned to a non-target group, i. e. mammals: *Bo*s (7 hits), *Hom*o (10), *Ovi*s (1) and in three cases bat DNA remains, which were assigned to the *Myoti*s species, *M. emarginatus and M. capaccinii* (Tab. 2). Except for the latter, these species are very common contaminants, often present at low concentrations in reagents and plastic ware (cf. Leonard *et al*., 2007) or – in the case of human DNA – can be introduced during experiments despite all precautions taken. Due to our highly sensitive PCR set up with 60 cycles, also smallest trace amounts of DNA can serve as templates and lead to amplicons that can be successfully sequenced. Approaches to avoid this type of contamination have been developed (Champlot *et al*., 2010), but due to the pilot nature of our study – we wanted to investigate if fish DNA, normally no common contaminant, was present at all in the samples – we did not employ these approaches.

In six samples, however, the resulting consensus sequences could be identified as originating from fish species: three from Bulgaria and three from Germany (Tab. 2). The complete DNA sequences assigned to fish including the positive controls are listed in supplementary information Tab. S2.

Fish species identification by DNA barcoding and biogeographical context

The generated DNA sequences assigned to fish had an expected length of 112 bps (COI) and 74 – 77 bp (16S), and could be assigned reliably only to genus level (supplementary information Tab. S2). Using the biogeographical context of the locality and the known fish species from this region it was, however, possible to reconstruct the species in two cases. Interestingly, the partial DNA barcode recovered from sample number 5 (a single *M. capaccinii* from northern Bulgaria) matched several DNA barcodes with 100% identity on BOLD that belong to chub (*Squalius* spp.) specimens from Bulgaria, Greece, and Turkey and form the BIN (Barcode Index Number, a species surrogate) BOLD: AAE3493 (as of January 30th 2017). According to the BIN system, the closest DNA barcode cluster (BOLD: ACF2121) is comprised of *Squalius laietanus* specimens from France and Spain and differs by 1.12% p-distance (this is the proportion (p) of nucleotide sites at which two sequences

Tab. 2. Overview of PCR and sequencing results with sample origin (species), target locus (16S or COI) and amount of DNA template used in each PCR (5.0 or 0.5 µl). The presence (visible agarose gel band, black square) or absence of PCR products (no agarose gel band, white square) is indicated by boxes. Sample no. 35 was a blank DNA extraction sample. Complete DNA sequences of detected fish species (including fish positive controls) are provided in supplementary information Tab. S2.

ID	species	origin	number of positive PCR products and used primers				record of fish DNA	taxonomic assignment	
			16S_RNA		COI			16S_RNA	COI
			0,5 µl	5,0 µl	0,5 µl	5,0 µl			
1	*M. capaccinii*	Bulgaria	□	□	□	□			
2	*M. capaccinii*	Bulgaria	□	□	□	□			
3	*M. capaccinii*	Bulgaria	□	□	□	□			
4	*M. capaccinii*	Bulgaria	■	■	□	□		*M. capaccinii*	
5	*M. capaccinii*	Bulgaria	■	■	■	■	×	*M. capaccinii*	*Squalius* sp.
6	*M. capaccinii*	Bulgaria	■	■	□	□		*H. sapiens*	
7	*M. capaccinii*	Bulgaria	■	■	□	■			*M. emarginatus*
8	*M. capaccinii*	Bulgaria	■	■	□	■	×	*H. sapiens*	*Gobio* sp.
9	*M. daubentonii*	Bulgaria	■	■	□	■	×	*H. sapiens*	*Cottus* sp.
10	*M. daubentonii*	Bulgaria	□	■	□	□		*H. sapiens*, *B. taurus*	
11	*M. dasycneme*	Germany	□	□	□	□			
12	*M. dasycneme*	Germany	□	□	□	□			
13	*M. dasycneme*	Germany	■	■	□	□		*H. sapiens*	
14	*M. dasycneme*	Germany	□	□	□	□			
15	*M. dasycneme*	Germany	□	■	□	□		*H. sapiens*	
16	*M. dasycneme*	Germany	□	□	□	□			
17	*M. dasycneme*	Germany	□	■	□	□		*H. sapiens*	
18	*M. dasycneme*	Germany	■	■	□	□		*H. sapiens*	
19	*M. dasycneme*	Germany	□	□	□	□			
20	*M. dasycneme*	Germany	□	■	□	□		*H. sapiens*	
21	*M. dasycneme*	Germany	□	■	□	□		—	
22	*M. dasycneme*	Germany	□	■	□	□		—	
23	*M. dasycneme*	Germany	□	□	□	□			
24	*M. dasycneme*	Germany	□	□	□	□			
25	*M. dasycneme*	Germany	□	□	□	□			
26	*M. dasycneme*	Germany	■	■	□	□	×	*Phoxinus phoxinus*, *H. sapiens*	
27	*M. dasycneme*	Germany	□	□	□	□			
28	*M. dasycneme*	Germany	■	■	■	■		*H. sapiens*, *B. taurus*	—
29	*M. dasycneme*	Germany	□	■	■	■		*B. taurus*	—
30	*M. dasycneme*	Germany	■	■	■	■		*B. taurus*	—
31	*M. dasycneme*	Germany	■	■	■	■		*B. taurus*	—
32	*M. dasycneme*	Germany	■	■	■	■		*B. taurus*	*B. taurus*
33	*M. dasycneme*	Germany	□	□	■	■		*H. sapiens*	—
34	*M. dasycneme*	Germany	■	□	■	■		*B. taurus*	*B. taurus*
35	blank		□	□	□	□		—	—
36	*M. dasycneme*	Germany	□	□	□	□		—	—
37	*M. dasycneme*	Germany	□	□	□	□		—	—
38	*M. dasycneme*	Germany	□	■	□	■	×	*Ovis aries*	*Leuciscus* sp.
39	*M. dasycneme*	Germany	□	□	□	□		—	—
40	*M. dasycneme*	Germany	□	□	□	□		—	—
41	*M. dasycneme*	Germany	□	■	□	□	×	*Barbatula barbatula*	—
42	*M. dasycneme*	Germany	□	□	□	□			
43	*M. dasycneme*	Germany	□	□	□	□			
44	*M. dasycneme*	Germany	□	□	□	□			
45	*M. dasycneme*	Germany	□	□	□	□			
46	*M. dasycneme*	Germany	□	□	□	□			

being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared and does not make any corrections). A final taxonomic assignment of the presumed prey DNA is not possible due to the still unresolved taxonomy of chubs in the Anatolian and Black Sea region with several morphologically similar species (Özuluğ & Freyhof, 2011).

The second fish-DNA positive match was recovered from a single bat (ID 8; a *M. capaccinii* from northern Bulgaria, s. Tab. 2) and could be unambiguously assigned to the gudgeon species complex *Gobio* spp. with 99.7% sequence identity. The DNA barcode based molecular taxonomic unit BIN BOLD: AAC5607 contains over 150 specimens of different *Gobio* species from throughout Central Europe with up to 1.12% p-distance (uncorrected genetic distances) between them, and the taxonomic resolution of the small COI fragment obtained does not allow an assignment to species level with high probability. A manual comparison to DNA barcodes belonging to the geographical closely occurring *Gobio kovatschevi* (BOLD: ACL7481) shows a similarity of 97.8%, which does not support conspecifity of the latter with the prey item. As there are several other *Gobio* lineages occurring in neighbouring Romania and other Balkan countries, the question, which species of gudgeon was probably consumed cannot be answered.

The third incidence of positive fish-DNA faecal pellets occurred in a mixed sample from two *M. daubentonii* (Bulgaria, s. Tab. 1), and indicates the presence of bullhead (*Cottus* sp.) remains in the faeces. A particular *Cottus* species cannot be identified based on the short COI fragment, which is also due to a general problem in delineating sculpin species with DNA barcodes, where it has been shown that several species share the same haplotypes (Geiger *et al*., 2014; Knebelsberger *et al*., 2015).

Of the three positively tested German samples stemming from mixed pellets of nursing colonies of *M. dasycneme*, one (ID 26) could be assigned to a minnow (probably the common minnow *Phoxinus phoxinus*) by comparing the obtained short 16S fragment with data from NCBI GenBank (98% sequence identity via a BLASTN search). The second faecal sample (ID 38) belonging to the Wismar-Müggenburg nursing colony that was found to contain DNA remains of fish indicates the potential ingestion of dace (*Leuciscus* sp.), as demonstrated by the 16S sequence fragment similarity of 100% to DNA sequences in GenBank. The exact species (*L. idus* or *L. leuciscus*) cannot be determined unambiguously, also because both species have exchanged mitochondrial sequences over large areas of their distribution (Costedoat *et al*., 2006). Finally, sample ID 41 from the same nursing colony as the former (*M. dasycneme*) was positively tested for the presence of 16S DNA fragments assigned to a nemacheilid loach (BLASTN identity 91% to *Barbatula barbatula* from Sweden).

Interpretation of the sequencing results: arguments for piscivory?

With six (out of 45) guano samples of trawling *Myotis* species revealing unambiguously DNA remains of fishes, we have provided evidence for fish DNA in their scat, but only in a limited number of samples (13%). Our technical approach with an extremely sensitive PCR appears to be highly vulnerable for accumulating errors during the PCR and thus the likelihood of amplifying residual fragments of environmental DNA is very high. This is indicated by the majority of the obtained DNA sequences not matching fish species, even if we used fish-specific primers (Tab. 2). It would be possible to circumvent these issues using several approaches. For example, to avoid amplification of the most common contaminant in our data set, not unexpectedly, human DNA, blocking primers (Boessenkol *et al*., 2012) could be used. Alternatively, hybridization capture could be used for target enrichment as it is both highly sensitive and comparatively promiscuous with regard to potential targets (Kozarewa *et al*., 2015). And third, it would be possible to avoid contamination from reagents (c.f. Champlot *et al*., 2010), but we caution that the majority of contaminant sequences (bat and human) likely derived from the bat excrements either directly (bat) or due to handling (humans), and would thus not be prevented by decontamination of reagents. Moreover, all these approaches are more time- and money-intensive than our simple, high-sensitive PCR and thus not ideal for pilot-study work. Finally, we should point out that absence of amplification products does not necessarily mean absence of fish DNA in a faeces sample. Thus, we are not able to offer an exhaustive molecular record of fish-DNA in the faecal pellets of the investigated trawling *Myotis* species. On the other hand, the fish species revealed by the DNA sequences fit to the typical environment of trawling *Myotis* species at the netting sites. Interestingly, all recovered fish species are typically associated with river systems and not with standing water such as ponds. The bullheads (*C. gobio*), Eurasian minnows (*P. phoxinus*) and nemacheilid loaches (probably *B. barbatula* as no other nemacheilid loach occurs in Germany) are associated with gravel to stone bottom and fast flowing cold water with elevated levels of oxygen. In contrast to that, ide or dace (*L. idus* or *L. leuciscus*) and chub (*Squalius sp*.) prefer usually the lower reaches of larger streams or rivers with lower water current. Thus, we view our results as a first step towards a more comprehensive investigation of trawling bats dietary spectrum.

Possible contamination with environmental DNA and transfer path of fish DNA

As fishes, fish larvae and also fish eggs are part of the complex food web of aquatic organisms, several transfer paths of fish DNA into the faeces of bats must be considered.

Besides the possibility that small fish or larvae (dead or alive) could have been picked up from the water surface during trawling, several other possibilities must be considered, much as in forensics, where secondary trace DNA transfer is an undisputed issue (e. g. Szuta *et al*., 2015). One possibility for contamination of the bat faecal pellets with fish DNA would be fish larvae (dead or alive) eaten (complete or partially) by piscivorous insects, which themselves became later on prey for the trawling bats and served as vectors for fish DNA. Water boatmen (Corixidae) are regularly found in the diet of *M. dasycneme* and *M. daubentonii* with a frequency up to 6% in the faecal pellets (Krüger *et al*., 2012, 2014). As Corixidae occasionally feed on fish and fish larvae (McCormick & Polis, 1982), it cannot be ruled out that specimens of this prey serve as vector for fish DNA and its consumption led to a secondary transfer of fish tissue to the stomach and gut content of trawling bats. Additionally, contamination with environmental DNA (eDNA) may be the reason for the fish DNA in the analysed pellets. It is known from recent studies that the analysis of water content using environmental metabarcoding is a powerful tool for aquatic species detection (Valentini *et al*., 2016). Free fish DNA or cells in the water column could adhere to the prey and pose a possibility for secondary contamination although it is questionable if such low amounts of free DNA would still be detectable after passage of the digestive system.

Conclusions

Our data do not provide a definitive molecular record of diet-derived fish DNA in the faecal pellets of the investigated trawling *Myotis* species, nor definitive proof for piscivory in *M. dasycneme* and *M. daubentonii*. Rather, we consider the amplified sequences of fish DNA from bat guano as genuine pilot data. However, as we also successfully amplified fish DNA from faecal pellets of *M. capaccinii*, which is known to feed on fish, we speculate that the fish DNA we amplified with the same molecular approach from the guano of *M. dasycneme* and *M. daubentonii*, are not unlikely to be an indication for piscivory in those species. Further studies with a more refined molecular genetic approach like faecal DNA metabarcoding (cf. Razgouret *et al*., 2011; Pompanon *et al*., 2012) or hybridization capture approaches should ultimately be able to answer the question of piscivory in *M. dasycneme* and *M. daubentonii*.

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Supplementary Information Supplementary information Tab. S1: Pellet sampling, sampling sites and methods. Investigated fecal samples of *Myotis dasycneme*, *Myotis capaccinii* and *Myotis daubentonii* from different regions of Europe. Legend: * stored at room temperature, abbreviations for sample collector: BS (B. Siemers), IH (I. Harms), FK (F. Krüger), RS (R. Sommer) and SS (S. Sommer). Sample no. 35 is not listed, because this ID position was used for a blank DNA isolation.

ID	species	sex	sampling	no. of bat individ.	no. pellets	pellet storing	Date of sampling	country	locality	sample collector
1	*M. capaccinii*	fem	netting	1	9	dried, –20°C	17.07.2008	Bulgaria	Russenski Lom	BS
2	*M. capaccinii*	male	netting	1	7	dried, –20°C	17.07.2008	Bulgaria	Russenski Lom	BS
3	*M. capaccinii*	male	netting	1	10	dried, –20°C	17.07.2008	Bulgaria	Russenski Lom	BS
4	*M. capaccinii*	male	netting	1	9	dried, –20°C	17.07.2008	Bulgaria	Russenski Lom	BS
5	*M. capaccinii*	male	netting	1	8	dried, –20°C	17.07.2008	Bulgaria	Russenski Lom	BS
6	*M. capaccinii*	male	netting	1	10	dried, –20°C	17.07.2008	Bulgaria	Russenski Lom	BS
7	*M. capaccinii*	male	netting	1	11	dried, –20°C	17.07.2008	Bulgaria	Russenski Lom	BS
8	*M. capaccinii*	male	netting	1	7	dried, –20°C	17.07.2008	Bulgaria	Russenski Lom	BS
9	*M. daubentonii*	male	netting	2	15	dried, –20°C	14.08.2008	Bulgaria	Russenski Lom	BS
10	*M. daubentonii*	male	netting	2	14	dried, –20°C	14.08.2008	Bulgaria	Russenski Lom	BS
11	*M. dasycneme*	male	netting	2	2	alcohol 70%*	17.06.2009	Germany	Rastorf	IH, FK, RS
12	*M. dasycneme*	fem	netting	2	2	alcohol 70%*	25.05.2009	Germany	Wahlstorf	IH, FK, RS
13	*M. dasycneme*	fem	netting	2	2	alcohol 70%*	27.08.2009	Germany	Wahlstorf	IH, FK, RS
14	*M. dasycneme*	male	netting	2	2	alcohol 70%*	25.05.2009	Germany	Wahlstorf	IH, FK, RS
15	*M. dasycneme*	fem	netting	2	2	alcohol 70%*	25.05.2009	Germany	Wahlstorf	IH, FK, RS
16	*M. dasycneme*	fem	netting	2	2	alcohol 70%*	25.05.2009	Germany	Wahlstorf	IH, FK, RS
17	*M. dasycneme*	male	netting	2	2	alcohol 70%*	17.06.2009	Germany	Rastorf	IH, FK, RS
18	*M. dasycneme*	male	netting	2	2	alcohol 70%*	01.07.2009	Germany	Postsee/Preez	IH, FK, RS
19	*M. dasycneme*	fem	netting	2	2	alcohol 70%*	17.06.2009	Germany	Rastorf	IH, FK, RS
20	*M. dasycneme*	male	netting	2	2	alcohol 70%*	15.07.2009	Germany	Wahlstorf	IH, FK, RS
21	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	22.06.2009	Germany	Westende	FK
22	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	22.06.2009	Germany	Westende	FK
23	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	22.06.2009	Germany	Westende	FK
24	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	22.06.2009	Germany	Westende	FK
25	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	22.06.2009	Germany	Westende	FK
26	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	22.06.2009	Germany	Westende	FK
27	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	22.06.2009	Germany	Westende	FK
28	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	15.07.2009	Germany	Westende	FK
29	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	15.07.2009	Germany	Westende	FK
30	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	15.07.2009	Germany	Westende	FK
31	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	15.07.2009	Germany	Westende	FK
32	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	15.07.2009	Germany	Westende	FK
33	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	15.07.2009	Germany	Westende	FK
34	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	15.07.2009	Germany	Westende	FK

Tab. S1 continued.

ID	species	sex	sampling	no. of bat individ.	no. pellets	pellet storing	Date of sampling	country	locality	sample collector
36	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	16./17.06.2009	Germany	Wismar-Müggenburg	RS, SS
37	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	16./17.06.2009	Germany	Wismar-Müggenburg	RS, SS
38	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	16./17.06.2009	Germany	Wismar-Müggenburg	RS, SS
39	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	16./17.06.2009	Germany	Wismar-Müggenburg	RS,SS
40	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	14./15.07.2009	Germany	Wismar-Müggenburg	RS, SS
41	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	16./17.06.2009	Germany	Wismar-Müggenburg	RS, SS
42	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	14./15.07.2009	Germany	Wismar-Müggenburg	RS, SS
43	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	14./15.07.2009	Germany	Wismar-Müggenburg	RS, SS
44	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	14./15.07.2009	Germany	Wismar-Müggenburg	RS, SS
45	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	14./15.07.2009	Germany	Wismar-Müggenburg	RS, SS
46	*M. dasycneme*	—	nursing colony	—	10	plastic bag*	14./15.07.2009	Germany	Wismar-Müggenburg	RS, SS

Supplementary information Tab. S2: Recovered DNA sequences that could be unambiguously assigned to fish via BLAST search including the positives controls (pos).

ID	sample origin	marker and recovered DNA sequence after manual trimming with length in bps (5'-3')
pos	*C. carpio*	COI (positive): TGCCTGAGCCGGGATAGTAGGAACCGCCTTAAGCCTCCTCATTCGGGCCGAACTTAGCCAACCCGGGTCGCTTCTAGGTGATGACCAAATTTATAACGTTATCGTCACNG (110, single rv. read)
pos	*G. gobio*	COI: (positive) TCCTCTTCGAGCTGAGTTGAGCCACCTGGCTCACTTCTAGGTGATGACCAAATTTATAATGTAATCGTTACTGCCCACGCCTTCGTAATAATT (93, single rv. read)
pos	*O. mykiss*	COI: (positive) TGCCTGAGCCGGGATAGTAGGCACCGCCCTGAGTCTACTGATTCGGGCGGAACTAAGCCAGCCGGGCGCTNCTNTGGGGGATGACCAAATCTATAACGTGATCGTCACAGC (110, single rv. read)
pos	*R. rutilus*	COI: (positive) TCCTTATTCGGGCCGAACTAAGCCACCCGGGTCACTTTTAGGCGATGACCAAATTTATAATGTCATCGTTACCGCCCACGCCTTCGTAATAATT (94, single rv. read)
5	*M. capaccinii*	COI: (*Squalius* sp.): GGGGACTGCCCTAAGCCTCCTTATTCGGGCCGAACTAAGCCAACCTGGGTCACTTTTAGGCGATGACCAAATTTATAATGTCNTTGTCNCCGCCCACGCCTTCGTAATAA TTTTCTTTATAGTAATGCCAATTCT (112, assembled)
8	*M. capaccinii*	COI: (*Gobio* sp.): GGGGACTGCTTTAAGCCTCCTCATTCGAGCTGAGTTGAGCCAACCTGGCTCACTTCTAGGTGATGACCNANTTTATAATGTAATCGTTACTGCCCACGCCTTCGTAATGATT (112, assembled)
9	*M. daubentonii*	COI: (*Cottus* sp.): AGGCACAGCTTTAAGCCTCCTAATTCGAGCAGAACTAAGCCAACCCGGCGCCCTTTTGGGGGACGACCAGATCTATAATGTAATTGTTACAGCCCATGCTTTCGTAATAATT (112, assembled)
26	*M. dasycneme*	16S: (*Phoxinus phoxinus*): GAGCTTAAGGTACAAACTTAATCACGTTAAACGACTTCTAAAGAGCAAGAACTTAGTGGCGATAAGACTTTACC (74, assembled)
38	*M. dasycneme*	16S: (*Leuciscu*s sp.): GAGCTTAAGGTACAAAGTTCAACCACGTTAAACGACTCCACAGAAAGCAAGAACTTAGTGGCAAATGAAACTTTACC (77, assembled)
41	*M. dasycneme*	16S: (*Barbatula barbatula*): GAGCTTAAGGTACAGGCCCAACCGCGTTAAACAACTTATTAAATAAGTCTTAAACATAGCGGAATGTGGGACCTTACC (78, assembled)