TECHNICAL NOTE

Development of D-Loop mitochondrial markers for amplification of prey DNA from wolf scat

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Received: 6 April 2020 / Accepted: 14 September 2020 / Published online: 23 September 2020
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Abstract
Analysis of wolves dietary is a currently important theme because of the discussion about wolves preying on livestock as sheep or goats. We developed molecular markers to especially amplify the DNA of the prey out of wolf scat. For this purpose, we used the mitochondrial D-Loop using public available sequences for wolf and seven potential prey species (even-toed ungulates). We developed special primers amplifying either the wolves DNA or the prey DNA. In a fragment of 223-225 basepairs (bp) length we identified 21 SNPs, two 1-bp indels and one 3-bp indel, and three microsatellites to separate seven prey species from each other. Validation of the markers was performed by sequencing the PCR products of 12 fresh prey tissues and 20 wolf scat samples using the different primer pairs.

Keywords Wolf · Prey · Ungulates · Molecular markers · Mitochondrial DNA · D-Loop

Discussion about the diet of wolves (Canis lupus) grew louder the last decades due to recolonising of the European countries, especially about wolves preying on livestock instead of deer. In a review nearly 25 years ago, Meriggi and Lovari (1996) concluded that a simultaneous increase (or reintroduction) of wild ungulates may reduce predation on livestock. This was confirmed by other authors from different countries (Meriggi et al. 2011; Imbert et al. 2016; Newsome et al. 2016; Janeiro-Otero et al. 2020). In this discussion human management perspectives as economic interests and on the other hand protection obligations, of course, lead to conflicts as well as ideas for a co-existence between humans and wolves (Herzog 2018; Bruns et al. 2020; Martin et al. 2020). Thus, in order to alleviate this conflict between wolves recolonising former habitats and local human communities and to design concrete strategies, scientific research and analysis methods are needed to provide fundamental data on predator’s diet (Meriggi et al. 2011; Newsome et al. 2016). Molecular methods are useful in a wide range of scientific research. A lot of literature is available on the wolf about population ranges, migration, population dynamics and classification (e.g. Pilot et al. 2010; Duleba et al. 2015; Kraus et al. 2015; Ersmark et al. 2016; Hindrikson et al. 2017; Hulva et al. 2018). For prey species of wolves, molecular species identification methods from fresh material are available (Fajardo et al. 2006; Hoffmann et al. 2015). Molecular methods for the identification of prey species from scat are discussed since long (Symondson 2002). For the identification of prey species in wolf scat, e.g. species-specific short fragments are amplified using specific primers for each prey species (Shores et al. 2015). This helps to avoid amplifying only the wolf DNA from tissue cells of the wolf’s digestive tract in the scat rather than the prey’s DNA. Here, we describe different methods to extract the DNA from wolf scat and the development of specific primer pairs to either amplify a range of potential prey species of the wolf (Capreolus capreolus, Cervus elaphus, Dama dama, Sus scrofa, Ovis aries, Capra hircus, Bos taurus) or the DNA of Canis lupus.

20 wolf scat samples were collected, 13 in a wolf enclosure in the Zoo Eberswalde (sample 1–13), six from a wild animal pack from a military training area “Heidehof” (sample 14–19) and one near the federal highway B96 (sample...
20), all in Brandenburg, Eastern Germany. As fresh reference material, we used 12 different fresh prey tissues from six species: 4× roe deer, 2× fallow deer, 1× red deer, 3× wild boar, 1× goat, and 1× sheep. Different DNA extraction methods were tested, (i) an ATMAB protocol by Dumolin et al. (1995), (ii) adding a phenol/chloroform step to this protocol, (iii) the QIAamp DNA Stool Kit, Qiagen (40724 Hilden, Germany) modified as follows: the homogenized samples were solved in 1.6 ml extraction buffer and 0.5 µg/ml Proteinase K was added, then incubated for 3 h at 50 °C. Afterwards we followed the protocol as specified by the manufacturer, (iv) the InnuPREP Forensic Kit, Analytic Jena (07745 Jena, Germany). Best results were obtained using the modified Qiagen Stool Kit.

For marker development, at least 10 public available (NCBI) sequences for each prey species from different authors, in total 78 sequences, have been used for an alignment to identify differentiating variations between the prey species. We chose highly variable regions between wolf and the prey species to design primers that have no mismatches for all prey species and a high number of mismatches for the wolf sequence for amplifying the prey species and vice versa (Table 1).

In total in the 223-225 bp long mitochondrial D-Loop fragment 21 SNPs, two 1-bp indels and one 3-bp indel, and three microsatellites were identified based on 78 public available sequences. 18 out of the 21 SNPs are species-specific as well as all Indels (Table 2).

The in silico developed markers were first validated using 12 fresh prey tissues. After confirmation of the success of the markers with fresh material, the markers were applied to identify the prey species in the 20 wolf scat samples. In samples 1–13 either roe deer (one time), goat or cattle was found. For two samples amplification failed. The owner of the zoo confirmed that occasionally deer and mostly goat or cattle are fed to the wolves. Samples 14–20 contained either roe deer or red deer, which is the main game in the sampling area. Wolf DNA was only amplified and successfully sequenced when using the special D_Loop_wolf primers.

There is only a very small amount of literature about the use of molecular methods for the identification of prey species using scat (Monterroso et al. 2019). Especially for the molecular identification of the prey species of wolves, the assortment is even smaller. Thus, the fast and cost-efficient method described here will help to identify whether wolves prey on livestock or deer.

| Primer name       | sequence       | Annealing temp | Fragment length |
|-------------------|----------------|----------------|-----------------|
| D_Loop_prey_F    | GAT CCC TCT TCT CGC TCC GG | 55°C           | 223-225 bp      |
| D_Loop_prey_R    | GCT GAG TCC AAG CAT CCC C  | 55°C           |                 |
| D_Loop_wolf_F    | TGT CCC TCT TCT CGC TCC GG | 55°C           | 232 bp          |
| D_Loop_wolf_R    | TGA GTG ATA GCA GAT TCC CC  | 55°C           |                 |
Acknowledgements We are grateful to the colleagues from the Thünen Institute of Forest Ecosystems in Eberswalde, especially Frank Tottewitz and the corresponding hunters for sampling of the wolf scats. And we thank all our hunting colleagues for providing us with fresh material from wild ungulates.

Funding Open Access funding enabled and organized by Projekt DEAL.

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Table 2 Prey species identifying DNA variations

| SNP* | Cc | Dd | Ce | Ss | Oa | Ch | Bt |
|------|----|----|----|----|----|----|----|
| 389  | C  | T  | T  | C  | T  | C  | C  |
| 398  | A  | A  | A  | T  | A  | A/C| C  |
| 399  | G  | G  | G  | T  | A  | G  | G  |
| 404  | T  | T  | T  | T  | T  | T  | C  |
| 405  | T  | T  | T  | T  | T  | T  | C  |
| 406  | A  | A  | A  | G  | A  | A  | A  |
| 417  | T  | T  | T  | A  | A  | T  | C/T|
| 421  | A  | A  | A  | G  | G  | A  | G  |
| 434  | T  | T  | T  | A  | T  | T  | T  |
| 435  | C  | C  | T  | C  | C  | C  | C  |
| 458  | T  | T  | T  | T  | T  | T  | C  |
| 459  | C  | C  | C  | C  | C  | C  | G  |
| 461  | C  | C  | C  | C  | C  | C  | C  |
| 468  | T  | T  | C  | T  | T  | T  | T  |
| 471  | C  | C  | G  | C  | C  | C  | C  |
| 473  | T  | T/C| A  | C  | T/C| T  | T  |
| 476-478 | TAA | TAA | ::: | TAA | TAA | TAA | TAA |
| 519  | T  | T  | T  | T  | C  | T  | T  |
| 522  | C  | C  | C  | C  | :  | C  | C  |
| 532  | T  | T  | T  | A  | T  | T  | T  |
| 534  | G  | G  | G  | G  | G  | C  | C  |
| 536  | G  | G  | G  | T  | G  | G  | G  |
| 541  | A  | A  | A  | A  | G  | A  | A  |

From 551 6xT 5xT 6xT 6xT 6xT 7xT

From 559 5xT 6xT 5xT 5xT 5xT 4xT 4xT

From 564 5xG 5xG 6xG 4xG 4xG C.4xG 5xG

Cc Capreolus capreolus, Dd Dama dama, Ce Cervus elaphus, Ss Sus scrofa, Oa Ovis aries, Ch Capra hircus, Bt Bos taurus

*The numbering of nucleotide sites refers to a sequence of Capreolus capreolus (NCBI, accession No.: KP659204.1; bp 359 to 583)
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