



Factors influencing genotyping success and genotyping error rate of Eurasian otter (*Lutra lutra*) faeces collected in temperate Central Europe

Marcia Sittenthaler^{1,2} · Eva Maria Schöll¹ · Christoph Leeb² · Elisabeth Haring^{2,3} · Rosemarie Parz-Gollner¹ · Klaus Hackländer¹

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Abstract

The use of non-invasively collected DNA source material for genetic and genomic applications is usually characterized by low target DNA concentration and quality, genotyping errors and cost-intensive lab procedures. However, for otters (*Lutrinae*) as elusive species of conservation concern, genetic non-invasive sampling has become an important tool to study their ecology and demography. To increase cost-efficiency of monitoring programmes and to promote the expansion of genomic approaches to non-invasive samples, we aimed to refine sample collection and preparation. Therefore, we examined the effects of intrinsic sample characteristics (including diet), environmental conditions in the field and sample treatment in the molecular laboratory on the success of genotyping and allelic dropout (ADO) rates using microsatellite markers in 1970 fresh Eurasian otter (*Lutra lutra*) scats. Using fresh samples only, we probably eliminated one of the most important impediments of genotyping DNA from otter faecal samples beforehand. But, we observed higher genotyping success and lower ADO rates for anal gland secretions and faecal samples containing high proportions of mucus. Moist conditions during sample collection may promote DNA degradation and PCR inhibition, leading to decreased genotyping success rates. ADO was further affected by the type of extraction kit. However, a high proportion of variance remaining unexplained by our models implied that additional parameters were acting (amount of PCR inhibitors, non-uniform distribution of intestinal cells, efficiency of PCRs, specific microclimate at marking sites). We summarized influential factors maximizing genotyping quality of otter scats and give recommendations for sample collection, storage and DNA extraction based on our results and current literature.

Keywords Allelic dropout · *Lutra lutra* · Microsatellites · Non-invasive genetic monitoring · Otter spraint · Scat

Introduction

The use of non-invasive genetic methods in wildlife research and management has gained on importance due to continuous advances in molecular genetic techniques (Schwartz et al. 2007; Beja-Pereira et al. 2009; De Barba et al. 2017; Andrews et al. 2018; Carroll et al. 2018). Especially for species of conservation concern and for elusive and nocturnal species, genetic non-invasive sampling facilitates their investigation without the need of disturbing, capturing and handling the animals (Waits and Paetkau 2005). Within the field of non-invasive genetic monitoring, faecal samples have been widely used as DNA from sloughed epithelial cells of the intestine wall of the defecating individual can be analysed (Kohn and Wayne 1997). Faeces are constantly produced by every animal in a population, and for many animal species, they are abundant in the field and relatively easy to detect.

Marcia Sittenthaler and Eva Maria Schöll contributed equally to this work.

✉ Marcia Sittenthaler
marcia.sittenthaler@boku.ac.at; marcia.sittenthaler@nhm-wien.ac.at

¹ Institute of Wildlife Biology and Game Management, Department of Integrative Biology and Biodiversity Research, University of Natural Resources and Life Sciences, Vienna, Gregor-Mendel Strasse 33, 1180 Vienna, Austria

² Central Research Laboratories, Natural History Museum Vienna, Burgring 7, 1010 Vienna, Austria

³ Department of Evolutionary Biology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

Another advantage is that sampling permissions are not required (Beja-Pereira et al. 2009).

Despite of the seductive advantages using non-invasive samples, there are certain limitations when using them as source of DNA. In particular, extracted target DNA from faeces is usually degraded, available in very low amounts, and finally, bacteria and enzymes may be present in considerable amounts (Taberlet et al. 1999). The latter is a common problem with faecal samples as such enzymes and bacteria may act as inhibitors in polymerase chain reactions (PCRs) in subsequent analyses.

These factors often may lead to erroneous genotypes and/or reduce the genotyping success and hence the effective sample size (Taberlet and Luikart 1999). Genotyping errors are primarily due to allelic dropouts (ADOs) and false alleles (Broquet and Petit 2004; Pompanon et al. 2005), which can lead to the creation of so-called ghost individuals or an inability to distinguish individuals (Taberlet et al. 1999; Taberlet and Luikart 1999). As genotyping data are often used in population studies, such errors could cause false results, e.g. a bias in abundance estimates (Lampa et al. 2015a). Therefore, rigorous standards for the analysis of non-invasive samples have been established to minimize genotyping errors (Navidi et al. 1992; Taberlet et al. 1996; Morin et al. 2001; Beja-Pereira et al. 2009; Hausknecht et al. 2010). However, this is at the cost of increased personnel and material expenses (De Barba and Waits 2010). Moreover, high sample dropout rates produce high costs, as more samples have to be collected and analysed to obtain an adequate sample size.

Besides traditional sequencing methods using genetic markers (e.g. microsatellites), also next-generation sequencing methods (e.g. RAD sequencing, DNA metabarcoding) depend on non-invasively collected sample material when dealing with elusive and protected animals to identify the consumer species and individual, and its diet (Monterroso et al. 2019). However, genomic approaches require large amounts of high-quality template DNA (Carroll et al. 2018), and generally, analysis costs for next-generation sequencing are still too high to allow high sample dropout rates due to PCR inhibition or low DNA concentrations. Thus, there is a need to optimize sample collection and DNA extraction of samples collected non-invasively to reduce negative samples and genotyping errors and increase the DNA yield.

DNA quality and quantity isolated from faeces may be influenced by multiple factors in the field, in the subsequent sample storage and lab procedures (Murphy et al. 2003; Piggott and Taylor 2003; Waits and Paetkau 2005; Murphy et al. 2007; Broquet et al. 2007). Several studies on different species and regions have already identified various factors influencing DNA quality including environmental conditions (temperature, humidity, sun exposure) (e.g. Nsubuga et al. 2004; Murphy et al. 2007; Agetsuma-Yanagihara et al. 2017), sample preservation method (e.g. Frantzen et al.

1998; Santini et al. 2007; Bubb et al. 2011), DNA extraction method (e.g. Hájková et al. 2006), storage duration until extraction of DNA (e.g. Soto-Calderón et al. 2009; Bourgeois et al. 2019) and diet of the defecating animal (Murphy et al. 2003; Panasci et al. 2011). Broad consensus was found regarding the age of the scat samples: genotyping success rates and sample quality were highest using the freshest samples (Piggott 2004; Murphy et al. 2007; Brinkman et al. 2010; Vynne et al. 2012; Bourgeois et al. 2019). However, since texture of faeces, diet and environmental conditions may differ between species and populations, influencing factors can vary with different animal species and even within one species occurring in different habitats (Panasci et al. 2011). Generally, carnivores seem to be the most challenging group regarding genetic analysis of faecal material (Ramón-Laca et al. 2015). Within the group of carnivores, fish-eating species seem to be the most problematic (Reed et al. 1997; Murphy et al. 2003). Hence, it is not surprising that the genetic analysis of otter (*Lutrinae*) faeces is deemed as notoriously tricky (Lerone et al. 2014; Klütsch and Thomas 2018) and characterized by high dropout rates (about 50%; e.g. Hájková et al. 2009; Mowry et al. 2011; Silveira Trinca et al. 2013; Vergara et al. 2014; Sittenthaler et al. 2015; Biffi and Williams 2017; Sittenthaler et al. 2020). Nevertheless, when studying otter species, faeces are the sample material of choice, as otters are elusive living animals depositing scats (so-called spraints) regularly at conspicuous marking sites close to the water body (Kruuk 2006). All but one (*Lontra canadensis*) of the 13 otter species worldwide are of conservation concern (IUCN 2020), and non-invasive genetic sampling methods have become an important research tool, which has been integrated in a large number of otter monitoring schemes.

Previous studies investigating genotyping success, amplification rate and error rate of faecal samples in otters have tested a variety of different storage media and extraction kits (Fike et al. 2004; Hájková et al. 2006; Lampa et al. 2008; Lerone et al. 2014). Some of these studies also considered the effect of sample type, sample age, storage time, temperature and humidity during collection on genotyping quality, yielding the best results for fresh samples (Jansman et al. 2001; Hájková et al. 2006; Lerone et al. 2014) extracted soon after collection (Lampa et al. 2008). Many studies showed that slimy secretions of anal scent glands of otters (so-called anal jellies) outperformed typical otter faeces (Coxon et al. 1999; Fike et al. 2004; Hájková et al. 2006; Lampa et al. 2008; Mowry et al. 2011; Lerone et al. 2014). Focusing on the influence of temperature and humidity during collection of the samples, some studies led to contradicting results (Hájková et al. 2006; Lerone et al. 2014; Martin et al. 2017). However, most of these studies were restricted by low sample sizes and/or could include only a limited number of variables in their statistical analyses. Additionally, none of the studies investigated the effect of diet (e.g. crayfish-rich diet) on DNA

quality. In the present study, we used a large data set consisting of 1970 faecal Eurasian otter (*Lutra lutra*), hereinafter referred to as otter, samples collected in temperate Central Europe and analysed the effects of a set of variables on nuclear DNA (microsatellite) genotyping success and genotyping error rate (allelic dropout), both representing quantity and quality of target DNA. The variables included (i) intrinsic sample characteristics (diet composition, sample type, sex of the otter individual), (ii) environmental conditions in the field during sample collection (ambient temperature, humidity, season) and (iii) sample treatment in the molecular laboratory (sample storage time until DNA extraction, type of extraction kit). Although we did not apply genomic approaches, but focused on marker-based analysis, our study aimed to refine and optimize the collection and preparation of non-invasive samples for both traditional and modern genetic approaches.

Material and methods

Faecal sample collection and preservation

We collected otter scats in seven different study sites (one pond area, six salmonid streams) located in northern and eastern parts of Austria (Table 1; Online Resource 1: Fig. S1). Initially, we defined sampling sites along the river and pond banks, representing typical otter marking sites. As the age of a scat has already been identified as crucial factor for DNA degradation and hence genotyping success (Nsubuga et al. 2004; Hájková et al. 2006; Lerone et al. 2014), we used only otter scats deposited within the last 24 h before collection. Hence, we removed all old otter scats from a sampling site and inspected every site repeatedly on three to six consecutive days to ensure a collection of fresh samples only.

Otter scats appeared in various shapes and consistencies, mainly differing by the amount of mucus, and hence potentially the number of excreted cells of the intestinal mucosa. We classified them into three distinct types: spraints (consisting of prey remains and almost no mucus), jellyspraints (consisting mainly of mucus and to a lesser extent of prey remains) and anal jellies (slimy secretion of anal scent glands with hardly any prey remains). All samples were stored in a sample buffer (for details, see Sittenthaler et al. 2015) at minus 20 °C until DNA extraction. The time span between collection and DNA extraction of the sample was defined as storage time.

Climate data and seasons

According to the collection date, samples were assigned to meteorological seasons: spring (March–May), summer (June–August), autumn (September–November) and winter

(December–February). In addition, for each study area and sampling year, data on ambient temperature and relative humidity of the closest meteorological station were provided by Zentralanstalt für Meteorologie und Geodynamik (www.zamg.ac.at), Amt der Niederösterreichischen Landesregierung (www.noe.gv.at) and Höhere Lehranstalt für Umwelt und Wirtschaft Yspertal (www.hluwyspertal.ac.at). The combination of high temperatures and humid conditions lead to higher enzyme and bacteria activity in a faecal sample and promote DNA degradation (Kohn et al. 1995). To account for this effect, we included hourly measured maximum values of ambient temperature (°C) and relative humidity (%) in our analysis. The climate data covered the time frame between dusk the day before collection (when otters start to get active, Kruuk 2006) and 2 p.m. on the day of sample collection (when field work was finished).

DNA extraction and genotyping of otter scats

Faecal DNA was extracted using two different DNA extraction kits, the standard QIAamp DNA Stool Mini Kit and the redeveloped Fast QIAamp DNA Stool Mini Kit (both Qiagen, Germany) following the manufacturer's protocol with slight modifications, i.e. an increase of final incubation time to 5 min before elution of the DNA and elution of the DNA with 100 µl AE buffer (Qiagen, Germany). Samples were genotyped at 11 (dinucleotide and tetranucleotide) microsatellite loci established by Dallas and Piertney (1998): Lut435, Lut457, Lut615, Lut701, Lut717, and Lut833 (multiplex set 1) and Lut453, Lut604, Lut715, Lut733, and Lut832 (multiplex set 2), using the protocol and PCR conditions described in Sittenthaler et al. (2015). For characteristics of microsatellites used in this study, we refer to Online Resource 1: Table S1. Fragment length analysis was performed on an ABI PRISM® 377 DNA sequencer (Perkin Elmer, Germany) and an ABI PRISM® 3130xl Sequence Analyser (Applied Biosystems, USA). Alleles were analysed using automatic allele recognition and manually reviewed with GENESCAN 3.1.2, GENOTYPER 2.5 and GENEMAPPER 5.0 software (Applied Biosystems, USA).

Initially, all samples were amplified in independent quadruplicates for the first multiplex set to identify samples which failed to amplify in all of the six loci. Subsequently, remaining samples, i.e. samples that showed amplification for multiplex set 1, were processed with marker set 2 and up to eight additional PCR reactions for both sets were performed to determine a sample's consensus genotype (referring to the multiple tube approach by Taberlet et al. 1996), according to the following rules: a heterozygote genotype was accepted when each of both alleles was recorded at least twice and a homozygote genotype after at least three independent replications of a single allele. Sex identification was performed as described in Sittenthaler et al. (2015), also in independent

Table 1 Otter scat collection information on location and study sites, including the years when faecal sampling took place, start and end point of river stretch, location of the closest meteorological station and number of otter marking sites where faecal samples were found. Ranges (minimum and maximum) of ambient temperature and relative humidity are given as maximum value of sampling hours at the specific sample date

Study site	Sampling year(s)	Site type	Start point [°N, °E]	End point [°N, °E]	Meteorological station [°N, °E]	Marking sites	Temperature (min; max) (°C)	Relative humidity (min; max) (%)
Romaubach	2013/2014	Pond area	48.94167, 15.16022	48.88463, 15.14435	48.86567, 15.12081	73	- 4.4; 23.5	63; 99
Ysper	2011; 2014/2015	Stream	48.37679, 15.04382	48.19599, 15.00254	48.28646, 15.06279	94	- 3.7; 30.3	57; 99
Kleiner Kamp	2011	Stream	48.42438, 14.98504	48.52596, 15.09440	48.61743, 15.202797	22	1.3; 19.6	94; 98
Piesting	2014/2015	Stream	47.80497, 15.81149	47.87823, 15.90626	47.87550, 15.89118	47	3.2; 27.5	58; 100
Ois	2014/2015	Stream	47.77278, 15.14875	47.85732, 15.02441	47.85961, 15.03061	24	1.1; 32.0	92; 100
Feistritz	2014/2015	Stream	47.58602, 15.86474	47.58689, 16.10007	47.55353, 16.08959	41	8.1; 23.5	87; 100
Loichbach	2014/2015	Stream	47.96306, 15.41604	48.01313, 15.39445	48.01740, 15.59567	24	5.4; 24.0	56; 100

quadruplicates. Meeting the standards of working with non-invasive DNA samples, negative controls were included throughout the extraction procedure and PCR amplifications to check for possible contaminations. Furthermore, pre- and post-PCR pipetting was carried out in different laboratory rooms.

After laboratory analysis, we evaluated the genotyping success for every sample (positive/negative). We counted a sample as positive when at least nine of 11 microsatellites (adequate number of loci to differentiate even between closely related individuals according to Sittenthaler et al. 2020) could be scored unambiguously in the consensus genotype. We further calculated genotyping error rates (ADO) for every positive sample across loci based on the consensus genotype obtained from repeated genotyping and multiple recaptures of the same otter individuals. ADO rate was quantified as the number of allelic dropouts over the total number of successful amplifications of heterozygote loci for a given sample according to Broquet and Petit (2004).

Otter diet composition

For a subset of our samples ($N = 714$; study areas *Ysper*, *Piesting* and *Ois*), we analysed hard prey remains in otter scats (for details of dietary analysis of otter faeces, see Sittenthaler et al. 2019). This subset consisted only of spraints and jellyspraints, as anal jellies did not contain any prey remains. We performed a hierarchical cluster analysis to classify the samples in different diet categories, based on presence/absence data of hard prey remains of the main prey categories (fish, crayfish, amphibians, birds, reptiles, mammals, insects). We used the R package *cluster* (Maechler et al. 2019), and applied the Ward clustering algorithm (Ward 1963).

Statistical analysis and modelling

To analyse the effects of sample characteristics, environmental conditions in the field and sample treatment in the molecular analysis on genotyping success and allelic dropout rate of a sample, we set up four different models. As our response variables were either binary (genotyping success: positive/negative) or proportional (allelic dropout rate 0–100%) and due to the fact that otter scats were repeatedly collected at marking sites from the same individuals, we applied generalized linear mixed models (GLMMs) with a binomial distribution and logit link function.

In our first model, we fitted a GLMM with *genotyping success* (positive and negative samples, $N = 1970$) as response variable. We included the following fixed covariates: *season*, *sample type*, *extraction kit*, *storage time*, *temperature* and *humidity*, as well as an interaction term between the latter two. The *sample location*, i.e. marking site, was included as a random intercept.

For our second model, we used the same model parameters as in model 1, but reduced the data set (positive and negative samples, $N = 714$) to include *diet* as an additional fixed explanatory variable. Additionally, we had to exclude the covariate *extraction kit*, as within the reduced data set, we only used one DNA extraction kit.

In the third model, we fitted a GLMM for all positive samples ($N = 1049$) with the proportion of *allelic dropout* (0–100%) per sample as response variable. As fixed covariates, we used *season*, *extraction kit*, *sample type* and *sex* of the corresponding otter individual. As according to Lampa et al. (2015b), male otters are supposed to leave more anal jellies than females, we included an interaction term between the variables *sample type* and *sex*. Further, we included the fixed covariates *storage time*, *temperature* and *humidity* (and again an interaction term between the latter two). In addition to *sample location*, we also included the *otter individual* as random intercept.

In our fourth model, we principally used the same model parameters as in model 3, but again used a reduced data set, including only those positive samples for which we had analysed diet composition ($N = 340$). Consequently, in model 4, we used *diet* as additional fixed factor and again had to exclude the covariate *extraction kit* (see model 2).

Before fitting the four models, we followed the protocol by Zuur et al. (2010) for data exploration to check specifically for outliers in the data sets, multicollinearity of the explanatory variables and independence of the response variable. There was no multicollinearity between variables; further, we found no indications for the inclusion of more interactions than the ones we mentioned above. We used the R package *lme4* (Bates et al. 2015) to generate a global model for each response variable including all parameters. To identify important explanatory variables, we applied a multimodel inference approach (Burnham and Anderson 2002; Burnham et al. 2011) using the R package *MuMIn* (Bartón 2018). For a set of models representing all possible combinations of explanatory variables, we calculated the Akaike information criterion (AIC_c) (Hurvich and Tsai 1989), and finally selected those models with a $\Delta AIC_c < 2$ (Burnham et al. 2011). Model-averaged coefficients were calculated based on the subset of models, and conditional averaging was used (Grueber et al. 2011; Symonds and Moussalli 2011). Further, for each parameter of the final model, we calculated relative importance values and corresponding upper and lower bounds of the 95% confidence intervals (CIs). Model parameters not including zero within their CI were considered to be informative (Grueber et al. 2011). The amount of variance explained by the models is presented as marginal and conditional R^2 for GLMMs (Nakagawa and Schielzeth 2013). All statistical analyses were performed using the software R 3.5.1 (R Core Team 2018).

Results

We included a total of 1970 otter faecal samples (1401 spraints, 407 jellyspraints, 162 jellies) in our analysis, of which 1049 revealed a positive genotyping result (overall genotyping success rate of 53%). ADO rates across all loci were not normally distributed (Shapiro-Wilk normality test: $W = 0.872$, $p < 0.001$), and the overall median was 17.2% with a minimum value of 0.0 and a maximum of 96.9% (Table 2). For a total of 714 samples, diet analysis was conducted, while the number of positive samples including information on diet totalled 340 (Table 2). Hierarchical cluster analysis revealed a classification of otter samples into 5 diet categories: (1) samples containing amphibian bones, $N = 79$; (2) samples containing bird remains, $N = 75$; (3) samples consisting of fish remains only, $N = 432$; (4) samples containing mammalian and crayfish remains, $N = 58$; and (5) samples containing mainly crayfish, $N = 70$. Maximum ambient temperature at sampling dates ranged between -4.4 and 32.0 °C (mean \pm SD 12.9 ± 7.9 °C), maximum relative humidity between 56 and 100% (mean \pm SD $92.1 \pm 9.4\%$) and storage time covered the range between 0 and 28 days (mean \pm SD 3.8 ± 1.9 days).

For modelling the genotyping success (model 1), the subset of models with a $\Delta AIC_c < 2$ consisted of nine models (Table 3). The final averaged model included the variables *sample type*, *extraction kit*, *storage time*, *season*, *temperature* and *humidity*. However, the confidence intervals for the parameter estimates of *extraction kit*, *storage time*, *temperature* and *humidity* included zero and importance values that were below 0.5, indicating little evidence that these predictors affected genotyping success of otter samples (Table 4). With an importance value of 1, *sample type* had a significant effect on genotyping success. We found lowest genotyping success rates for spraints and highest rates for jelly samples (Fig. 1a, Table 4). Coefficients from the final model showed that using spraints or jellyspraints instead of jelly samples for genotyping, the odds for a positive result decreased by 15 and 27%, respectively (Table 4). Although we detected higher genotyping success rates in summer compared with autumn (Table 4), an importance value of 0.09 indicated little evidence that the variable *season* actually affected genotyping success.

For our second model analysing genotyping success with the reduced data set, the subset of models comprised only two models, including the variables *humidity*, *sample type* and *storage time*, whereas the latter had no effect (Tables 3 and 4). With an increase of 10% in maximal humidity, the odds of a positive genotyping result decreased by 82%. Also in the reduced data set, *sample type* affected genotyping success: the odds of obtaining a positive genotyping result using spraints as source material decrease by 17% and 44% compared with jelly samples and jellyspraints, respectively (Table 4).

Modelling proportion of ADO rate including all positive samples (model 3), the multimodel inference approach

Table 2 Microsatellite genotyping success and error rates (ADO (allelic dropout)) of otter scats collected in seven study areas in Austria. Total number of samples and number of successfully genotyped samples for which diet analysis was conducted are given, and number and sex of otter individuals identified

Study site	Genotyped samples	Sample type (jelly/jellyspraint/spraint)	Positive samples (N; %)	ADO (median; min; max) (%)	Samples for diet analysis (total; genotyped)	Otter individuals (total; males/females)
Romaubach	488	27/82/379	287; 58.8	8.3 (0; 90.9)	-	21 (11/10)
Ysper	510	51/116/343	241; 47.2	12.0 (0; 96.9)	293; 128	23 (12/11)
Kleiner Kamp	68	1/12/55	32; 47.1	7.4 (0; 69.2)	-	5 (3/2)
Piesting	386	41/55/290	224; 58.0	25.0 (0; 88.9)	291; 152	8 (4/4)
Ois	179	15/52/112	88; 49.2	35.2 (0; 94.7)	130; 60	10 (5/5)
Feistritz	207	24/61/122	119; 57.5	16.0 (0; 76.0)	-	13 (6/7)
Loichbach	132	3/29/100	58; 43.9	42.9 (0; 71.4)	-	4 (1/3)
Overall	1970	162/407/1401	1049; 53.2	17.2 (0; 96.9)	714; 340	84 (42/42)

revealed a model subset consisting of four models (Table 3). The variables *extraction kit*, *sample type*, *sex*, an interaction of the latter two, *storage time* and *temperature* were included in the final averaged model. The confidence intervals for the parameter estimates of *sex*, *storage time*, *temperature* and

the interaction term included zero, while *extraction kit* and *sample type* affected ADO rate, also indicated by the importance values of 1 (Table 4). Compared with the redeveloped Fast QIAamp DNA Stool Mini Kit, lower allelic dropout rates were generated with the standard stool extraction kit (Table 4).

Table 3 Subsets of generalized linear mixed effect models with $\Delta AIC_c < 2$ explaining genotyping success and allelic dropout (ADO) rate of genotyped otter scat samples based on a multimodel inference approach. $R^2_{GLMM(m)}$ = marginal R^2 ; amount of variance explained by fixed factors; $R^2_{GLMM(c)}$ = conditional R^2 ; amount of variance explained by the entire model including random factors

Model	Response	Model subsets	AIC _c	ΔAIC_c	AIC _c weight	$R^2_{GLMM(m)}$	$R^2_{GLMM(c)}$	
1.1	Genotyping success (positive/negative) N = 1970	= Intercept + sample type	2618.69	0.00	0.19	0.079	0.120	
1.2		= Intercept + extraction kit + sample type	2618.72	0.03	0.19	0.080	0.117	
1.3		= Intercept + sample type + storage time	2619.29	0.60	0.14	0.079	0.118	
1.4		= Intercept + sample type + season	2620.19	1.50	0.09	0.081	0.116	
1.5		= Intercept + extraction kit + sample type + temperature	2620.22	1.53	0.09	0.080	0.117	
1.6		= Intercept + extraction kit + sample type + storage time	2620.29	1.60	0.08	0.080	0.117	
1.7		= Intercept + sample type + temperature	2620.43	1.74	0.08	0.079	0.120	
1.8		= Intercept + extraction kit + humidity + sample type	2620.56	1.87	0.07	0.080	0.118	
1.9		= Intercept + humidity + sample type	2620.65	1.96	0.07	0.079	0.121	
2.1	Genotyping success (positive/negative) N = 714	= Intercept + humidity + sample type	966.98	0.00	0.66	0.059	0.066	
2.2		= Intercept + humidity + sample type + storage time	968.33	1.35	0.34	0.060	0.064	
3.1	ADO rate (0–100%) N = 1049	= Intercept + extraction kit + sample type + sex	984.43	0.00	0.38	0.120	0.120	
3.2		= Intercept + extraction kit + sample type + sex + storage time	985.08	0.65	0.27	0.120	0.120	
3.3		= Intercept + extraction kit + sample type + sex + sample type/sex	985.70	1.27	0.20	0.128	0.129	
3.4		= Intercept + extraction kit + sample type + sex + storage time + temperature	986.30	1.88	0.15	0.121	0.121	
4.1		ADO rate (0–100%) N = 340	= Intercept + sample type	409.23	0.00	0.32	0.072	0.072
4.2			= Intercept + diet + sample type + season + temperature	410.63	1.40	0.16	0.129	0.129
4.3	= Intercept + sample type + storage time		410.87	1.64	0.14	0.073	0.073	
4.4	= Intercept + diet + humidity + sample type + season + temperature		411.01	1.78	0.13	0.135	0.138	
4.5	= Intercept + sample type + sex		411.02	1.79	0.13	0.073	0.073	
4.6	= Intercept + humidity + sample type		411.17	1.94	0.12	0.072	0.072	

Table 4 Model-averaged summary statistics of generalized linear mixed effect models explaining genotyping success and allelic dropout (ADO) rate of genotyped otter scat samples: estimates of coefficients and standard error (SE), lower and upper limits of the 95% confidence intervals (CI 95%), odds ratios and relative importance values of the model parameters. Model averaging was implemented on models with a $\Delta AIC_c < 2$. Significant parameters are marked with an asterisk

Model	Response	Variables	Estimate	SE	CI 95%	Odds ratio	Relative importance
1	Genotyping success (<i>N</i> = 1970)	(Intercept)	1.740	0.315	1.123; 2.356		
		Sample type*					1.00
		Jellyspraint*	- 1.296	0.247	- 1.781; - 0.811	0.274	
		Spraint*	- 1.907	0.232	- 2.362; - 1.452	0.148	
		Extraction kit_standard stool kit	0.158	0.118	- 0.073; 0.389		0.43
		Storage time	0.026	0.027	- 0.027; 0.079		0.22
		Season*					0.09
		Spring	0.097	0.151	- 0.199; 0.393		
		Summer*	0.301	0.15	0.007; 0.594	1.351	
		Winter	0.170	0.152	- 0.129; 0.468		
		Temperature	0.004	0.006	- 0.008; 0.016		0.17
Humidity	- 0.002	0.005	- 0.012; 0.009		0.14		
2	Genotyping success (<i>N</i> = 714)	(Intercept)	3.375	1.006	1.403; 5.348		
		Humidity*	- 0.020	0.009	- 0.038; - 0.002	1.020	1.00
		Sample type*					1.00
		Jellyspraint	- 0.968	0.591	- 2.127; 0.190		
		Spraint*	- 1.781	0.565	- 2.888; - 0.674	0.170	
3	ADO rate (<i>N</i> = 1049)	Storage time	- 0.040	0.049	- 0.136; 0.055		0.34
		(Intercept)	- 2.019	0.371	- 2.747; - 1.291		
		Extraction kit_standard stool kit*	- 1.015	0.213	- 1.433; - 0.598	0.362	1.00
		Sample type*					1.00
		Jellyspraint*	0.949	0.392	0.181; 1.717	2.583	
		Spraint*	1.334	0.351	0.647; 2.021	3.800	
		Sex_male	- 0.334	0.331	- 0.983; 0.315		1.00
		Storage time	- 0.057	0.051	- 0.156; 0.042		0.42
		Sample type:sex					0.20
		Jellyspraint:male	- 0.797	0.727	- 2.221; 0.627		
4	ADO rate (<i>N</i> = 340)	Spraint:male	- 0.157	0.644	- 1.418; 1.104		
		Temperature	0.010	0.011	- 0.011; 0.031		0.15
		(Intercept)	- 3.069	1.438	- 5.888; - 0.249		
		Sample type					1.00
		Jellyspraint	1.631	1.084	- 0.494; 3.755		
		Spraint	2.048	1.061	- 0.031; 4.127		
		Diet*					0.29
		Diet birds	- 0.821	0.530	- 1.861; 0.218		
		Diet fish*	- 1.281	0.439	- 2.141; - 0.421	0.278	
		Diet mammals_crayfish	- 0.832	0.656	- 2.117; 0.454		
		Diet crayfish	- 0.955	0.558	- 2.049; 0.140		
		Season*					0.29
		Spring	- 0.617	0.450	- 1.500; 0.266		
		Summer	- 0.421	0.437	- 1.277; 0.436		
		Winter*	1.123	0.460	0.221; 2.025	3.074	
Temperature*	0.072	0.028	0.016; 0.127	1.075	0.29		
Storage time	- 0.048	0.074	- 0.193; 0.096		0.14		
Humidity	0.013	0.016	- 0.019; 0.044		0.25		
Sex_male	- 0.132	0.249	- 0.621; 0.356		0.13		

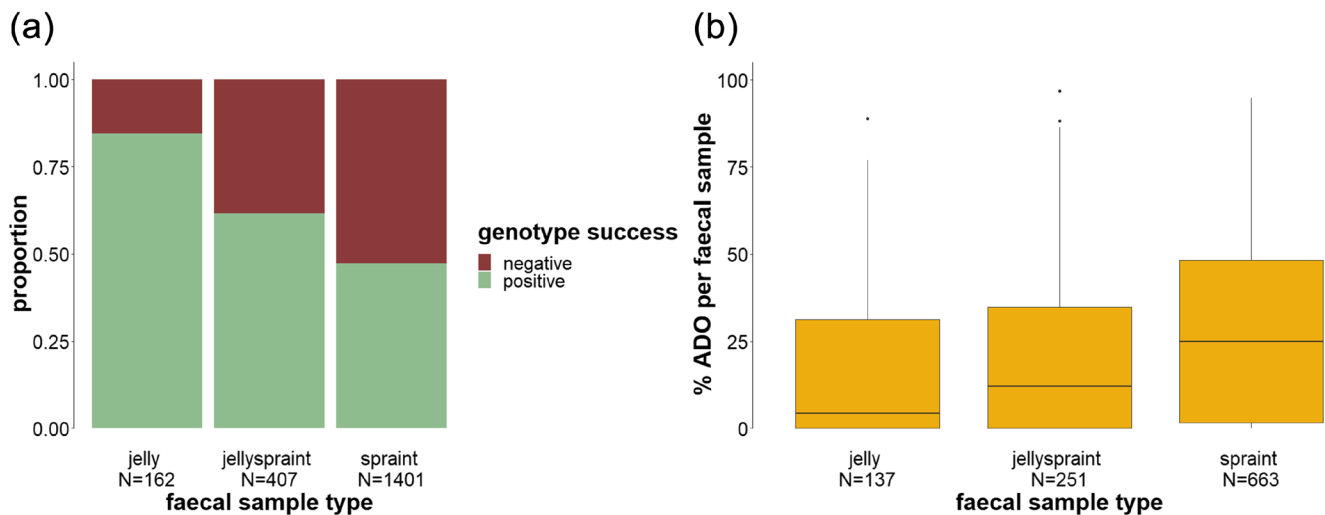


Fig. 1 Proportion of genotyping success (a) and boxplots showing median values, quartiles and maximum values of allelic dropout (ADO) rates (b) for three different otter faecal sample types: anal jellies, jellyspraints and spraints

Regarding *sample type*, higher ADO rates were produced by using jellyspraint and spraints samples, with odds increasing 2.6 times and 3.8 times compared with anal jellies (Fig. 1b, Table 4).

For model 4 (modelling proportion of ADO rate with the reduced data set), we obtained a subset consisting of six models (Table 3). Here, seven variables were included in the final model, but only the variables *diet*, *season* and *temperature* had a confidence interval not including zero. Nevertheless, the low importance values (0.29) of these model parameters indicated little evidence that these predictors affected ADO rate much.

For all models, variance explained as shown by $R^2_{GLMM(m)}$ and $R^2_{GLMM(c)}$ ranged between 0.06 and 0.14 and indicated little variance explained by the final models.

Discussion

In the present study, we examined factors impacting microsatellite genotyping success and allelic dropout rates of otter scats collected in temperate Central Europe. Of the tested variables, only few had a considerable influence on these two parameters indicating the quality of the DNA. Most studies on genotyping animal scats collected in the field highlighted the importance of using fresh scats to increase DNA quality (e.g. Murphy et al. 2007; Vynne et al. 2012; Lerone et al. 2014; Bourgeois et al. 2019). In the present study, we have already considered this factor and collected only otter faeces from the previous night. Thereby, we probably eliminated one of the most important impediments for genotyping DNA from otter faecal samples beforehand. We showed that genotyping

success rate depended mostly on the type of sample and humidity during sample collection, whereas the latter was only significant in the reduced data set (model 2). Allelic dropout was mainly affected by sample type and extraction kit. Using the reduced data set, none of the considered variables was of high relevance explaining ADO rate.

In accordance with previous studies on different otter species using scats for genetic analyses, we observed significantly higher genotyping success rates and to a lesser extent lower allelic dropout rates for anal jelly samples (Fike et al. 2004; Hájková et al. 2006; Lampa et al. 2008; Mowry et al. 2011; Silveira Trinca et al. 2013; Lerone et al. 2014; Biffi and Williams 2017). Furthermore, as already pointed out by Hájková et al. (2006) and Lerone et al. (2014), not only when using anal jellies, but also otter scats containing high proportions of mucus (jellyspraints) led to significant better genotyping results compared with otter scats mainly consisting of prey remains and less mucus.

Concerning climate conditions during sample collection, similar studies investigating DNA quality of scats originating from various animal species and regions concluded that either dry and/or very cold climatic conditions preserve the DNA in the field best (Piggott 2004; Murphy et al. 2007; Brinkman et al. 2010; Demay et al. 2013; Agetsuma-Yanagihara et al. 2017; Nakamura et al. 2017; Klütsch and Thomas 2018). With otter scats deposited in the night before collection, our results indicated that ambient temperature seemed to have no impact on DNA quality, whereas the probability of a sample resulting in a positive genotype decreased with increasing humidity. However, this effect was not constantly significant in all our models. Although Hájková et al. (2006) found that PCR amplification success of otter scat samples was higher at colder

temperatures, the overall effect of temperature was weak compared with the impact of other factors (e.g. sample type). Contradictory to their findings, Lerone et al. (2014) observed higher genotyping success rates with increasing mean daily temperatures, but they assumed that the observed temperature effect was confounded with other undetected influential environmental factors. However, they also found lower PCR success rates with increasing humidity, which was confirmed by the results of the present study. Summarizing, ambient temperature during the collection of otter scats for genetic analyses may play a tangential role when sample collection takes place within 24 h after scat deposition, while moist conditions seem to offer potential to high enzyme and bacteria activity resulting in DNA degradation and PCR inhibition (Kohn et al. 1995; Lampa et al. 2008).

The DNA extraction kits we used in this study were specifically designed for extracting DNA from stool samples; hence, both of them include procedures to remove some of the enzymes and bacteria present in faeces, which can act as PCR inhibitors in downstream analyses. As there was no effect of extraction kit on genotyping success, we conclude that both kits removed PCR inhibitors to a comparable level. However, based on our results, the standard kit with the InhibiteEX Tablets (Qiagen, Germany) performed slightly better concerning DNA quality, as ADO rates were significantly lower (Table 4). To our knowledge, in our field of application, there does not exist another study comparing these two kits. However, other studies comparing the efficiency of various DNA extraction protocols for extracting DNA of faecal material reported higher DNA yield and genotyping success for faecal samples extracted with commercially available kits specifically designed for stool samples (Bhagavatula and Singh 2006; Lampa et al. 2008; Beja-Pereira et al. 2009). A reduction of genotyping errors leads to a reduction of material and personnel costs, as efforts to obtain a reliable genotype decrease. Hence, to ensure a high level of genotyping success and low error rates, we recommend to use DNA extraction kits designed to remove PCR inhibitors in faecal samples.

In accordance with most previous studies, the time span between sample collection and DNA extraction neither had an effect on genotyping success, nor on ADO rate (Hájková et al. 2006; Lerone et al. 2014). However, Lampa et al. (2008) reported a significant decrease in amplification success rate by increasing storage time with highest declines after 1 week. Studying the effects of storage type and time on DNA amplification success in tropical ungulate faeces, Soto-Calderón et al. (2009) also recommended to extract the DNA within 1 week after collection to ensure optimal recovery of DNA. As the effect of storage time may also depend on how samples have been preserved, these contradictory results may have been arisen due to the usage of different sample storage media. Otter faecal DNA samples stored in a buffer solution or ethanol (Hájková et al. 2006; Lerone et al. 2014) seemed to be

preserved over a longer time span compared with samples deep-frozen without a special storage buffer (Lampa et al. 2008). This assumption is supported by our samples, which have been stored at minus 20 °C in a self-made buffer solution comparable with the RNAlater® stabilization reagent, which is considered to be one of the best preservation techniques for faecal sample material over longer storage periods (Soto-Calderón et al. 2009; Beja-Pereira et al. 2009). As all studies specifically testing the effect of storage time covered not more than a few months of storage, future studies should investigate if and how long the DNA will still be sufficiently preserved after sample collection. In a study on otter population dynamics, Bonesi et al. (2013) extracted the DNA of otter scat samples stored in ethanol 2 years after sample collection and yielded a relatively low genotyping success rate of only 19%. However, apart from long storage periods, the authors discussed several factors that may have contributed to the poor result (namely, sample collection during warm periods, wrong sample preservation and few anal jellies among the analysed samples).

We assumed that different diets containing varying proportions of undigestible dietary components will differ in their digestion time and in their intestinal cell slough rates and therefore influence the amount of abraded cells containing target DNA in a faecal sample (Murphy et al. 2003). In the present study, this variable was included for the first time in comparable analyses on genotyping quality of otter faeces. However, based on our results, different prey types (e.g. crayfish containing high proportion of undigestible dietary components) consumed by otters in stream and pond habitats in temperate Central Europe did not impact genotyping success. Although there was a significant effect of diet on ADO rate, relative importance was very low, indicating a very weak effect on DNA quality.

The generally low genotyping success rates in otters compared with other mammal species may be caused by their diet, which is usually based on fish (Kruuk 2006; Krawczyk et al. 2016). Murphy et al. (2003) reported significantly lower DNA amplification success rates in bears when they were fed on salmon. They assumed that the high lipid content and the low dietary fibre content of fish may lead to less abrasion of intestinal cells that contain target DNA or that there may be an interference with extraction chemicals. The latter might explain why different prey types did not impact genotyping quality, as in a free-ranging otter's digestive system there will be always a certain proportion of fish and its chemical components potentially hampering efficiency of DNA extraction or promoting DNA degradation.

We included the sex of the otter individuals and the interaction with sample type in our models to take into account that a certain sample type and its DNA quality is linked to either males or females. While Lampa et al. (2015b) found that male otters leave more anal jellies than

females, we did not observe a sex difference in deposition rate of sample types, and consequently we did not observe any sex bias due to higher genotyping success rates of anal jellies and jellyspraints compared with spraints.

Due to a large data set, we were able to include a large number of parameters that potentially affect the quality of genotyping otter scats. However, it is important to note that our models explained only a small amount of variance of genotyping success and ADO rates (Table 3). Unfortunately, it is not possible to draw comparisons with results of previously published studies, since most of them did not report values for the goodness of fit of their models. However, the high proportion of variance remaining unexplained by our models implies that additional parameters were acting, which were not included in our models because they were difficult or even impossible to quantify. One factor that could have potentially affected genotyping success and ADO rate is a non-uniform distribution of intestinal cells in our samples. A successful extraction of DNA molecules may be depended on the location on the scat where the sample for extraction was taken. The impact of sampling location within a faeces on DNA quality of two carnivores was shown by Stenglein et al. (2010), who reported significantly higher genotyping quality when taking samples from the tip (grey wolf, *Canis lupus*) and the inside (brown bear, *Ursus arctos*) of the scat. Compared with other carnivore scats, it is not always possible to distinguish between front end, back end and middle of an otter scat, especially when the proportion of mucus is high making its shape formless. An adoption of the extraction protocol in order to use larger volumes of initial faecal material may help to increase the chance of capturing enough intestinal cells for DNA extraction (Sittenthaler et al. 2018). Alternatively, conducting more than one extraction per sample was suggested by Frantzen et al. (1998) and Nsubuga et al. (2004), though leading to an extra time and financial effort.

Another factor, which is difficult to identify and measure, is the presence and amount of PCR inhibitors related to diet consumed or the underground where the scat was deposited by the animal. Some authors suggested swabbing protocols instead of collecting the whole scat sample in order to optimize target DNA recovery and limit the presence of PCR inhibitors to a minimum (Klüttsch and Thomas 2018; Bourgeois et al. 2019). However, other studies found no differences in amplification success and genotyping error rate between the different sampling protocols (Velli et al. 2019) or even better results with the traditional approach (Miles et al. 2015). Lampa et al. (2008, 2015b) applied a swabbing protocol to otter samples, but yielded similar genotyping success rates like others collecting the whole scat (e.g. this study; Hájková et al. 2009; Vergara et al. 2014). Martin et al. (2017) used swabs and yielded an overall success rate of only 23%, which is about the half compared with the others.

Moreover, also factors like the efficiency and specificity with which PCR primers anneal or other PCR conditions, as

well as secondary structures in template DNA might influence amplification success and genotyping error rates.

Although it is commonly assumed for the genetic analysis of non-invasive samples that longer fragments show lower amplification success rates and higher error rates (e.g. Sefc et al. 2003; Buchan et al. 2005; Broquet et al. 2007), this was not the case with our microsatellite markers selected for this study (see Table S1 in Online Resource 1; Hájková et al. 2009; Hausknecht et al. 2010; Sittenthaler et al. 2015). Also, Lerone et al. (2014) found no relationship between molecular weight and genotyping error rate, but a decrease in PCR success rate with increased median allele size.

In order to correctly analyse the impact of temperature and humidity, it would have been beneficial to measure the specific microclimate at each sampling point rather than using climate data of meteorological stations for the whole study area. Especially along water bodies there might be large differences in microclimate on a very small scale. The same may be valid for UV light exposure of the scat sample. Another reason why we were not able to detect a temperature effect could be the lack of knowledge on the exact time period for how long a deposited scat was exposed to the environmental conditions. However, measuring meteorological parameters at marking sites (especially, on stones with sun exposure) is almost impossible. Therefore, we suggest ex situ experiments under controlled conditions in a lab, which would allow to adjust parameters and simulate field scenarios. Such experiments may shed light on the influence of climatic conditions on genotyping quality of otter faeces.

Despite of the relatively low genotyping success rates of faecal otter samples (about 50%; Lanszki et al. 2008; Janssens et al. 2008; Hájková et al. 2009; Bonesi et al. 2013; Vergara et al. 2014; Lerone et al. 2014; Sittenthaler et al. 2015; Martin et al. 2017) compared with faeces of other semi-aquatic mammals (e.g. 67% Pyrenean desman, *Galemys pyrenaicus* (Gillet et al. 2017); 89% American mink, *Neovison vison* (Velando et al. 2017)) and other animal species (e.g. 96% Black grouse, *Tetrao tetrix* (Sittenthaler et al. 2018); 66% brown bear *Ursus arctos* (Frosch et al. 2014)), the usefulness of otter spraints as non-invasive source of DNA for genetic and population demography analyses is unquestioned. However, it seems that one has to accept and allow for sample dropout rates of about 50%, which may be related to factors difficult or impossible to control. In the light of increasing the cost-efficiency of DNA-based conservation studies and monitoring programmes (e.g. Ferreira et al. 2018), as well as enabling the application of non-invasive genetic methods in the expanding field of genomics, we here summarize influential factors maximizing genotyping quality of otter scats and give recommendations for sample collection, storage and DNA extraction:

1. Samples collected should be as fresh as possible, i.e. maximum 24-h-old samples, to limit DNA degradation.

2. Sample design has to account for high dropout rates (about 50%) regarding sample sizes. Thus, multiple samples, at least twice as many samples as needed for respective statistics, should be taken from the same location to maximize the chances to detect all individuals present.
3. Even if anal jellies provide the most promising genotyping results, one should not only focus on the collection of anal jellies. Firstly, they are available in the field to a lesser amount than other sample types, and secondly, they pose potential risk of a certain male bias (Lampa et al. 2015b). In addition to jelly samples, spraints containing a high proportion of mucus (jellyspraints) also showed above-average genotyping success rates.
4. Although there are contradictory results concerning temperature and humidity during sampling, we recommend to plan sample collection during dry periods. As long as fresh samples are taken only, it does not matter if sampling takes place during warm or cold seasons—at least in temperate climatic zones as it was the case of the current study.
5. Use commercially available DNA extraction kits specifically designed to remove PCR inhibitors which may be present in faecal samples.
6. To be on the safe side, storage times (i.e. the time span from collection to DNA extraction of a sample) should be as short as possible, not exceeding 1 week. However, when using a buffer solution, length of storage time may not be of relevance, at least as samples will be extracted within a few months.
7. Allow for more extractions per sample, if necessary. Further, the use of larger volumes of stool material in DNA extraction may maximize the chances to capture enough sloughed cells and hence DNA.

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