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DNA Quantity and Quality in Remnants of Traffic-Killed Specimens of an Endangered Longhorn Beetle: A Comparison of Different Methods

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ABSTRACT. The sampling of living insects should be avoided in highly endangered species when the sampling would further increase the risk of population extinction. Nonlethal sampling (wing clips or leg removals) can be an alternative to obtain DNA of individuals for population genetic studies. However, nonlethal sampling may not be possible for all insect species. We examined whether remnants of traffic-killed specimens of the endangered and protected flightless longhorn beetle *Iberodorcadion fuliginator* (L., 1758) can be used as a resource for population genetic analyses. Using insect fragments of traffic-killed specimens collected over 15 yr, we determined the most efficient DNA extraction method in relation to the state of the specimens (crushed, fragment, or intact), preservation (dried, airtight, or in ethanol), storage duration, and weight of the sample by assessing the quantity and quality of genomic DNA. A modified cetyltrimethyl ammonium bromide method provided the highest recovery rate of genomic DNA and the largest yield and highest quality of DNA. We further used traffic-killed specimens to evaluate two DNA amplification techniques (quantitative polymerase chain reaction [qPCR] and microsatellites). Both qPCR and microsatellites revealed successful DNA amplification in all degraded specimens or beetle fragments examined. However, relative qPCR concentration and peak height of microsatellites were affected by the state of specimen and storage duration but not by specimen weight. Our investigation demonstrates that degraded remnants of traffic-killed beetle specimens can serve as a source of high-quality genomic DNA, which allows to address conservation genetic issues.

Key Words: CTAB method, DNA quantity and quality, qPCR, microsatellite, *Iberodorcadion fuliginator*

Empirical data of conservation genetics permit estimation of parameters that carry management implications, including levels of genetic diversity within and among populations, magnitude of gene flow between populations, biological parentage and kinship, and levels of introgressive exchange between hybridizing species (Avise and Hamrick 1996, Kajtoch et al. 2013). However, in rare and/or endangered insect species, it is often difficult to obtain adequate sample sizes without increasing the extinction risk of small populations. Several recent studies have explored nonlethal genetic sampling for insects, which generally involve wing-clips and leg removal (e.g., Holehouse et al. 2003, Hadrys et al. 2005). However, the impact of tissue sampling on the survival and behavior of the individuals concerned has rarely been assessed. The few studies addressing fitness-related effects of nonlethal genetic sampling provided contrasting results. Some studies demonstrated that wing-clips and leg or partial antennae removals had no noticeable effects on survival and behavior, especially in butterflies and bees (Hamm et al. 2010, Koscinski et al. 2011, Marschalek et al. 2013, Oi et al. 2013), while others found increased mortality and lower mating success after leg removal in other insect groups (Starks and Peters 2002, Vila et al. 2009). Thus, leg removal in small-sized, flightless insects is likely to decrease the fitness of individuals.

Insect mortality from collisions with motor vehicles along roads is a common phenomenon and a serious management concern in endangered and protected species (Seibert and Conover 1991, Riffel 1999, Rao and Girish 2007). Insect casualties occur frequently on roads intersecting grasslands (Hayward et al. 2010). However, so far it has not been assessed whether degraded traffic-killed insect specimens or fragments of them can still be used for conservation genetic studies. Insects crushed on the road are immediately colonized by bacteria and fungi, which rapidly degradate tissue and DNA resulting in difficulties to obtain large amounts of high-quality DNA from the target specimens.

We used traffic-killed specimens of the rare, highly endangered and protected longhorn beetle *Iberodorcadion fuliginator* (L., 1758) (formerly *Dorcadion fuliginator*) collected over 15 yr to explore genomic DNA preservation in degraded specimens and to assess their potential utility for future genetic studies. This flightless beetle inhabits different types of extensively managed dry grasslands and suffers from a dramatic decline of suitable habitat throughout its distribution area (Coray et al. 2000, Baur et al. 2002).

The first aim of our study was to determine the most efficient DNA extraction method with respect to DNA quantity and quality for degraded traffic-killed *I. fuliginator* specimens preserved for 1–15 yr. Second, we used three different approaches to assess the quality of the DNA extracted from traffic-killed specimens or remains of them in relation to the state of the specimens (crushed, fragments, or intact), storage duration, and weight of the sample. The three approaches included a direct DNA quantification using a spectrophotometer, quantitative polymerase chain reaction (qPCR) of a small portion of mtDNA, and DNA amplification of microsatellite markers.

Materials and Methods

Study species. The distribution of the grass-feeding flightless longhorn beetle *I. fuliginator* extends from the Iberian Peninsula through Central Europe to eastern part of Germany and from southern Holland to the northern border of Switzerland (Horion 1974, Villiers 1978, Vives 1983, Althoff and Danilevsky 1997). Destruction and degradation of extensively managed dry grasslands combined with increasing levels of fragmentation decreased dramatically its abundance in the past 40 yr (Horion 1974, Klausnitzer and Sander 1978, Coray et al. 2000, Baur et al. 2002). Because of its rarity and endangerment, the species is protected by law in Switzerland, Germany, and the Netherlands.

The beetle has a life cycle of 2 yr (Baur et al. 1997). Females deposit their eggs in stems of grass, preferably *Bromus erectus*, their main larval host plant, in late March to May. The larvae hatch in May or June, feed on grass roots, and pupate after 13.5–14.5 mo (including one hibernation in a late larval stage). Adults (14–17 mm body length) emerge from the pupae after 2–3 wk in July or August, but rest in the soil until the end of the second hibernation. Depending on weather conditions, adults emerge from the soil in March or April and are sexually active for 2–4 wk (Baur et al. 2005). A mark–release–resight study revealed that individuals move 20–100 m, mainly along habitat edges and verges of field tracks (Baur et al. 2005). Tarmac roads can be crossed, but then most individuals are killed by vehicles (B.B., unpublished data).

Provenance and storage of specimens. Twenty (16 traffic killed and four dead but undamaged) specimens of *I. fuliginator* sampled in population 16 near Istein (Germany) in 2000 were used for the evaluation of the different DNA extraction methods (Supp Table 1 [online only]). Designation of localities follows Coray et al. (2000). All specimens were stored dry until DNA extraction in 2013 (Evaluation of different extraction methods).

Another 56 specimens of *I. fuliginator* were collected in 16 localities in Switzerland, France, and Germany between 1998 and 2013 (Supp Table 1 [online only]). The samples consisted of more or less intact specimens found dead in the field ($N = 25$), traffic-killed or otherwise crushed specimens ($N = 17$), or fragments of individuals (e.g., an elytra or single legs, $N = 14$). The state of preservation varied across specimens, locations, and years (Supp Table 1 [online only]). The majority of the specimens ($N = 48$) were stored dry, but few were kept in ethanol ($N = 4$) or in airtight tubes ($N = 4$). These specimens were used to test three methods of genomic amplification and to examine the potential influences of state of specimen, type of storage, and age of material on genome amplification and the quality of the amplified DNA. DNA of the 56 specimens was extracted in 2013 using the modified cetyltrimethyl ammonium bromide (CTAB) method and the dry weight of each sample was determined.

Evaluation of different DNA extraction methods. Three different DNA extraction methods were tested to determine the most efficient DNA extraction from traffic-killed and degraded *I. fuliginator* specimens (Supp Table 1 [online only]). For this purpose, each of the 20 specimens from the first group was cut into three pieces of similar weight allowing a comparison of three methods with tissue from same individuals. The three pieces consisted of similar proportions of head, thorax, and abdomen tissue from an individual. The weight of each sample was determined. For the first DNA extraction method, we used the spin-column system DNeasy Blood & Tissue Kit (Qiagen, Switzerland) following the manufacturer protocol except a slightly modification (two final elutions with 50 µl each). Overnight lysis was conducted for 14 h. The second extraction method was the Fast DNA Spin Kit (MP Biomedicals, Switzerland). We followed the procedure described in the manufacturer manual. The third method was a modified CTAB extraction method (Milligan 1998) with a puffer of 2% CTAB diluted in 100 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid, 1.4 M NaCl, and 0.2% β-mercaptoethanol. For this method, each sample was grounded using a pestle in a mixture of 525 µl CTAB puffer, 15 µl Proteinase K (10 mg/ml), and 10 µl RNase (10 mg/ml). After incubation at 65°C for 90 min, the suspension was extracted with 500 µl chloroform/isoamyl alcohol (25:1) and centrifuged at $12,000 \times g$ for 10 min. The supernatant was transferred into a new 1.5 ml tube, and 450 µl Isopropyl alcohol was added to precipitate the DNA. After 30 min incubation at 4°C, the sample was centrifuged at $12,000 \times g$ for 10 min, and the supernatant was removed. The pellet was washed with 300 µl of 70% ethanol and centrifuged at $12,000 \times g$ for 10 min. After removing the supernatant, the pellet was dried in an Eppendorf Vacfuge at 37°C for 15 min and resuspended in 100 µl sterile water.

We assessed DNA quantity and quality using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Inc., Washington). The DNA concentration (ng/µl) was measured at 260 nm. The quality of the DNA was assessed using the absorbance ratios 260/280 nm and 260/230 nm. Pure samples of DNA are characterized by ratios ranging from 1.8 to 2.2 (NanoDrop Manual, NanoDrop Technologies, Inc., Washington).

Genome amplification and quality of DNA. Three different approaches were tested to evaluate DNA quality in traffic-killed and degraded specimens of *I. fuliginator* and to assess the quality of the amplified DNA: 1) DNA quantification using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Washington), 2) qPCR, and 3) DNA amplification of microsatellites.

Quantitative PCR. In a first step, DNA template was diluted to obtain similar concentrations in all samples. A qPCR based on a small portion of mitochondrial genome (18 S gene) was used to assess the quantity of *I. fuliginator* DNA that can be amplified. The amplification was conducted in 10 µl PCR mix containing 1x LightCycler 480 SYBR Green I Master (Roche, Rotkreuz, Switzerland), 1.8 µM of each primers (18Sai, Whiting et al. 1997 and 18Sb5.3: 5'-GGGTTTAGAG TGTACTCATTCC-3'), 0.5 µM of a dyed probe (5'-FAM-CTCCCG GCACGGGGAGGTAGTGACG-BHQ-1-3'), and 2 µl of DNA template with thermal cycles following the guidelines of the manufacturer (pre-incubation at 95°C during 5 min, followed by 45 cycles of 95°C during 10 s, 60°C during 10 s, and 72°C during 20 s) with a LightCycler 480 Real-Time PCR System (Roche). Three independent amplifications were conducted for each sample. When the concentrations widely differed among the three replicates (i.e. the standard error was larger than 20% of the mean value), three additional replicates were conducted, and all six repeats were averaged. To calibrate each run, a larger portion of the 18S was previously amplified with the primers 18Sa0.3 (5'-GTTGTAACGGGTAACGGG-3') and 18Sb5.0 (Whiting et al. 1997), subsequently diluted between 1×10^4 and 1×10^{10} times, and added as relative quantification.

Microsatellite amplification. To test the different DNA extracts for PCR amplification of microsatellites, a core set of nine microsatellite markers (Dorful_000213, Dorful_001410, Dorful_010423, Dorful_014284, Dorful_024913, Dorful_025921, Dorful_029315, Dorful_031273, and Dorful_032392, Supp Table 2 [online only]) was used following the protocol provided by Qiagen (see below).

To assess the quality of the DNA extracts, three microsatellite markers (Dorful_001410, Dorful_010423, and Dorful_014284, Supp Table 2 [online only]) were amplified in a 10 µl volume using the Type-it Microsatellite PCR kit (Qiagen) following the protocol provided by Qiagen (preincubation at 95°C during 5 min, followed by 28 cycles of 95°C during 30 s, 56°C during 90 s and 72°C during 30 s, and finally 60°C during 30 min). The L-primers were dyed, allowing to detect the product of the amplification on an ABI 3730xl sequencer (Applied Biosystems, Zug, Switzerland). Each microsatellite marker amplification was conducted separately. PeakScanner v 1.0 (Applied Biosystems) was used to visualize the extent of amplification and the height of the pick was recorded.

Statistical analyses. Statistical analyses were performed in the R environment (version 2.15.1, R Development Core Team 2012). Storage duration of *I. fuliginator* specimens ranged from 1 to 15 yr. We therefore assigned the specimens to three groups according to storage duration. Group 1 consisted of specimens stored for 1–5 yr ($N = 18$), group 2 of specimens stored for 6–10 yr ($N = 9$), and group 3 specimens stored for 11–15 yr ($N = 29$). To examine the potential effects of both the state of specimen (intact, crushed, or only fragments) and storage duration on the amount of DNA extracted and DNA quality, we used analyses of covariance with specimen state and storage duration as factors and sample weight as cofactor. A similar analyses of covariance model with specimen state, storage duration as factors and DNA quality, and sample weight as cofactors was used to evaluate the potential effects of specimen state and storage duration on the relative qPCR concentration and microsatellite peak height. If necessary, data were log- or sqrt transformed to obtain normally distributed residuals. Statistical models were stepwise reduced by deleting nonsignificant interactions as recommended by Crawley (2007).

Results

Evaluation of different DNA extraction methods. The comparison of the three extraction methods revealed that the modified CTAB method was the most efficient one for extraction of total genomic DNA from traffic-killed, crushed, and degraded *I. fuliginator* specimens (Table 1). The recovery rate of the CTAB method was 3–9 times higher than those of the two commercial DNA extraction kits tested (Table 1). Furthermore, a 3–10 times higher yield of total DNA was obtained by the CTAB method compared with the two other methods (Table 1). The quality of DNA (OD 260/280) did not differ among the extracts of the three methods (Table 1). However, DNA extracted with the CTAB method showed successful amplification of microsatellites in a significantly higher proportion than DNA extracts from the two commercial kits (Table 1).

Genome amplification and quality of amplified DNA. For all the following tests, DNA was extracted using the modified CTAB extraction method. Neither DNA quantity nor DNA quality was affected by the state of specimen (intact, crushed, or only fragments), storage duration, and the weight of the sample (Table 2).

Relative qPCR concentration and peak height of microsatellites were affected by specimen state and duration of storage (Table 2, Fig. 1). Unexpectedly, relative qPCR concentration and microsatellites peak height were lower in 6–10-yr-old specimens than in both younger (1–5-yr old) and older (11–15-yr old) specimens (Fig. 1). Furthermore, the relative qPCR concentration and microsatellites peak height were lower in the DNA extracts from fragments of *I. fuliginator*, than in extracts of intact or traffic-killed, crushed specimens, irrespective of sample weight (Table 2, Fig. 1). In addition, microsatellite peak height tended to affected by DNA quality (Table 2). Correlation analysis showed that peak height of microsatellites increased with increasing DNA quality ($r_s = 0.38, N = 56, P = 0.005$).

Discussion

This study shows that a large amount of high-quality DNA can be extracted from remnants of traffic-killed specimens of a longhorn beetle. Independent of the type of preservation and storage duration, the extracted DNA can successfully be used for microsatellite and qPCR analyses. Remnants of traffic-killed specimens represent therefore a so far unexplored resource for population genetic studies, especially in rare and protected insect species.

Our comparison of different extraction methods of total genomic DNA revealed that a slightly modified CTAB method is the most efficient one providing both the highest recovery rate and the largest amount of high-quality DNA from traffic-killed crushed specimens. This finding is in line with the results of several studies demonstrating that the CTAB method is the most appropriate one for extraction of DNA in various insect species (Gamba and Arrivillaga 2009, Chen et al. 2010, Wang and Wang 2012, Oi et al. 2013). Reineke et al. (1998) showed that the CTAB method provides the required amount of high-quality DNA for AFLP analysis in several insect species. The CTAB method was originally developed for DNA extraction in plants and

fungi, which contain high amounts of polysaccharides and secondary compounds inferring with DNA extraction (Milligan 1998). The increasing use of the CTAB method for DNA extraction in insects and crustaceans may be due to the fact that CTAB is also removing degraded DNA and decomposition products from the samples yielding a high DNA quality. The chelex extraction protocol used in several studies can be an even more efficient and cheaper method for DNA extraction in insects than the CTAB method (Strange et al. 2009, Gould et al. 2011, Holloway et al. 2013). However, this extraction method does not allow to store the DNA solution for a longer period, which is a clear disadvantage for population genetic studies (Hoy 2013).

Our study showed that both the quantity and quality of DNA obtained by the CTAB method are neither affected by the type of preservation nor by the storage duration and sample weight (Table 2). In general, dried insect tissues contain secondary compounds and degraded DNA (Lindahl 1993). The degree of DNA degradation is affected by the type of preservation and both the storage condition and duration (Lindahl 1993, Dillon et al. 1996). Living specimens frozen in liquid nitrogen and stored at −80°C yielded a large amount of high-quality DNA (Quick et al. 1999). However, this can rarely be applied in the field or for threatened species. Therefore, several alternative preservation methods have been developed including different types of storage solvents (Frampton et al. 2008, Stoeckle et al. 2010). To our knowledge, DNA quality has so far not been assessed in insect tissue affected by bacterial and fungal decomposition processes in the field and later preserved in different ways. Our findings are therefore hardly comparable with those of studies assessing the effects of different storage solutions or storage conditions for DNA analysis (e.g., Dean and Ballard 2001, Frampton et al. 2008, Zimmermann et al. 2008, Stoeckle et al. 2010).

We showed that qPCR and microsatellites were successfully amplified in all specimens examined independent of type of preservation and storage duration. In contrast, others studies showed that the success of DNA amplification was affected by these two factors (Dean and Ballard 2001, Deagle et al. 2006, Watts et al. 2007, Strange et al. 2009). However, differences in the DNA extraction method used and in the type of preservation and storage duration should be taken into account when different studies are compared. Discrepancy among studies could be a result of lower DNA quality obtained by other DNA extraction methods than in our study. Furthermore, species-specific and microsatellite-specific responses in DNA amplification were reported in bumble bee specimens stored for similar periods of time as in this study (Strange et al. 2009). Monroe et al. (2010) showed that the types of sampling (noninvasive vs. nonlethal) and preservation (dried vs. ethanol) were the most important factors determining the amplification success of microsatellites in an endangered Odonata species. Similarly, microsatellite and qPCR product quantities were both affected by the type of preservation and storage duration in our study. The finding that amplification products of both qPCR and microsatellites were reduced in 6–10-yr-old specimens (Fig. 1) could be due to differences in the state of specimens, because a high proportion of these specimens were

Table 1. Recovery rate and characteristics of genomic nuclear DNA from traffic-killed specimens of *I. fuliginator* obtained by three different extraction methods

DNA extraction method	N	Specimens with successful DNA extraction (%)[a]	DNA quantity (ng/μl)[b]	DNA quality[b] 260/280	DNA quality[b] 260/230	Proportion of successful microsatellite amplification[a]
DNeasy blood and tissue kit	20	2 (10)a	110 (84–135)a	1.93 (1.92–1.94)a	0.72 (0.67–0.77)a	2/9a
MP fast DNA spinkit	20	5 (25)a	377 (270–439)b	2.02 (1.98–2.04)a	1.94 (1.89–2.07)b	4/9a
CTAB	20	19 (95)b	1,088 (220–2,464)c	1.82 (1.38–2.03)a	1.74 (1.49–2.06)b	7/9b

Median and range (min, max) are shown. Different letters indicate significant differences among DNA extraction methods ($P < 0.05$). Tissue from the same 20 specimens was used for each extraction method (see Materials and Methods). N, sample size.
[a] Pair-wise proportion test.
[b] Pair-wise Wilcoxon rank-sum test.

Table 2. Summary of the analyses of covariance of the effects of specimen state, storage duration, DNA quality, and sample weight (mg) on the amount of DNA (ng/μl), DNA quality (260/280), relative qPCR concentration, and peak height of microsatellites in *I. fuliginator*

	Amount of DNA (ng/μl)			DNA quality (OD 260/280)			Relative qPCR concentration			Microsatellite peak height		
	df	F	P	df	F	P	df	F	P	df	F	P
Specimen state (ST)	2,47	1.41	0.257	2,47	1.30	0.284	2,47	11.04	**< 0.001**	2,50	11.00	**<0.0001**
Storage duration (D)	2,47	2.15	0.127	2,47	1.29	0.283	2,47	4.05	**0.024**	2,50	8.77	**0.001**
DNA quality (OD 260/280)	—	—	—	a	a	a	—	—	—	1,50	3.23	0.078
Weight of specimen	—	—	—	—	—	—	—	—	—	—	—	—
ST x D	4,47	2.81	**0.036**	4,47	3.05	**0.026**	—	—	—	—	—	—

Significant *P* values (<0.05) are in bold. —, cofactor was excluded from the model.
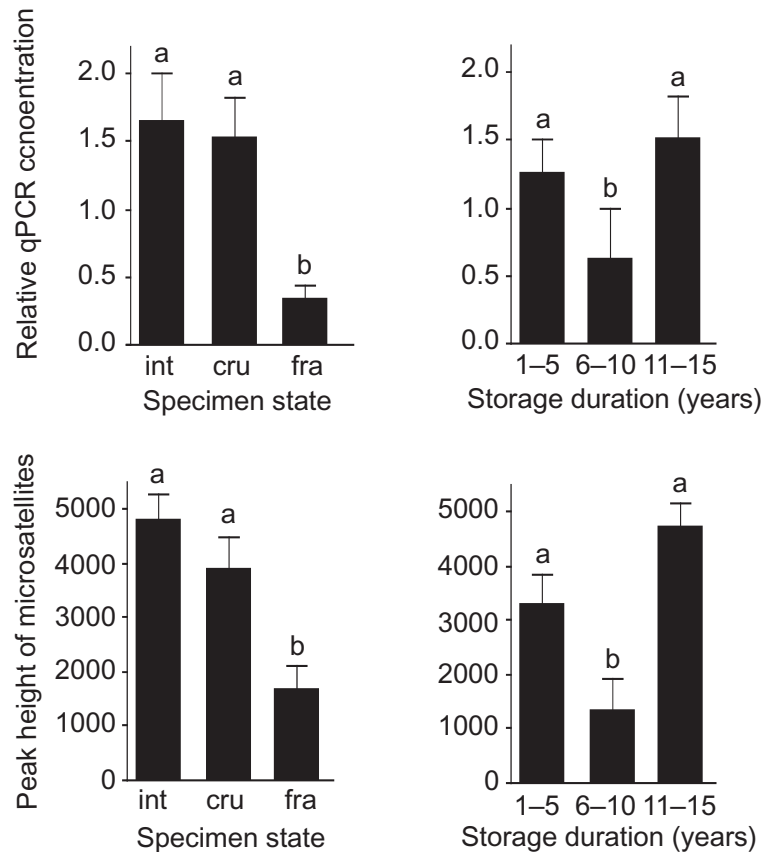[a]Not considered in the model.

Fig. 1. Effects of specimen state and storage duration on the relative qPCR concentration (a) and peak height of microsatellites (b) in *I. fuliginator*. Different letters indicate significant differences among specimen state and storage (int, intact; cru, traffic-killed, crushed, and fra, fragments; Tukey's HSD, *P* < 0.05).

fragments of individuals, whereas the 1–5 yr-old and 11–15-yr-old specimens were mainly traffic-killed or otherwise crushed specimens.

Specimens from natural history collections are nowadays often used for population genetic studies (Wandeler et al. 2007, Rowe et al. 2011). Thus, material from museum collections can play a key role in understanding genetic population structures, especially if large numbers of specimens have been sampled from the same populations in different years (Habel et al. 2014). The recent development of DNA extraction methods, which cause no damage to the morphology of the specimens, will increase the accessibility of museum collection for population genetic studies (Philips and Simon 1995, Rohland et al. 2004, Gilbert et al. 2007). However, in the case of rare species, museum collections may consist of only a small number of individuals sampled in a single population.

We developed another approach by using remnants of traffic-killed specimens which have no value for museums collections. We showed that it is possible to extract large amounts of high-quality DNA of the target species from degraded tissue colonized by bacteria and fungi. The procedure described can easily be adjusted to other insect species. DNA obtained from traffic-killed specimens allows to address certain conservation issues without further increasing the risk of local population extinction in rare and endangered insect species.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

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