A reference allelic ladder for Western Capercaillie (*Tetrao urogallus*) and Black Grouse (*Tetrao tetrix*) enables linking grouse genetic data across studies

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Abstract

Rapid anthropogenic climate change and progressing habitat degradation are considered top threats to biodiversity. The employment of demanding umbrella species as indicators for ecosystem health is a popular and cost-effective strategy that facilitates continuous monitoring and evaluation within a long-term conservation management scheme. The Western Capercaillie (*Tetrao urogallus*) and the Black Grouse (*Tetrao tetrix*) are both considered viable candidates due to their extensive habitat requirements, the possibility for conservative, non-invasive sampling, and their broad popular appeal. Regional population surveys based on genetic data from Short Sequence Repeat (SSR) analysis are being conducted throughout the Palearctic. However, to ensure reliable comparability among laboratories, standardization is required. Here, we report a catalogue of fifty fully characterized reference alleles from twelve SSR loci and the construction of a customizable allelic ladder for genotyping and individualization in Western Capercaillie and Black Grouse. This methodological improvement will help to cost-efficiently generate and collate supraregional data from different grouse surveys and thereby contribute to conservation management. Reference alleles and ladders can be obtained on demand.

Keywords Allelic ladder · Conservation · Grouse · Microsatellite · SSR · Umbrella species

Introduction

Climate change is one of the most pressing global concerns of the twenty-first century, and, together with anthropogenic habitat degradation and loss, poses a major threat to biodiversity (Pereira et al. 2010). To promote ecosystem resilience in the face of this prospect, habitat protection as integral part of conservation management is key (Segan et al. 2016). In this, conservation management is encouraged

to not only provide ‘conservation life support’ but rather monitor, anticipate, and counteract habitat deterioration proactively (McMahon et al. 2014). The use of surrogate taxa can be a cost-effective strategy for habitat evaluation. Within this scheme, surrogate species serve as indicators for regional biodiversity, and their occurrence is linked to general biome health (Andelman and Fagan 2000). Members of the pheasant subfamily Tetraoninae, *vulgo* grouse, are viable candidates to meet these criteria (Pakkala et al. 2003; Runge et al. 2019; Pilliod et al. 2020).

The grouse family comprises 20 currently recognized species (BirdLife International 2019). Most of its members are considered habitat specialists that occupy a broad variety of ecosystems throughout the Northern hemisphere, including prairies, different stages of forest succession, as well as Alpine and Arctic zones. Due to their specific habitat preferences and demanding life-history requirements, grouse are often considered indicators for habitat integrity (Storch 2007). Furthermore, grouse have been a cultural and economic factor throughout human history. Although their relevance as game birds has in general shifted to

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restricted trophy hunting and seasonal subsistence hunting, their broad popular appeal has remained high (Storch 2007). Evidently, such a high level of popularity raises public and, thus, political awareness towards associated conservational issues, making the most charismatic representatives of the grouse family viable flagship candidates (Andelman and Fagan 2000).

Over the last decades, research on grouse has increasingly shifted towards conservation-related topics. On the species level, the Western Capercaillie (*Tetrao urogallus*) and the Black Grouse (*Tetrao tetrix*) have been the two most-studied grouse in the 2000s (Moss et al. 2010). While both Old-World woodland grouse are listed as Least Concern on the IUCN Red List, their population numbers are in decline on a global scale with higher risk for extinction on a regional level (IUCN 2020). This downtrend is expected to continue unless conservational measures are taken (Jahren et al. 2016). In light of the surrogate species concept, conservational efforts could be beneficial to their entire habitats (Andelman and Fagan 2000).

There is evidence that the Western Capercaillie fulfils several criteria of an umbrella species for subalpine and boreal forests. In addition to its demanding habitat requirements, it is considered an indicator of avian biodiversity and presumably linked to invertebrate species richness (Suter et al. 2002; Pakkala et al. 2003). The Black Grouse is deemed to be an indicator species for boreal treeline ecotones (Patthey et al. 2012). Furthermore, the possibility for non-invasive sampling of feces and feathers provides a particularly conservative monitoring scheme devoid of disturbances to the animals and their life cycle (Vallant et al. 2018). Short Sequence Repeat (SSR) analysis based on low-quality sample material has been explored and refined over the last two decades (Horváth et al. 2005; Regnaut et al. 2006; Jacob et al. 2010; Vallant et al. 2018), and grouse population surveys that employ non-invasive sampling schemes have been performed regionally throughout the Palearctic (e.g., Rodríguez-Muñoz et al. 2007; Larsson et al. 2008; Jacob et al. 2010; Höglund et al. 2011; Rutkowski et al. 2018; Sittenthaler et al. 2018).

However, data sets from different laboratories and surveys are difficult to collate as only relative data are obtained from SSR analysis via capillary electrophoresis (Tu 1998). To ensure reliable comparability, the use of a reference standard, also known as *allelic ladder*, is strongly recommended (Bär et al. 1997). These standards were first introduced into human forensics and have since been adopted for animal forensics and wildlife management, such as deer and fisheries management (Poetsch et al. 2001; LaHood et al. 2002). Allelic ladders comprise the most common alleles across the range of a locus and allow for reproducible allele alignment and designation (Bär et al. 1997).

The aim of our study is to introduce a basic set of reference alleles across twelve loci for both Western Capercaillie and Black Grouse genotyping. Fifty alleles from both species were identified, isolated, and characterized regarding sequence and structure. The twelve loci and their allelic ranges were chosen to cover an already well established monitoring project in Tyrol, Austria (Vallant et al. 2018). From this basic catalogue of single alleles, customized allelic ladder mixes can be composed. To showcase its application, we constructed three multilocus allelic ladders to fit the multiplex PCR setup currently used in the aforementioned Western Capercaillie and Black Grouse monitoring. Thereby, we provide a methodological improvement to Palearctic monitoring, with the prospect of supraregionally comparable and, thus, comprehensive data. Additionally, having at hand reference alleles for Western Capercaillie and Black Grouse will increase the genotyping success of poor-quality DNA obtained from non-invasive sampling as generally used for both species.

Materials and methods

Samples

Genomic DNA samples were obtained from the stock of a publicly funded grouse monitoring project in Tyrol, Austria. In the frame of this project, non-invasive sampling of feathers and droppings took place 2011–2014 (Lentner 2017) as well as 2016–2019 (Lentner 2019), and was accomplished through transect mapping of four reference areas located in Tyrol. Genomic DNA was extracted following the protocol in Vallant et al. (2018).

Allele selection

Twelve SSR loci for the genetic individualization of Western Capercaillie and Black Grouse were subdivided into sets of four loci each to generate three multilocus allelic ladders for use in established multiplex PCR assays (Table 1). Species allocation was based on indicative size ranges of three loci, that is, BG15, BG18, and sTuT2, as well as on the Black-Grouse-exclusive marker BG19 (Vallant et al. 2018). Suitable alleles were selected to form evenly-spaced allelic ladders across the allelic ranges of both the Western Capercaillie and Black Grouse (Table 2).

PCR amplification and isolation of alleles

PCR amplification was carried out for each allele individually in 5 µl reactions, containing 2.5 µl 2× Multiplex PCR Kit master mix (Qiagen, Hilden, Germany), 0.75 µg bovine serum albumin, MgCl₂ 1 mM, forward and reverse primers

Table 1 Multiplex PCR assays, primer sequences, labelling (FAM, HEX, NED, Atto565), repeat motifs, and references

Multiplex-PCR	Locus	Forward primer	Reverse primer	SSR motif	References
ML1	sTuT2	FAM-TCTCCAAACTAGATA TGGAAACCAG	CAAAGCTGTGTTTCATTA GTTGAAG	GATA	Jacob et al. (2010)
	mTuT1	GGTCTACATTTGGCTCTG ACC	HEX-GCACAGGAACAGCAA TAGATGG	CTAT	Jacob et al. (2010)
	BG18	NED-CGCCATAACTTAACT TGCACTTTC	CTTCCTGATACAAAGATG CCTACAA	CTAT	Piertney and Höglund (2001)
	sTuT3	GCCTCAACTAATCACCCC TTTATC	Atto565-GAGGGATTATG CATGCTGCTAG	TATC	Jacob et al. (2010)
ML2	BG15	FAM-GAATAAATATGTTTG CTAGGGCTTAC	GATCTTACATTTTTCATT GTGGACTTC	CTAT	Piertney and Höglund (2001)
	sTuD6	HEX-AGCCTTTTACTGCAC TACTTGC	GGTGTGTGGGAAATGAGG AC	CA	Jacob et al. (2010)
	sTuT4	NED-TGGGAGCATCTCCCA GAGTC	ACAAACAAGGCAGCAGCA TG	TATC	Jacob et al. (2010)
	sTuD1	Atto565-ATTTGCCAGGAA ACTTGCTC	CCTTTGCCTCCTTATGAA ATCC	CA	Jacob et al. (2010)
ML3	TTT2	GTGAATGGATGGATGTAT GAA	FAM-AGTCTGTCAATGAAC TTCTTGG	GATA	Caizergues et al. (2001)
	BG19	HEX -CAAGGCGCAACATTA AGATTTC	TGTATTTTGGAACTCTG TGTGC	GATA	Piertney and Höglund (2001)
	BG20	NED-AAGCACTTACAATGG TGAGGAC	TATGTTTTCTTTTCAGT GGTATG	GATA	Piertney and Höglund (2001)
	TTT1	Atto565-TGCAGTCCAGCC TTATTTCA	TCAGTGCTTCACTAACCT CTT	GATA and TAAA	Caizergues et al. (2001)

each 80 nM, and 0.5 µl of template DNA. Thermal cycling was performed as described by Vallant et al. (2018) on a UnoCycler 1200 thermal cycler system (VWR International, Radnor, Pennsylvania, USA), with a uniform annealing temperature of 56 °C. Amplification success was assessed by agarose gel electrophoresis, and the obtained amplicons were subsequently cloned into either a pTZ57R/T InsTAclone or a pJET1.2/blunt cloning vector (both ThermoFisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. After transformation of a chemically competent *E. coli* DH5α host strain and growth of positive transformants for 24 h, plasmids were extracted by alkaline lysis (Sambrook et al. 1989). Sanger sequencing of all inserts was performed by a commercial contractor (Eurofins Genomics, Ebersberg, Germany) to confirm size, SSR motifs, and number of repeat units. The obtained sequences are accessible via the GenBank sequence database (National Center for Biotechnology Information, Bethesda, MD, USA) under the accession numbers MT119884–MT119933 (Table 2).

Ladder preparation and balancing

All plasmid stocks were diluted to approximately 0.5 ng/µl to obtain single-allele working stocks for mixing and balancing the three multilocus ladder cocktails ML1, ML2, and ML3. For multiplex PCR, the ladder blends were treated

as standard genotyping samples and amplified on a 10 µl scale as described in the previous section. Capillary electrophoresis was performed by the Comprehensive Cancer Center DNA Sequencing & Genotyping Facility (University of Chicago, IL, USA). Balancing was achieved iteratively through multiple rounds of readjusting allele concentrations, followed by capillary electrophoresis and evaluation. Stock solutions and ladder cocktails were stored at – 20 °C. *Escherichia coli* JM109 cells transformed with sequenced plasmids were stored in 50% glycerol at – 80 °C to provide a means of rapid allele production.

Results and discussion

This study aimed to create a set of reference alleles for standardized and comparable genotyping of Western Capercailie and Black Grouse across twelve loci. From this basic catalogue of single alleles, three multilocus allelic ladders consisting of four loci each were assembled. PCR amplification encountered certain limitations owed to minute amounts of low-quality DNA, which is a common pitfall of non-invasive sampling (Taberlet and Waits 1998). Hence, we pursued a feasible compromise of ladder complexity and allelic resolution. Each ladder was generated with three to six quasi-evenly spaced allelic rungs across the complete

range of the surveyed populations of both species (Table 2). To represent overlapping regions, suitable alleles were selected from either Western Capercaillie or Black Grouse and complemented by species-specific alleles outside the shared size spectrum.

The true size of all ladder alleles, including primer sites, flanking regions, and repeat sequence, was determined by Sanger sequencing to correct for potential fragment size offset in CE analysis. Additionally, SSR motifs were examined regarding structure and number of repeat units (Table 2). Allelic designations were assigned according to the recommendations of the European DNA Profiling Group (Gill et al. 1997). Following these guidelines, complete and partial repeats are represented by the number before and the digits after the decimal separator, respectively, and the values of the digits thus indicate the number of bases an incomplete repeat comprises.

The structure types were characterized as either *simple repeats*, *simple with non-consensus repeats*, *compound repeat sequences with non-consensus repeats*, or *complex repeat sequences* (Urquhart et al. 1994), showing various degrees of complexity across loci. Minor variations in SSR motifs within the same locus were found between both grouse species as well as intraspecifically, for example in BG18, BG19, and TTT2 (Table 2). However, for each locus, the maximal accumulation of SNPs (single-nucleotide polymorphisms) and InDels (Insertion–Deletion mutations) found in the conserved flanking regions of both species amounted to less than 4% of the shortest fragment length in the allelic range (sequencing data available at GenBank, accession number: MT119884–MT119933). Inter- and intraspecific variations both contributed in similar shares. This implies a certain degree of variability throughout these loci that may lead to mild deviations from the expected electrophoretic behaviour and should be considered for allele calling. It also highlights the need for an allelic ladder that provides predefined reference points of > 95% sequence identity across the allelic range of each locus. Given the low level of interspecific variation, we argue that a hybrid ladder assembled from both Western Capercaillie and Black Grouse alleles is a viable length standard for both species. Field trials have been successfully performed in a multiplex PCR setup.

The multiplex PCRs (ML1, ML2 and ML3) of the three ladder sets yielded strong signal intensities above 2500 relative fluorescent units (RFU) and distinct peaks, except for the three alleles sTuD1/15, sTuD6/16, and sTuD6/27 (Fig. 1). These alleles exhibited stutter peaks of significant intensity between approximately half and two thirds of the original peaks' heights. Stutter artefacts are usually one repeat unit shorter or longer than the corresponding alleles and can be explained through polymerase slippage during amplification (Strand et al. 1993). SSRs derived from dinucleotide

repeat loci were shown to exhibit slippage at a higher rate than tri- and tetranucleotide repeats (Kruglyak et al. 1998). Furthermore, Klintschar and Wiegand (2003) described a positive correlation between stutter occurrence and number of uniform repeats. Both phenomena likely contribute to the observed stuttering as the respective alleles are all derived from *simple repeat* dinucleotide loci and comprise at least 15 repeat units.

A comparison of allele lengths as determined by CE (Fig. 1) and sequencing (Table 2) revealed a fragment size offset in multiple loci. Migration shifts occur due to varying operating conditions and differences in sequence and labelling of sample and generic standard fragments (Zhang and Yeung 1996). An allelic ladder composed of reference alleles that closely resemble the samples in composition enables size-offset correction and facilitates reliable genotyping.

Conclusions

The arrival of population genomics has caused a shift in the scientific conservationists' community towards techniques that provide extensive data at the genomic level, inaccessible by traditional population-genetic analyses (McMahon et al. 2014). However, the latter still pose a viable tool for studying population structure, effective size, and migration rates, parameters heavily consulted in conservation genetics. In this context, microsatellite analysis offers a cost-effective combination of informational depth, practicability, and robustness. Furthermore, as a PCR-based method, it is effectively applicable to low-quantity and/or poor-quality DNA samples (Hodel et al. 2016). This is particularly advantageous to grouse monitoring schemes that rely on non-invasive sampling.

We established a microsatellite-based reference system for genotyping and individualization of Western Capercaillie and Black Grouse. This basic catalogue comprises twelve common genotyping loci and covers the allelic ranges of the Tyrolian populations. A comparison with grouse population analyses in the Swiss Alps revealed a very high resemblance of allele-size ranges for all loci in common usage (Jacob et al. 2010). This indicates immediate relevance to neighbouring populations with similar genotype composition. However, for differentiated populations with shifted allelic ranges, reference allele sets have to be adjusted accordingly. All standards are available as plasmid-based single-allele master stocks and can be combined into customized allelic ladders. It is therefore conveniently possible to expand this reference catalogue by adding single alleles or even entire loci as needed. Standardization of allelic designation is highly recommended as it facilitates interlaboratory data exchange and reproducibility (Budowle et al. 2005). Comprehensive and

ML1

ML2

ML3

Fig. 1 Capillary electrophoresis of the three multilocus allelic ladders comprising 12 SSR loci: sTuT2, mTuT1, BG18, and sTuT3; BG15, sTuD1, sTuD6, and sTuT4; TTT2, BG19, BG20, and TTT1. Ladder alleles are denoted by their respective allelic designations (Table 2).

X-axis: fragment size in base pairs. Y-axis: signal intensity in relative fluorescent units. Ladders were amplified in three multiplex PCR reactions: ML1, ML2, and ML3 (Table 1)

Table 2 Loci and allelic ranges, allelic designations (AD), SSR structure motif, sizes (bp, basepairs) and GenBank accession number (Acc. Number)

Locus and range	AD	SSR motif structure	Size (bp)	Acc. number	Locus and range	AD	SSR motif structure	Size (bp)	Acc. number
sTuT2	6	(GATA)6	123	MT119884	mTuT1	12.2	(CTAT)3 AT (CTAT)9	170	MT119888
123–171	9	(GATA)9	135	MT119885	166–206	15.2	(CTAT)3 AT (CTAT)12	182	MT119889
	12*	GATA GACA (GATA)2 GACA (GATA)7	148	MT119886		18.2*	CCAT CTAC (CTAT)2 AT (CTAT)13 CTAC	194	MT119890
	17*	GATA GACA (GATA)2 (GACA)2 (GATA)11	167	MT119887		20.2	(CTAT)3 AT (CTAT)17	202	MT119891
BG18	12	(CTAT)12	153	MT119892	sTuT3	10*	(TATC)10	98	MT119897
149–216	15	(CTAT)15	165	MT119893	86–122	12*	(TATC)12	106	MT119898
	18	(CTAT)3 TTAT (CTAT)14	177	MT119894		15	(TATC)15	118	MT119899
	22*	(CTAT)3 TTAT (CTAT)5 CTGT TTAT (CTAT)11	196	MT119895					
	25*	(CTAT)3 TTAT CTAT CCAT (CTAT)3 CTGT TTAT CTAT CTAC (CTAT)12	208	MT119896					
BG15	10*	CTAT CTAC (CTAT)8	142	MT119900	sTuD1	10*	(CA)2 CT (CA)7	155	MT119913
138–214	13*	(CTAT)12 TTAT	154	MT119901	153–167	12*	(CA)12	159	MT119914
	15*	(CTAT)15	162	MT119902		15*	(CA)15	165	MT119915
	18	(CTAT)12 CCAT (CTAT)4 TTAT	174	MT119903					
	24	(CTAT)12 CCAT (CTAT)5 CCAT (CTAT)5	198	MT119904					
	26	(CTAT)14 CCAT (CTAT)5 CCAT (CTAT)5	206	MT119905					
sTuD6	5	(CA)5	146	MT119906	sTuT4	11*	(TATC)2 TGTT (TATC)8	123	MT119910
144–190	10	(CA)10	156	MT119907	119–147	14*	(TATC)2 TGTT (TATC)4 TGTT (TATC)6	135	MT119911
	16*	(CA)12 CG (CA)3	168	MT119908		16	(TATC)16	143	MT119912
	27*	CA CG (CA)25	191	MT119909					
TTT2	16.3	(GATA)3 GAT (GATA)9 GACA GATA (GACA)2	180	MT119916	BG19	9.3	(GATA)9 GAT	168	MT119922
176–227	19.3	(GATA)3 GAT (GATA)12 GACA GATA (GACA)2	192	MT119917	160–192	12.3	(GATA)12 GAT	180	MT119923
	19.33*	(GATA)3 GAT (GATA)2 GGTA GATA GAT (GATA)7 GACA GATA GGTA (GACA)2	195	MT119918		14.3	(GATA)5 GACA AATA (GATA)7 GAC	188	MT119924

Table 2 (continued)

Locus and range	AD	SSR motif structure	Size (bp)	Acc. number	Locus and range	AD	SSR motif structure	Size (bp)	Acc. number
	21.33*	(GATA)3 GAT (GATA)2 GGTA GATA GAT (GATA)9 GACA GATA GGTA (GACA)2	203	MT119919					
	22.33*	(GATA)3 GAT (GATA)2 GGTA GATA GAT (GATA)2 GGTA (GATA)7 GACA GATA GGTA (GACA)2	207	MT119920					
	26.33*	(GATA)3 GAT (GATA)2 GGTA CATA GAT (GATA)14 GACA GATA GGTA (GACA)2	223	MT119921					
BG20	11.13*	GATG (GATA)6 A GATA GTTA (GATA)2 GAT	126	MT119925	TTT1	11.2*	(GATA)5 GA (TAAA)6	212	MT119929
126–155	13.13*	(GATA)9 A GATA GTTA (GATA)2 GAT	134	MT119926	212–256	13.2*	(GATA)6 GA (TAAA)7	220	MT119930
	15.3	(GATA)15 GAT	141	MT119927		16.2	(GATA)9 GA (TAAA)7	232	MT119931
	18.3	(GATA)16 GACA GATA AAT	152	MT119928		18.2	(GATA)10 GA (TAAA)8	240	MT119932
						21.2	(GATA)9 GACA GA (TAAA)11	252	MT119933

Amplification source was *Tetrao tetrix* except for alleles denoted by *, for which it was *Tetrao urogallus*

supraregional data across the ongoing Palearctic grouse monitoring projects provide a means to track individual and genetic exchange across borders and reference areas, evaluate existing networks of stepping stones and corridors, and identify beneficial and detrimental factors to grouse conservation management.

The allelic ladders and single reference alleles can be obtained upon request at <https://molecular-ecology.at/> or <https://birgit.schlick-steiner@uibk.ac.at>.

Author contributions Conceptualization: Birgit C. Schlick-Steiner, Florian M. Steiner, Wolfgang Arthofer; Methodology: Philipp Andesner; Formal analysis and investigation: Philipp Andesner, Thomas Seeber; Writing—original draft preparation: Philipp Andesner; Writing—review and editing: Philipp Andesner, Stephanie Vallant, Reinhard Lentner, Wolfgang Arthofer, Florian M. Steiner, Birgit C. Schlick-Steiner; Funding acquisition: not applicable; Resources: Stephanie Vallant, Reinhard Lentner, Florian M. Steiner, Birgit C. Schlick-Steiner; supervision: Wolfgang Arthofer, Florian M. Steiner, Birgit C. Schlick-Steiner.

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Data availability Data are accessible on NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>; accession number: MT119884-MT119933).

Software for data evaluation Data from capillary electrophoresis was evaluated using GeneMarker (SoftGenetics, PA, USA).

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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