University of Natural Resources
and Life Sciences, Vienna

Genetic diversity and structure of Alpine black grouse (*Tetrao tetrix)* populations

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Declaration in lieu of oath

I herewith declare in lieu of oath that this thesis has been composed by myself without any inadmissible help and without the use of sources other than those given due reference in the text and listed in the list of references. I further declare that all persons and institutions that have directly or indirectly helped me with the preparation of the thesis have been acknowledged and that this thesis has not been submitted, wholly or substantially, as an examination document at any other institution.

10.05.2017	
Date	Signature

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Abstract

Austrian Alpine black grouse, *Tetrao tetrix*, populations are among the largest black grouse populations in Central Europe and thus are important for conservation issues in this species. These Alpine black grouse populations are described to be metapopulations and migration barriers further aggravate gene flow. For management programs of the black grouse, knowledge of the genetic structure is crucial.

In this master thesis, populations from the eastern (Styria) and western (Tyrol) Austrian Alps were analyzed using nine microsatellite loci. Analysis from faeces, feathers and tissue samples as well as calibration of the data set of Tyrol were successful. Genetic diversity of the two alpine regions did not show much difference. Applying Bayesian clustering and multivariate analyses did not reveal a clear distinction between Styria and Tyrol. Results indicate ongoing gene flow, expressed in a gradient of shared genotypes over the Austrian Alps. Populations overlap genetically, with certain subpopulations being important for population connectivity.

This study improves our understanding for conservation measures for Alpine black grouse. Small spatial scale as well as large-scale considerations are important. This study should further encourage Alpine-wide research on this species.

Zusammenfassung

Die Birkhuhn-Populationen, *Tetrao tetrix*, der Alpen zählen zu den größten Populationen in Zentraleuropa und sind bedeutend für den Schutz dieser Art. Diese Populationen sind jedoch Metapopulationen. Migrationsbarrieren erschweren zudem den Genfluss zwischen den Populationen. Um erfolgreiche Managementkonzepte für das Birkhuhn zu ermöglichen sind Kenntnisse der genetischen Populationsstruktur unumgänglich.

In dieser Masterarbeit wurden Populationen aus der Steiermark und Tirol mit Hilfe von neun Mikrosatelliten untersucht. Die genetische Analyse von Losungs-, Feder- und Gewebeproben sowie das Kalibrieren mit den Tiroler Daten waren erfolgreich. Die zwei Regionen zeigten kaum Unterschiede in ihrer genetischen Diversität. Auch Bayesische und multivariate Analysen konnten keine klare Abgrenzung der Regionen nachweisen. Die Resultate der Studie deuten auf Genfluss zwischen den Regionen hin. Dies äußerte sich in einem Gradient gemeinsamer Genotypen über die österreichischen Alpen. Die Populationen überlappten sich in genetischen Clusteranalysen und einige Subpopulationen erschienen besonders wichtig für die Vernetzung.

Diese Studie trägt zum besseren Verständnis alpiner Birkhuhn-Populationen bei. Naturschutzmaßnahmen sollten kleinräumig (innerhalb von Metapopulationen) als auch großräumig (zwischen weiter entfernten Regionen) angedacht werden.

Directory

1. Introduction

The black grouse, *Tetrao tetrix*, is one of four Austrian grouse (*Tetraoninae*) species. It inhabits a wide range of landscapes from structured boreal forests at higher latitudes in northern Eurasia up to forest combat zones at higher altitudes in Central Europe (Fig. 1) (BirdLife International 2016; Storch and Segelbacher 2000). Although its distribution is widespread, habitat requirements do contain essential structural elements. Black grouse is a typical ecotone species, preferring forest edges and transition areas between forests and open land. Diverse forests at early stages of succession with corresponding undergrowth or moorland habitats are inhabited by the species. In mountainous areas, habitats are situated around the tree line. These environments frequently show an increase in habitat suitability with newly occurring open spaces due to avalanches, forest calamities or anthropogenic land use like alpine pastures (Klaus et al. 1990; Storch and Segelbacher 2000).

Fig. 1 Distribution of black grouse (BirdLife International 2016)

1.1. General status of black grouse populations

On a large scale, black grouse appear in high numbers compared to other grouse species (Storch 2007) and thus is considered “least concern” by the International Union for Conservation of Nature and Natural Resources IUCN (BirdLife International 2016). It shows a continuous distribution over large northern boreal forests (Caizergues et al. 2003b; Höglund et al. 2003) (Fig. 1). However, Central European black grouse

occurrences are fragmented after major declines and shifts in range mainly after 1970 (Storch 2007). Populations in Britain have contracted and Irish populations went extinct (Watson and Moss 2008; Höglund 2009), while populations in the Netherlands dropped from more than 10 000 individuals to numbers below 30 (Höglund et al. 2007 after Jansman et al. 2004; Larsson et al. 2008). Black grouse in Denmark went extinct (Höglund et al. 2007 after Holst-Joergensen 2000) whereas populations in Germany decreased significantly (Ludwig et al. 2009), following the ongoing trend for lowland populations (Klaus et al. 1990). Those populations are assumed to be isolated from each other as distances exceed the assumed maximum black grouse dispersal distance (Höglund et al. 2003). This results in decreased genetic diversity and a higher impact of genetic drift and inbreeding, with populations eventually facing higher risks of extinction (Höglund et al. 2007).

The largest, still stable Central European populations can be found in the Alps (Storch 2007), where occurrences are constrained to tree line and mountain ridge habitats, often associated with alpine pastures (Storch and Segelbacher 2000). This patchy distribution of suitable habitats is paired with aggravated migration conditions. Black grouse seem to be reluctant to cross mountain ridges exceeding their natural habitat in altitude (Caizergues and Ellison 2002), but also human infrastructure and changed land-use inhibit a strong connection of populations (Ingold 2005; Nopp-Mayr and Grünschachner-Berger 2011). The resulting fragmentation of populations becomes evident in terms of a higher genetic differentiation compared to the continuous occurrences, with at the same time lower genetic variability (Caizergues et al. 2003b). However, populations still show some minor connection through dispersal of individuals (Höglund et al. 2007). Dispersal distances resemble 2 km for males and 5 to 10 km for females with maximum distances of 30 km under certain conditions (Caizergues and Ellison 2002; Warren and Baines 2002). Populations therefore are assumed to be contiguous rather than continuous and can be described as metapopulation systems (Caizergues et al. 2003b; Höglund et al. 2003).
The term metapopulation depicts a group of partially isolated subpopulations with an ongoing but minor exchange of individuals, respectively gene flow. As a consequence, subpopulations do fluctuate in population size, even extinction and re-colonialization events can occur occasionally (Klug et al. 2007; Frankham et al. 2010).

Reasons for decline and extinction of black grouse populations are well described: Storch (2007) lists habitat degradation due to changes in land use and human infrastructure as the major cause. However, also fragmentation, disturbance, small population size, predation, exploitation and climate change impact population trends with various intensities depending on specific habitat situations (BirdLife International 2016; Nopp-Mayr and Grünschachner-Berger 2011). In mountainous regions, besides habitat degradation, also fragmentation and the resulting isolation and small subpopulation sizes are problematic in particular (Höglund et al. 2003). Also, artificial disturbances like anthropogenic recreational activities show negative influence on black grouse (Ingold 2005; Arlettaz et al. 2007).

1.2. Situation in the Austrian Alps

In Styria, black grouse still occur at reasonable numbers according to lek counts of the hunting association (Grünschachner-Berger 2013; Sittenthaler et al. 2016), but Wöss and Zeiler (2003) showed that especially subpopulations at the south-eastern border of the Alpine distribution range declined and expired over the past centuries. Besides habitat degradation by human infrastructures (Zeiler and Grünschachner-Berger 2009; Nopp-Mayr and Grünschachner-Berger 2011), also aggravated migration due to fragmentation and barriers like the Mur-Mürz valley (Sittenthaler et al. 2016) are major causes for the decreasing population sizes. Therefore, black grouse is listed as “vulnerable” in Styrian red list (Sackl and Samwald 1997).

In Tyrol, black grouse is listed as “almost vulnerable” by Landmann and Lentner (2001), occurring all over Tyrol in slightly rising numbers compared from 2005 to 2010 (Reimoser and Habe 2011). As these estimates rely on lek counts, Vallant (2014) established population extrapolation based on capture-recapture models of genetic data.

1.3. Conservation need of Austrian Alpine populations

Compared to other Alpine countries, Austria harbors the second largest black grouse population, being the only country where population sizes are assumed to be stable (Storch 2007). Internationally, black grouse is listed both in annex 1 and annex 2 of the

European Bird Directive (Directive 2009/147/EC of the European Parliament and of the Council of 30 November 2009 on the conservation of wild birds), urging member states and federal states to conduct special conservation measures to ensure survival and reproduction. Additional measures have to be done as black grouse is considered as a game species in Austria according to annex 2 part B policy. Cocks are legally hunted as stated in the federal hunting laws. In Styria, cocks are hunted from the 1st until the 31th of May (Auer- und Birkwild-Verordnung 14. April 2008 LGBl. Nr. 40/2008) according to an authorized hunting schedule, based on last years estimated population numbers (Steiermärkisches Jagdgesetz 1986 LGBl. Nr. 23/1986). For Tyrol, yearly hunting season is also published via administrative orders (Tiroler Jagdgesetz 1983 LGBl. Nr. 41/2004) and concentrates on a fifteen day period within the 1st of May until the 15th of June (Tiroler Jägerverband s.a.). To justify usage, member states and therefore federal states have to ensure sustainability according to the European Bird Directive. This requires a comprehensive understanding of population size, genetic composition, exchange and resilience. Explicitly mentioned hereby is the importance of observing trends and variations of populations and subpopulations.
On a national level, black grouse is listed as “vulnerable” in the Austrian red list (Bauer 1994).
Facing these status assumptions, a conservation need is undeniably given.

1.4. Conservation genetics of black grouse

In order to design and manage valid conservation actions for threatened species, understanding and considering of genetic issues is important (Frankham et al. 2010). Genetic diversity and population structure have an impact on survival and persistence (Höglund 2009), representing the evolutionary potential of a species. Especially small populations or subpopulations in a metapopulation context are prone to genetic effects, as genetic drift and inbreeding do have higher impacts and can lead to extinction of individual subpopulations or the whole metapopulation. This so-called extinction vortex is enhanced in fragmented populations due to habitat fragmentation or migration inhibition, as gene flow is aggravated. As a consequence, genetic diversity is lost and population structure increases, significantly impairing already small populations (Frankham et al. 2010). Conformingly, such functional chains have been observed in isolated European black grouse populations (Höglund 2009). Therefore,

understanding to what extent exchange between populations and subpopulations occurs is crucial for conservation planning (Storch and Segelbacher 2000).

In the last decades, microsatellites or simple sequence repeats (SSR) were the most commonly used genetic markers in conservation genetics (Holderegger and Segelbacher 2016). SSRs are short base sequences (like CA, CTAT) repeated several to hundreds of times (Frankham et al. 2010), typically situated in the non-coding region. SSRs are assumed to lose or gain whole repeat units at high rates (Dieringer and Schlötterer 2003), altering the fragment lengths of the specific loci which ultimately represent the different alleles.
Applying several SSR loci can reveal the genetic structure, representing the genome. Hence, population structure and other genetic parameters of populations can be analyzed (Höglund 2009; Segelbacher and Höglund 2009; Frankham et al. 2010).

So far, Austrian populations and metapopulations had been studied separately without combining data sets in a broader genetic context encompassing the Alps (Vallant 2014, Sittenthaler et al. in prep.). Hence, a comprehensive view over Austrian Alpine population structure is lacking. Caizergues et al. (2003b) generally described the Alpine populations to be contiguous. Höglund et al. (2007) adopted this classification, however could not find any difference in genetic diversity between continuous and contiguous populations, which was attributed to low statistical power (Höglund 2009). Larsson et al. (2008) however assumed Austrian Alpine populations to be continuous, comparable with Norwegian populations.

2. Aims of this study

In this study, population genetic patterns of Austrian Alpine black grouse were investigated. Thereby, the aims of this master thesis were:

(1) to extract DNA from tissue, faeces and feathers and to apply nine SSR loci previously characterized

(2) to calibrate and to combine data sets from Styria and from Tyrol obtained by Vallant (2014)

(3) to analyze the differences in genetic diversity and population structure between the two study sites in the eastern (Styria) and western (Tyrol) part of the Austrian Alps.

Consequently, an insight into Austrian Alpine black grouse genetic patterns should be given. Addressing larger spatial scales instead of small-scaled single-site studies, this study should provide the basis for comprehensive future research and conservation implications.

3. Methods

3.1. Study area Styria

Styrian black grouse occur in the inner Alpine region (Northern Limestone Alps and Central Eastern Alps) on the north-western part as well as its border areas (Styrian Pre-Alps), broadly divided by the Mur-Mürz valley. Within the Inner Alps, black grouse occur along the tree line whereas at the lower mountain regions they are limited to small sites at the highest mountain ridges. Several of these exterior subpopulations already became extinct (Wöss and Zeiler 2003). Recently there are still noteworthy subpopulations south of the Mur-Mürz valley (Grünschachner-Berger 2013).

The Styrian metapopulation was divided into eleven subpopulations (Fig. 2, Table 1) based on topographical criteria described in Sittenthaler et al. (2016) and Grünschachner-Berger (2013). The subpopulations were further assembled into four groups indicated by the cardinal directions (north-west and north-east, south-west and south-east).

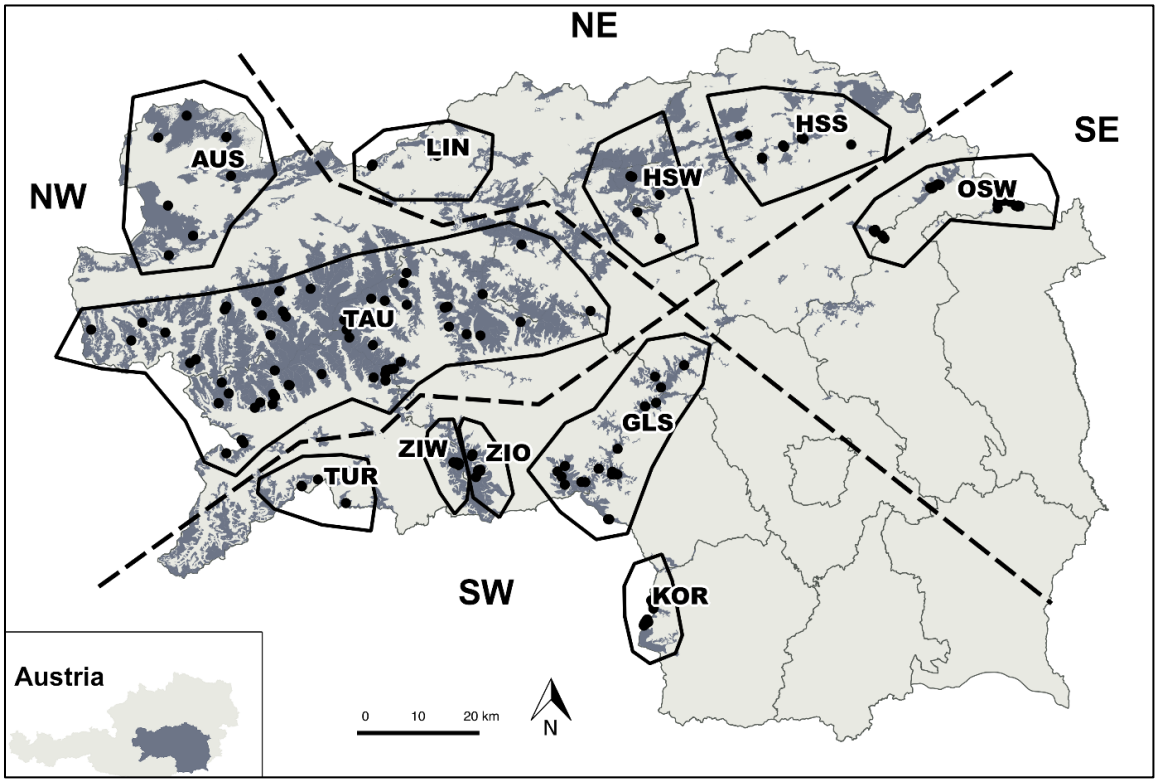


Fig. 2 Styrian black grouse metapopulation classification. Mur-Mürz valley equals the division between the northern part and the southern part and follows up southwards between Turrach (TUR) and Zirbitz West (ZIW) (Sittenthaler et al. in prep.)

Table 1 Subpopulations, abbreviations and sample sizes N in Styria and Tyrol

nr	subpopulation	abbreviation	N
1	Aussee/Regionen	AUS	7
2	Liezen Nord	LIN	5
3	Hochschwab Süd	HSS	13
4	Hochschwab West	HSW	13
5	Tauern	TAU	56
6	Oststeiermark/Wechsel	OSW	41
7	Turrach	TUR	4
8	Zirbitz West	ZIW	10
9	Zirbitz Ost	ZIO	7
10	Gleinalm/Stubalm	GLS	23
11	Koralm	KOR	15
12	Tyrol	TIR	94

Faeces and feather samples were collected non-invasively from March to May 2013, 2015 and 2016. Altogether 307 samples were acquired. Faeces were collected straightway after days of snowfall to obtain preferably intact DNA from fresh droppings as well as taken out of recently melted snow caves (black grouse overnight quartiers in winter) as the cooling effect of snow decelerates DNA degradation (Vallant 2014). They were stored into 15 ml tubes and frozen at -40 °C until extraction. Feather samples were moulted feathers as well as remains from preyed birds, and frozen at -40°C in paper envelopes for storage. Tissue samples were received from legally shot birds during the hunting season. Samples were taken from internal organs or muscle, put into ethanol (> 96 %) and frozen at -40 °C until extraction.

3.2. Study area Tyrol

The study area was situated in the Central Eastern Alps of Tyrol in the Ötztal Alps, mainly between Kauner valley and Pitz valley (Fig. 3). Black grouse faeces and feather sampling was done in smaller, so-called areas for intense field collection (accumulating to 980 ha), based on specific habitat models described in Vallant (2014).

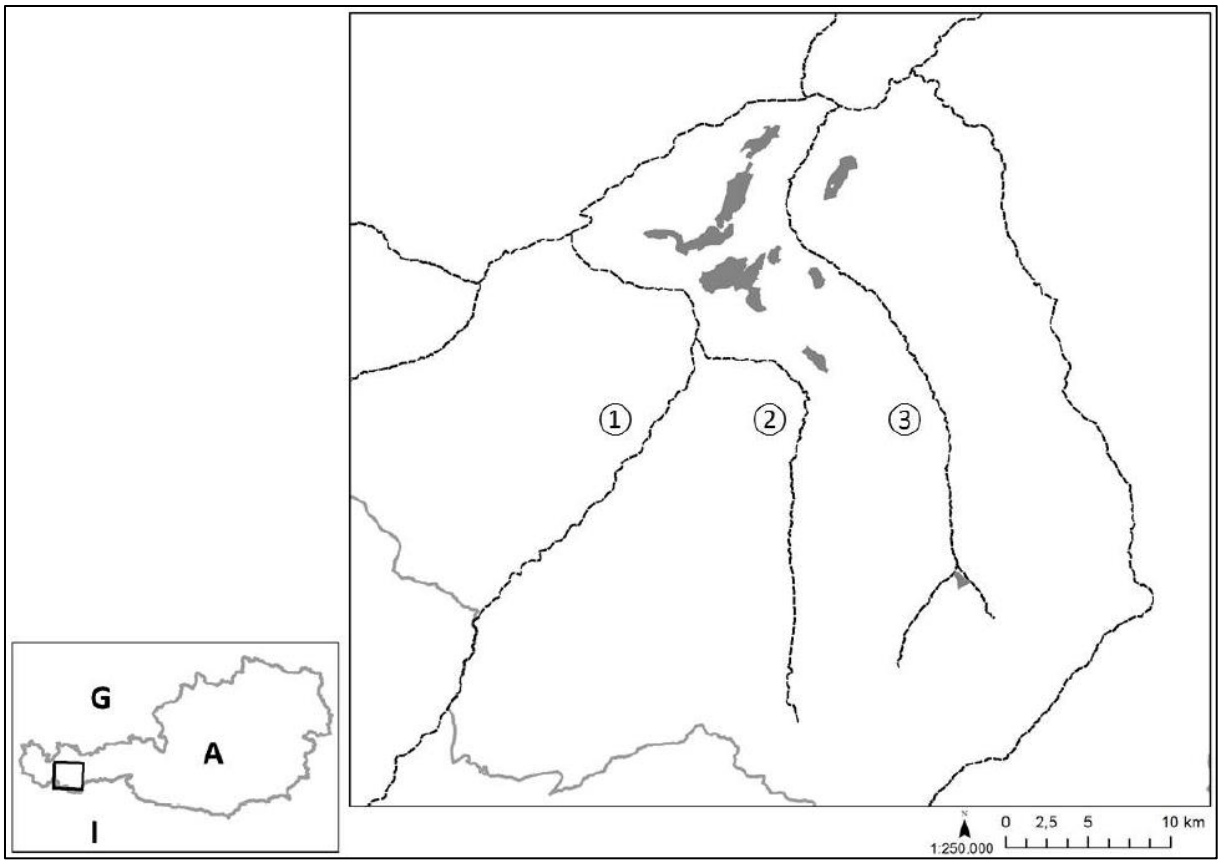


Fig. 3 Areas of intensive field collection, Tyrol. Shaded areas consist of black grouse as well as capercaillie (*Tetrau urogallus*) sampling areas (1 = Inn valley; 2 = Kauner valley; 3 = Pitz valley; G = Germany; A = Austria; I = Italy) (Vallant 2014)

The sampling took place in spring 2012 as described in Vallant (2014) and resulted in 214 samples, of which 116 different genotypes could have been achieved.

Tyrolean study site was not designed to represent a predefined subpopulation. However, it was later included into the dataset at the same hierarchy level as a subpopulation in Styria as the extent of the sample area is small, comparable to a Styrian subpopulation. Thus, genetic analysis could have been done respectively to compare genetic diversity, address population structure on a finer scale and answer questions about population differentiation and gene flow.

3.3. Extraction and amplification of SSRs

DNA from faeces was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's standard protocol. To ensure higher quality in DNA, modifications to the protocol were implemented. The frozen faeces were cut off to approximately one to two centimeters, hashed, and put into 10 ml tubes containing 4 ml of buffer ASL (Qiagen). Urine acid covered parts were avoided as urine (specifically urea) contains additional polymerase chain reaction (PCR) inhibitors (Khan et al. 1991). Lysis was performed over night at 55 °C under constant shaking, afterwards the samples were centrifuged and further steps were conducted according to the standard protocol. Elution of DNA was done two times in a row with 70 µl buffer AE (Qiagen) after 5 min of incubation at room temperature.

The extraction of feathers was done using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Small pieces from the root end as well as the afterfeather rudiment were cut into pieces and crushed in a ball mill for 3 min. After adding of 20 µl SIGMA proteinase K (Sigma-Aldrich), the samples were incubated under constant shaking for 24 h and further steps were done according to the adapted manufacturer's standard protocol. Elution was done two times in a row with 50 µl and subsequently 30 µl of provided elution solution (Sigma-Aldrich) after 5 min of incubation at room temperature.

Tissue samples were extracted using the same GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the adapted standard protocol. DNA quality and quantity was measured on a NanoDrop 2000c spectrophotometer (PeQLab) determining the necessary dilution before conducting PCR.

To avoid and check for contamination, extraction and PCR amplification were carried out in different rooms and negative controls were included throughout the whole extraction process.

The samples were analyzed using nine SSR loci, based on Sittenthaler et al. (2016). The SSRs BG6, BG15, BG16, BG18, BG19 were described by Piertney and Höglund (2001) and TUD6, TUT1, TUT2, TUT3 were described by Segelbacher et al. (2000).

Multiplex PCR (multiplex 1: TUT1, TUT2 TUT3; multiplex 2: BG15, BG16, BG18; multiplex 3: TUD6, BG19, BG6; multiplex 4: BG19, BG16, BG6) was conducted on a 2720 Thermal Cycler (Applied Biosystems) and a Primus 96 advanced (PeQLab) as

well as a Primus 25 advanced (PeQLab) with a total reaction volume of 10 µl. The mixture contained 2 µl of autoclaved, filtered water, 1 µl bovine serum albumin 20 mg/ml (Sigma-Aldrich), 5 µl of SuperHot Mastermix 2x (Genaxxon bioscience), 1 µl of prepared primer mix containing each labeled forward and reverse primer adapted for the multiplex and 1 µl of the extracted DNA per sample. The SuperHot Mastermix 2x contains inactivated *Taq* DNA polymerase (M3307), PCR buffer, $MgCl_2$, dNTPs and additives. It is designed to launch the PCR reaction after a 15 min heat activation step to possess higher sensitivity, especially improving multiplex PCR. Two different thermal cycling conditions were tested and used to achieve optimal results. Both conditions generated evaluable PCR results, no differences were noticed. The first thermal cycling condition comprised of an initial denaturation step of 95 °C for 15 min, followed by 38 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for multiplex 1/ 53 °C for multiplex 2/ 57 °C for multiplex 3 for 1 min and extension at 72 °C for 1 min, ending with a final extension step at 72 °C for 30 min. The second thermal cycling condition conforms the standard protocol for SuperHot Mastermix 2x (Genaxxon bioscience) and consists of an initial denaturation at 95 °C for 10 min, followed by 36 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for multiplex 1/ 53 °C for multiplex 2 for/ 57 °C for multiplex 3/ 54 °C for multiplex 4 for 45 sec and extension at 72 °C for 45 sec, ending with a final extension step at 72 °C for 10 min. Number of cycles varied by one for both thermal cycling conditions to account for the differing DNA concentration of samples.

The amplification of the sexing markers 1237L and 1272H (Kahn et al. 1998) was carried out in a 10 µl reaction mixture, composed of 5.9 µl of autoclaved, filtered water, 1 µl of buffer S for taq-polymerase (PeQLab), 1 µl of bovine serum albumin 20 mg/ml (Sigma-Aldrich), 0.2 µl of dNTP mix (PeQLab), 0.3 µl of each forward and reverse primer solution, 0.1 µl of peqGOLD taq-DNA-polymerase (PeQLab) and 1.2 µl of template DNA per sample. PCR thermal cycling conditions were led by an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 49 °C for 45 sec and extension at 72 °C for 1 min, ending with a final extension step at 72 °C for 10 min.

To avoid contamination, pre PCR and post PCR pipetting was carried out in different rooms. Every PCR approach contained at least one negative control without DNA to test for overall contamination.

3.4. Extension and calibration of the Tyrolean dataset

For Tyrol, DNA samples as well as a data set consisting of six genotyped loci (TUT1, TUT2, TUT3, BG15, BG18, TUD6) (Vallant 2014) per individual could have been obtained. The missing SSRs BG16, BG19 and BG6 were amplified and analyzed from the obtained DNA and then added to the dataset. Thus, both study sites were analyzed for the same nine loci.

Pre-existing Tyrolean data had to be calibrated as microsatellite fragment length data is a relative quantity, depending on electrophoretic mobility due to temperature, fluorescent labels and others (de Valk et al. 2009) as well as on laboratory factors such as internal size standard, laboratory equipment or even experience of the researcher (Moran et al. 2006; Ellis et al. 2011). Additionally, BG19 also had to be calibrated as for Tyrol a NED labeled primer was used, which differs in fragment lengths from the FAM labeled primer used for the Styrian samples. Therefore, nine to fifteen samples per locus were repeatedly re-analyzed using the same method as applied to the Styrian samples. The differences in fragment length were recorded and afterwards applied onto the Tyrolean dataset. Later during analysis, the adapted Tyrolean dataset was merged with the Styrian dataset, treating Tyrol at the hierarchy level of a subpopulation. This resulted in a consistent dataset divided into twelve subpopulations.

3.5. Genotyping

Fragment length was analyzed on an ABI PRISM 310 automatic sequencer (Applied Biosystems) by a commercial provider (Comprehensive Cancer Center DNA Sequencing Facility, Chicago). Size standard LIZ500 (GeneScan) was used for allele length scoring in PeakScanner 2.0 (Applied Biosystems). As noninvasive samples have different quantity and quality of DNA and therefore can lead to false genotype assignment, genotyping was done using a multi-tubes approach (Navidi et al. 1992; Taberlet et al. 1996). A genotype was only recorded if at least three independent repeats showed the same allele combination. For heterozygote loci, one allele missing in one of the accepted repeats was still considered assured. Occurrence of a new allele at an otherwise homozygote locus resulted in calling NA, as distinction between false allele or dropout in such cases is not possible.

Individuals with three or more loci missing due to amplifying errors or insufficient assurance were excluded from further analyses.

3.6. Analysis of genetic diversity

Microsatellite toolkit 3.1.1 (Park 2001) was used to find and exclude samples with matching genotypes. Samples with identical alleles at all loci and sex were considered to originate from the same individual. Probability of identity (PI) and probability of identity for siblings (PI_{SIB}) were calculated using GenAlEx 6.502 (Peakall and Smouse 2006, 2012) to check the power of the applied markers to identify individuals. Genepop 4.2 web application (Raymond and Rousset 1995; Rousset 2008) was used to check the datasets for deviations of Hardy-Weinberg-equilibrium and linkage disequilibrium. Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) was then applied to check for occurrence of genotyping errors due to null alleles, short allele dominance and stuttering, as these errors can cause deviations from Hardy-Weinberg proportions. Standard summary parameters of genetic variation were calculated using GenAlEx 6.502 (Peakall and Smouse 2006, 2012). Allelic richness, a measurement to compare the number of alleles corrected for differences in sample size using a rarefaction method, was computed by FSTAT 2.9.3.2 (Goudet 1995) (per locus) and HPrare 1.1 (Kalinowski 2005) (per subpopulation). Differences in allelic frequency distribution between the regions per locus were tested using Fishers exact tests in R 3.3.2 (R Development Core Team 2016) on the absolute allele counts.

3.7. Analysis of population structure

Analysis of molecular variance (AMOVA), F-statistics and pairwise F_{ST} values were computed using Arlequin 3.5.2.2 (Excoffier et al. 2005). All tests for significance were conducted at a significance level of $\alpha = 0.05$, adjusted for multiple testing via Bonferroni correction. A systematic Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000) was applied to delineate clusters of individuals based on differences in their genotypes. Admixture models with correlated allele frequency with and without LOCPRIOR information (the division into subpopulations) were run with a burnin of 200 000 repeats, followed by 600 000 Markov-Chain-Monte-Carlo (MCMC)

repeats. Styria and Tyrol were analyzed separately as well as combined. For each K (Styria: 1 to 13; Tyrol: 1 to 10; combined: 1 to 13), 30 iterations were computed. As computation time for the combined dataset of Styria and Tyrol was not feasible, for K = 7 to 13 slightly reduced parameters of burnin of 100 000 repeats, 400 000 MCMC repeats and 20 iterations were run after a pilot study resulted in K to be less than 5. Results from STRUCTURE 2.3.4 (Pritchard et al. 2000) were analyzed for best fitting K using the web application of Structure Harvester (Earl and VonHoldt 2012), determined by highest ΔK and specific patterns of L(K) according to Evanno et al. (2005). Additionally, the results of the next higher values for K were considered too because of their supplementary information about patterning when forcing more clusters onto the data sets. Averaging over all iterations and visualization via barplots was done with the web application CLUMPAK (Kopelman et al. 2015). Population structure was further analyzed using a principal component analysis (PCA) as well as a discriminant analysis of principal components (DAPC) (Jombart et al. 2010) as implemented in the package adegenet 2.0.1 (Jombart 2008; Jombart and Ahmed 2011) for R 3.2.2 (R Development Core Team 2016). When conducting a DAPC, prior information about group membership is necessary. DAPC first conducts a PCA on the data, followed by a linear discriminant analysis (DA) on the retained principal components generating new synthetic variables, so called discriminant functions. Those maximize between group variation while within group variation is minimized (Jombart et al. 2010). Afterwards, the groups can be plotted against the discriminant functions.

The multivariate methods PCA and DAPC differ from Bayesian clustering approaches as they don’t make assumptions about Hardy-Weinberg, linkage disequilibrium or underlying population models (Jombart et al. 2010).

4. Results

4.1. Extraction and amplification success

Out of 83 black grouse samples collected in Styria in the year 2016, genotypes were determined for 81 samples only, nine of which were duplicates and neglected for this study. One feather and one faecal sample had to be excluded due to amplification failure caused by degraded DNA. Sexing markers revealed 64 males and 19 females. The high amount of males is explained by the limitation of hunting allowed only on males. Taking only faeces and feathers, 28 males and 19 females were detected.
The 72 individual genotypes were included into the Styrian genotype dataset, already consisting of 37 genotypes sampled in 2013 and 85 genotypes sampled in 2015. Altogether, 194 individual genotypes divided into eleven subpopulations represent the Styrian black grouse metapopulation dataset.

Amplifying the additional three SSR loci of the Tyrolean DNA samples succeeded for 81 individuals, with furthermore thirteen individuals missing one and seven individuals missing two loci due to amplifying errors. Eleven individuals failed to amplify all SSRs and four individuals were not processed as sample DNA was not provided. Altogether, 94 individual genotypes represent the Tyrolean dataset.

4.2. Calibration of SSR data sets

Detecting the differences for calibration of the pre-existing genotype data was successful at altogether 84 loci. Only one locus showed shifted results and was excluded. For most of the loci, all existing alleles were covered at least once (Table 2). The differences between the same alleles of the six SSR loci as well as of BG19 due to the used labeled dye were recorded and calibration was then undertaken by adding the exact difference Δ to the corresponding locus of the Tyrolian genotypes in order to adjust Tyrolean genotypes to Styrian genotypes.

Table 2 Differences Δ in allele lengths between Tyrol and Styria. *Difference Δ* is the shift, which was summed with the Tyrolean genotypes to equate the Styrian genotypes. The row *individuals* shows the number of analyzed individuals per microsatellite used for calibration, the row *alleles* displays how many of the existing alleles were covered in the calibration

	TUT1	TUT2	TUT3	BG15	BG18	TUD6	BG19
difference Δ	31	13	54	-4	-6	35	-4
individuals	9	10	10	15	15	13	12
alleles	9 (9)	3 (3)	7 (7)	9 (9)	7 (8)	6 (9)	7 (9)

For analyzing population structure, Styrian and Tyrolean datasets were pooled, resulting in 288 genotypes divided into twelve subpopulations.

4.3. Testing for Hardy-Weinberg, linkage disequilibrium and probability of identity

After testing all datasets for deviations from Hard-Weinberg-equilibrium and for linkage disequilibrium, all loci remained in the analysis. Tests were run for both Styria and Tyrol independently.

In Styria, TUT1 showed significant deviations from Hardy-Weinberg-equilibrium in two subpopulations after Bonferroni correction, the excess of homozygotes in most of the size classes suggested the presence of null alleles. Nevertheless, TUT1 remained in the analysis as it had no distorting effects on further analyses. Linkage disequilibrium was found in sixteen (4 %) of the possible 395 pairings of loci, after Bonferroni correction only three remained significant.

In Tyrol, significant deviations from Hardy-Weinberg-equilibrium after Bonferroni correction were present at TUT1, BG15 and TUD6. TUT1 and BG15 deviations were most likely due to occurrence of null alleles, similar to the Styrian dataset and displayed in a positive inbreeding coefficient F_{IS} of 0.51 for TUT1 and 0.18 for BG15. Deviation of TUD6 was presumably caused by allelic dropout and stuttering, as indicated by a shortage of heterozygote genotypes especially with alleles of one repeat unit difference. As the study site does not relate to a natural subpopulation distribution, deviations are also likely to emerge due to the Wahlund effect (Wahlund 1928; Frankham et al. 2010). Ultimately, all loci were kept in the analysis as the discovered

deviations did not call for immediate exclusion of those loci (Carlsson 2008; Waples 2014). In addition, results from Styria, representing a known metapopulation distribution, proved their proper functionality and utility. Also for Styria and Tyrol, re-runs in STRUCTURE without the deviating loci have been done in order to test if they possessed a biasing influence, which could not have been shown.
Significant linkage disequilibrium after Bonferroni correction was only found in two (5.6 %) out of 36 pairings, not corresponding to significant pairings found in Styria. Therefore, random association of alleles at different loci is given.

Probability of identity (Styria: $PI = 4.3 \times 10^{-9}$; Tyrol: $PI = 2.1 \times 10^{-9}$) was reasonable low as discussed by Mills et al. (2000) and Waits et al. (2001). Corrected for effects of potential population structure or related individuals, the calculated probability of identity between siblings (Styria: $PI_{SIB} = 5.5 \times 10^{-4}$; Tyrol: $PI_{SIB} = 4.1 \times 10^{-4}$) also assured exact recognition of individuals by genotype (Waits et al. 2001).

4.4. Genetic diversity in Styria and Tyrol per locus

For Styria, the number of alleles (A) per locus (Table 3) ranged from three (TUT2) to fifteen (BG6). With exception of TUT2, all loci showed a high polymorphism with an average of eight alleles. TUT2 was already known to show little diversity, Larsson et al. (2008) and Vallant (2014) found similar results. Expected heterozygosity (H_E) exceeded observed heterozygosity (H_O) at every locus, but not significantly. As mentioned above, the deviation of TUT1 seemed to be due to the appearance of null alleles, also implied by the positive fixation index (F_{IS}).
In Tyrol, genetic diversity per locus appeared in similar patterns as in Styria. The loci corresponded in number of alleles as well as in heterozygosity. Observed heterozygosity overall was lower than expected, TUT1 also showed an excess in homozygotes as discussed above. A difference was visible at BG19 and BG6. BG19 appeared in Tyrol in much higher observed and expected heterozygosity while at BG6 only half of the number of alleles in Styria were observed.
The same was true for the effective number of alleles (A_E), describing the number of alleles necessary for the same heterozygosity if all alleles appear at the same frequency. Thus, it is a measurement of distribution of individual alleles and robust against sample size differences (Frankham et al. 2010). While TUT1 to TUD6 again

seemed to be similar to Styria, BG19 had a much higher effective number of alleles, indicating a more even distribution of alleles. This also explained the higher observed heterozygosity. BG6 showed the same effective number of alleles as in Styria, while only half the number of alleles was observed. Therefore, although Tyrol displayed a more even distribution, Styria potentially harbored rare alleles at this locus. Comparing allelic richness (A_R) calculated for the smallest Styrian subpopulation size, general patterns were similar. However, allelic richness appeared to be higher at BG19 in Tyrol than expected, indicating not only a more even distribution but a higher diversity in general for this particular locus.

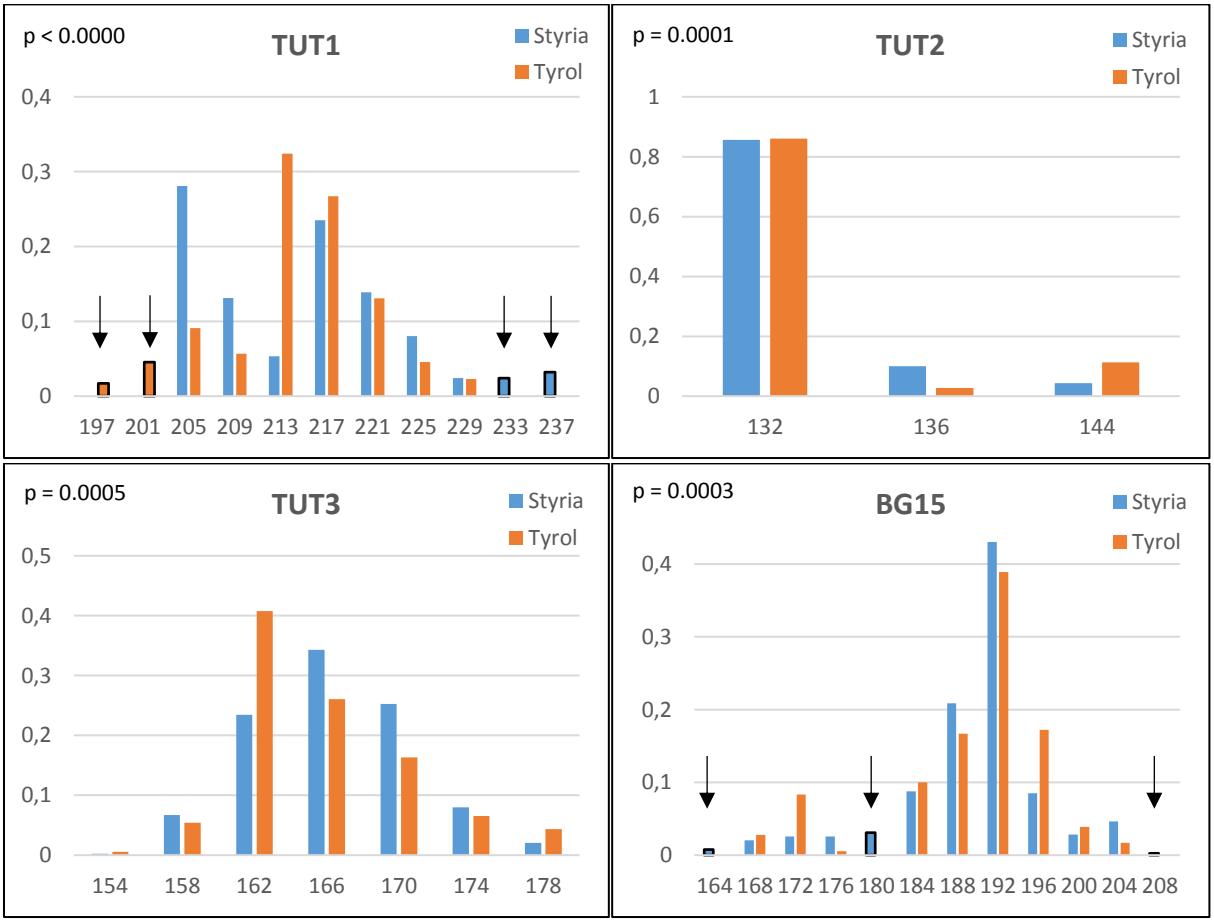
Table 3 Genetic summary statistics per SSR locus over all subpopulations for Styria and Tyrol. Rounded to two decimals. Number of alleles (A), effective number of alleles (A_E), allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS})

	TUT1 (CTAT)	TUT2 (GATA)	TUT3 (TATC)	BG15 (CTAT)	BG16 (CTAT)	BG18 (CTAT)	TUD6 (CA)	BG19 (GATA)	BG6 (GATA)	mean
Styria										
A	9	3	7	12	7	10	7	8	15	8.67
A_E	5.5	1.34	4.04	4.01	4.5	5.41	3.46	2.13	3.88	3.81
A_R	4.64	1.89	3.85	4.24	4.03	4.54	3.69	2.95	4.11	3.77
H_O	0.54	0.25	0.73	0.78	0.70	0.79	0.72	0.52	0.72	0.64
H_E	0.82	0.26	0.75	0.75	0.78	0.82	0.71	0.53	0.74	0.68
F_{IS}	0.35	0.01	0.03	-0.04	0.1	0.03	-0.01	0.03	0.03	0.06
Tyrol										
A	9	3	7	9	7	8	8	9	7	7.45
A_E	4.77	1.33	3.71	4.38	4.11	4.42	3.77	5.61	3.99	4.01
A_R	4.42	1.82	3.81	4.28	3.87	4.04	3.82	4.61	3.83	3.83
H_O	0.39	0.26	0.72	0.63	0.71	0.72	0.69	0.84	0.77	0.64
H_E	0.79	0.25	0.73	0.77	0.76	0.77	0.73	0.82	0.75	0.71
F_{IS}	0.51	-0.05	0.02	0.18	0.06	0.07	0.06	-0.03	-0.03	0.09

As the standard summary statistics presented per locus enabled the comparison of genetic diversity, a more detailed look into allelic frequency distribution per locus per region was necessary to identify potential shifts in allelic range. For all loci, allele frequency distribution of Styria and Tyrol appeared in corresponding patterns (Fig. 4). Only Locus TUT1 showed a clear shift in allelic range, yet the main distribution again overlapped. Distribution patterns also reflected previous results, especially at BG19 and BG6. However, for all loci allele frequency distributions differed significantly

between Styria and Tyrol. But differences were only due to certain alleles per locus not corresponding to the expectation of independence of the fisher exact test. Thus these results did not contradict the overall consequence of similar patterns.

As an additional information when comparing genetic diversity, occurrence of private alleles in general leads to differentiation. Private alleles are alleles only represented by one region. Altogether, 23 private alleles (27 %) were found. However, almost all of the private alleles occurred at very low frequencies, suspecting them to occur as a consequence of stochastic sampling effects as sample size for Tyrol was less than half of the sample size for Styria.



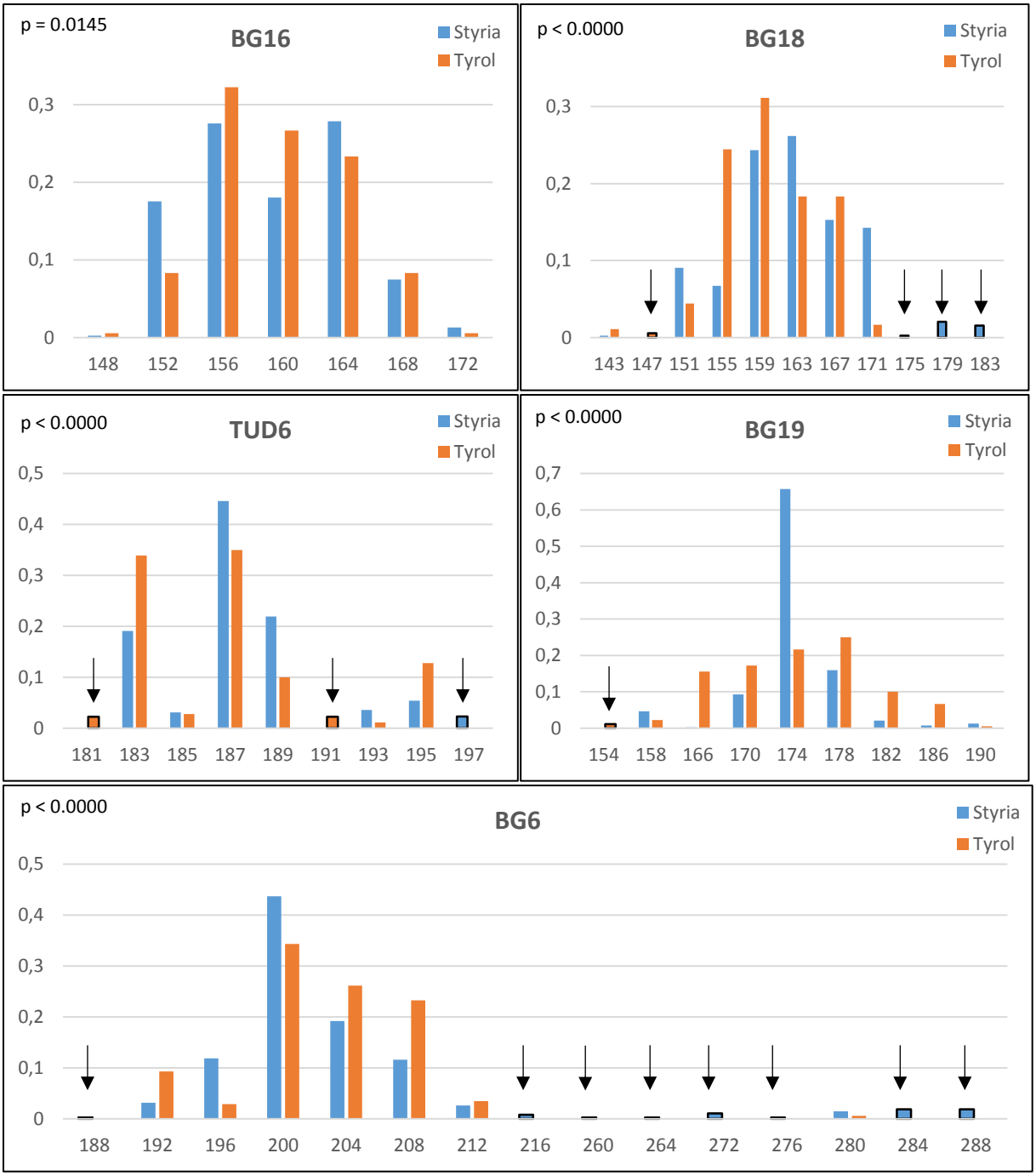


Fig. 4 Allele frequency distributions per locus compared for Styria and Tyrol. X-axes give the different alleles, y-axes give frequencies in percent (in different scales for improved perception). Alleles with no visible bars bear frequencies below 0.005 %, private alleles are bordered black and marked by an arrow. p-values are rounded to four digits

4.5. Genetic diversity in Styria and Tyrol per subpopulation

On a subpopulation level, an excess in observed heterozygosity was visible in Styria, however not significant (Table 4). The greater difference and consequently higher absolute value of the fixation index at Turrach (TUR) and Liezen Nord (LIN) was due to the small sample size of four respectively five. A bigger sample size generally resulted in higher numbers of alleles, with Tauern (TAU) having an average of 7.5 alleles per locus. Independent from sample size, the effective number of alleles averaged overall loci barley differed between Styrian subpopulations, indicating a resembling diversity in all subpopulations. All loci in all subpopulations except TUT2 in Zirbitz West (ZIW) were polymorphic.

In Tyrol, expected heterozygosity was higher than observed heterozygosity, as discussed above. Both appeared in similar patters as in Styria. The number of alleles corresponded best to the Tauern subpopulation (TAU), due to both subpopulations having high sample sizes. The effective number of alleles as well as allelic richness were a little higher than for Styrian subpopulations.

Table 4 Genetic summary statistics per subpopulation over all loci. Rounded to two decimals. Number of samples (N), number of alleles (A), effective number of alleles (A_E), allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}) and standard deviation (±)

	N	A	A_E	A_R	H_O	H_E	F_{IS}
Styria							
AUS	7	4.78	3.35	4.01	0.65 ± 0.09	0.62 ± 0.08	-0.06
LIN	5	3.89	2.87	3.72	0.69 ± 0.09	0.57 ± 0.07	-0.20
HSS	13	5.22	3.41	3.87	0.76 ± 0.05	0.68 ± 0.04	-0.12
HSW	13	5.22	3.63	3.97	0.68 ± 0.07	0.67 ± 0.06	-0.04
TAU	56	7.56	3.74	3.96	0.66 ± 0.06	0.68 ± 0.06	0.02
OSW	41	6.11	3.23	3.67	0.60 ± 0.07	0.64 ± 0.06	0.06
TUR	4	3.67	2.90	3.67	0.75 ± 0.09	0.62 ± 0.05	-0.21
ZIW	10	4.44	3.03	3.48	0.51 ± 0.13	0.54 ± 0.10	0.06
ZIO	7	4.67	3.33	4.00	0.60 ± 0.08	0.62 ± 0.07	0.02
GLS	23	5.78	3.46	3.82	0.62 ± 0.05	0.66 ± 0.05	0.06
KOR	15	4.67	2.87	3.39	0.61 ± 0.08	0.60 ± 0.06	-0.01
mean	17.5	5.09	3.26	3.78	0.65 ± 0.02	0.63 ± 0.02	-0.04
Tyrol							
TIR	94	7.45	4.01	4.02	0.64 ± 0.06	0.71 ± 0.06	0.09

4.6. Population structure of Styria and Tyrol separately

Population structure was first analyzed separately with an analysis of molecular variance (AMOVA), F-statistics as well as STRUCTURE analyses, in order to accomplish an insight into regional structure and distinguish from differentiation between the regions.

Analysis of molecular variance and F-statistics

In Styria, referring to the hierarchical analysis of molecular variance (AMOVA), 92.6 % ($p < 0.0000$) of the genetic variation was explained by differences within individuals and 4.1 % ($p = 0.0011$) among individuals of the same subpopulation. Only 3.3 % ($p < 0.0000$) of the total variance was attributable to differences between subpopulations, suggesting low population structure between subpopulations. Regarding the F statistics, the fixation index $F_{ST} = 0.0328$ and overall fixation index $F_{IT} = 0.0739$ showed similar results. Inbreeding coefficient $F_{IS} = 0.0426$, describing the mean reduction of heterozygosity of an individual due to non-random mating within subpopulations, resembled the separate values per population presented before and showed irrelevancy of inbreeding in Styria.

Tyrolean individuals were considered to represent one subpopulation, thus no hierarchical AMOVA could have been calculated as this analysis requires a pre-defined subdivision. Measurement of genetic variation between subpopulations (F_{ST}, F_{IT}) was also not possible. The inbreeding coefficient F_{IS} of 0.1068 corresponded to the Styrian value and denied inbreeding in the Tyrolean subpopulation.

STRUCTURE analysis

The STRUCTURE analysis for Styria without LOCPRIOR information resulted in no distinguishable metapopulation structure. The most probable number of clusters was K = 3. Based on genetic data only, no distinction of subpopulations was possible (Fig. 5).

Including LOCPRIOR information, Styrian individuals clustered most likely into K = 3 cluster as well, with results of K = 2 also possible, but differences between subpopulations became visible. The most eastern subpopulation Oststeiermark/Wechsel (OSW) was clearly separated. Also, the southern subpopulations Zirbitz Ost (ZIO), Gleinalm/Stubalm (GLS) and Koralm (KOR) represented a separable region displayed via the red cluster, with Zirbitz West (ZIW)

as an admixtured subpopulation and Turrach (TUR) belonging to Tauern (TAU). As outlier, Hochschwab West (HSW) seemed to differ slightly, indicating exchange with Oststeiermark/Wechsel (OSW). Comparable patterns were visible at Zirbitz West (ZIW), although no connection to Oststeiermark/Wechsel (OSW) was supposed.

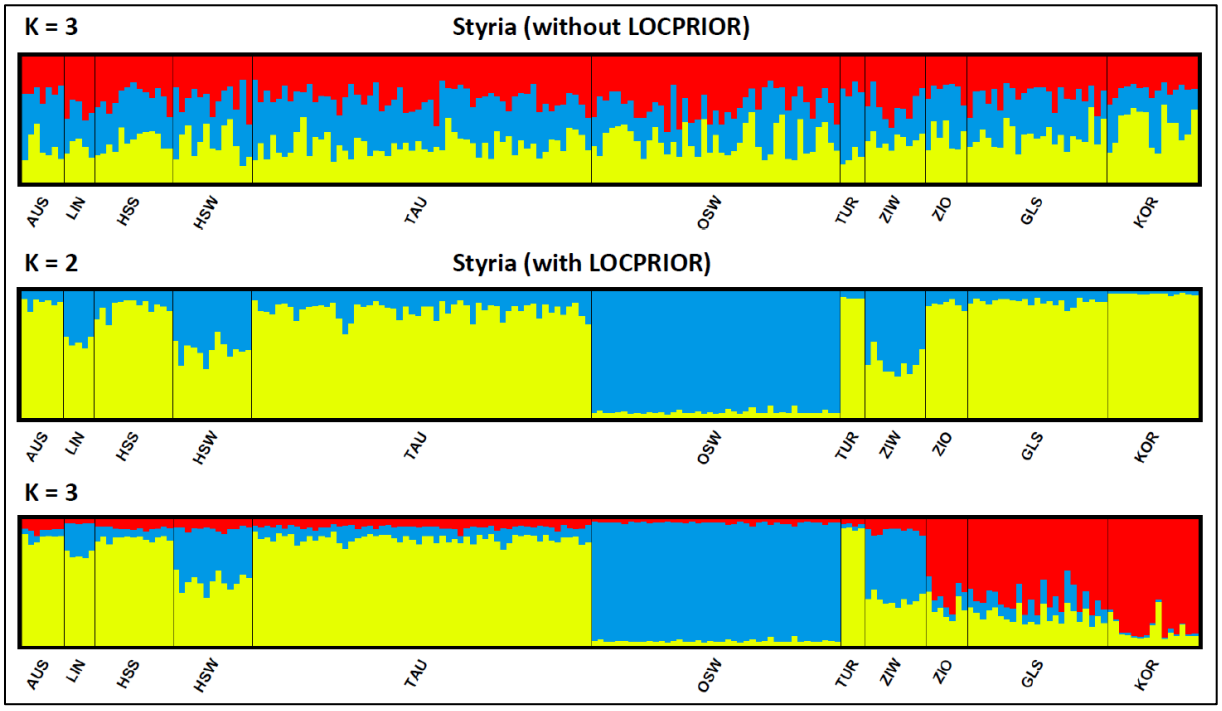


Fig. 5 STRUCTURE barplots for Styria. Without LOCPRIOR for K = 3 and with LOCPRIOR for K = 2 and K = 3

For Tyrol, STRUCTURE resulted in K = 3 as the most probable number of clusters with K = 2 also possible. Within Tyrol, no further distinction into subpopulations could have been drawn, supporting the precondition of treating Tyrolean data as one subpopulation (Fig. 6). Although, compared to Styrian results without LOCPRIOR, more differentiation was visible within Tyrol. Some individuals seemed to separate (Fig. 6, K = 3), but the differences were not significant enough to arrange those individuals into subpopulations regarding results from K = 2.

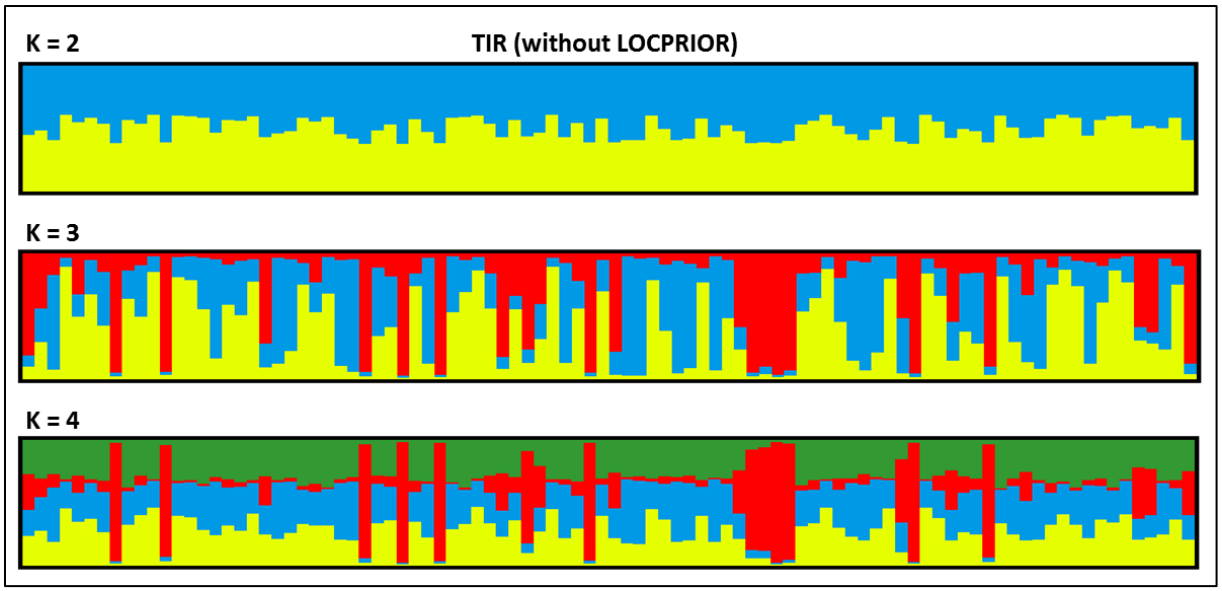


Fig. 6 STRUCTURE barplots for Tyrol for K = 2 to K = 4. Without LOCPRIOR information

4.7. Population structure of Styria and Tyrol combined

For the following analyses, Styrian and Tyrolean datasets were pooled and classified as regions, one hierarchy level above subpopulation level.

Analysis of molecular variance and F-statistics

According to the hierarchical analysis of molecular variance (AMOVA) with Tyrol and Styria classified as regions, 88.4 % ($p < 0.0000$) of total genetic variance was explained by differences within individuals and 6.1 % ($p < 0.0000$) among individuals within the same subpopulation. 3 % ($p < 0.0000$) of variance was based on differences among subpopulations in the same region and only 2.5 % ($p = 0.0941$) of total variance was explained by variation between regions, respectively Styria and Tyrol.

A hierarchical extension to the F statistics was computed via F_{SC} and F_{CT}. F_{SC}, describing the variance among subpopulations within regions, was 0.0308 while F_{CT} = 0.0255 described the variance among regions relative to the total variance. Together with the inbreeding coefficient F_{IS} = 0.0646 and the overall fixation index F_{IT} = 0.1165, F_{SC} showed similar results to the F_{ST} values calculated before for Styria and Tyrol separately. The F_{CT} value as an index of distance between the regions hinted to a possible distinction while still being relatively small.

Pairwise F_{ST} matrix

Pairwise F_{ST} values (Table 5) did not show a clear pattern of distinction between Styria and Tyrol based on absolute values. Regarding significant values, a differentiation within Styria was visible with Oststeiermark/Wechsel (OSW) as well as the southern subpopulations (ZIW, ZIO, GLS, KOR) being separated. Also Tyrol was differentiated, as nine of eleven comparisons showed significant F_{ST} values.

Table 5 Pairwise F_{ST} between subpopulations. Rounded to three decimals. Bold values are significant after correction for multiple testing (α = 0.00076)

	AUS	*LIN*	*HSS*	*HSW*	*TAU*	*OSW*	*TUR*	*ZIW*	*ZIO*	*GLS*	*KOR*
AUS											
LIN	0.015										
HSS	0.013	0.021									
HSW	0.023	0.037	0.014								
TAU	0.002	0.007	0.011	0.021							
OSW	0.042	0.03	**0.041**	**0.042**	**0.03**						
TUR	0.013	0.052	0.057	0.041	0.025	0.107					
ZIW	0.076	0.062	**0.097**	**0.071**	**0.047**	0.062	**0.141**				
ZIO	0.017	0.05	0.049	0.024	0.02	0.04	0.065	0.016			
GLS	0.011	0.039	0.012	0.012	0.012	**0.028**	0.06	**0.071**	0.003		
KOR	0.034	0.084	**0.056**	**0.059**	0.033	**0.076**	0.088	**0.111**	0.023	0.013	
TIR	**0.05**	0.053	**0.046**	**0.058**	**0.024**	**0.083**	0.044	**0.098**	**0.07**	**0.061**	**0.076**

STRUCTURE analysis

The STRUCTURE analysis without LOCPRIOR information showed the most probable number of clusters to be K = 2, same as for the analysis including LOCPRIOR information. Without LOCPRIOR information, it was not possible to distinguish between Styria and Tyrol properly (Fig. 7). There were individuals in every region showing a closer membership to the other region. Especially Tauern (TAU) was not classifiable to either belong to Styria or Tyrol as there were individuals assigned to Styrian (yellow) as well as Tyrolean (blue) cluster.

Including LOCPRIOR information for K = 2, a clear distinction between Styria and Tyrol in general was possible, especially Oststeiermark/Wechsel (OSW) as most eastern subpopulation was differentiated the most. The Tauern (TAU) subpopulation, situated in the west of Styria, partly shared the Tyrolean cluster. This was even more pronounced when a third cluster (Fig. 7, K = 3) was introduced. Tauern (TAU) and in

succession the north-western part of Styria (LIN, HSW, HSS) displayed a clear decline in shared cluster with Tyrol while the south-eastern subpopulations (ZIW, ZIO, GLS, KOR) were separated. Compared to the separate analysis of Styria, the differentiation within the region stayed the same with the minor difference of the northern subpopulations (TAU, HSW, HSS, LIN, AUS) also sharing a cluster (red) with the southwestern subpopulations. This pattern however was due to the limited number of clusters, when introducing a fourth cluster, differentiation within Styria stayed the same as in the separate analysis (data not shown).

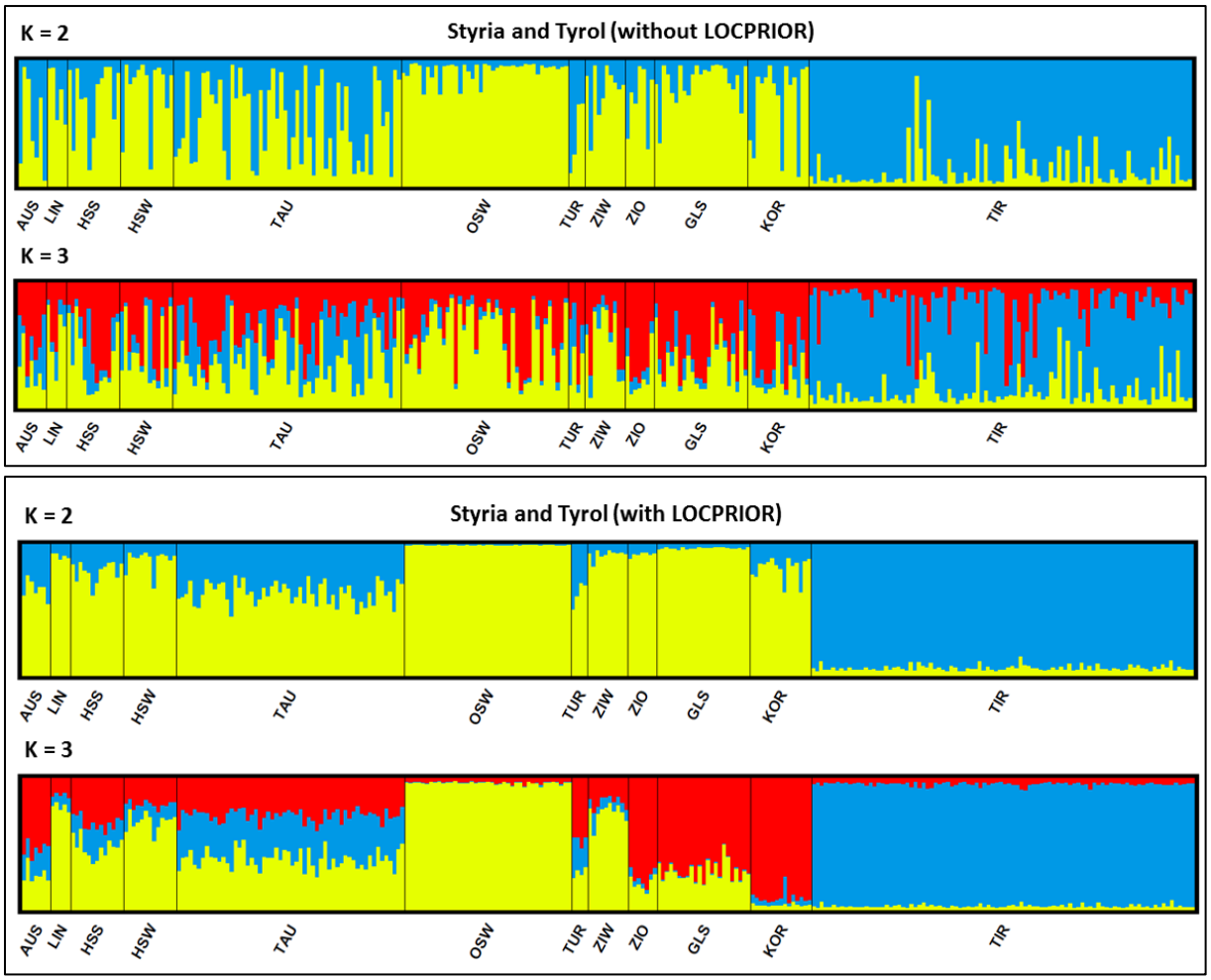


Fig. 7 Structure barplots for Styria and Tyrol combined. Without and with LOCPRIOR information for K = 2 and K = 3

Principal component analysis

The principal component analysis (PCA) resulted in 75 principal components, with little proportion of variance covered by the first 3 principal components, respectively 3.8 %, 3.2 % and 3.1 %. The first 22 principal components accounted for 50 %. The first three principal components were kept in the analysis as there was a significant decrease in eigenvalue visible. Covererd proportion of variance was low and therefore the power of interpretation of the PCA was reduced. Differentiation was almost not visible, as only principal component 1 was able to seperate subpopulations at all (Fig. 8). Tyrol was clearly differentiated from the most eastern subpopulation Oststeiermark/Wechsel (OSW), while overlapping with the other Styrian subpopulations. Therefore, no clear seperation between Styria and Tyrol was detectable. A gradient was visible from Tyrol into Styria up to Oststeiermark (OSW), corresonding to previously shown STRUCTURE results.

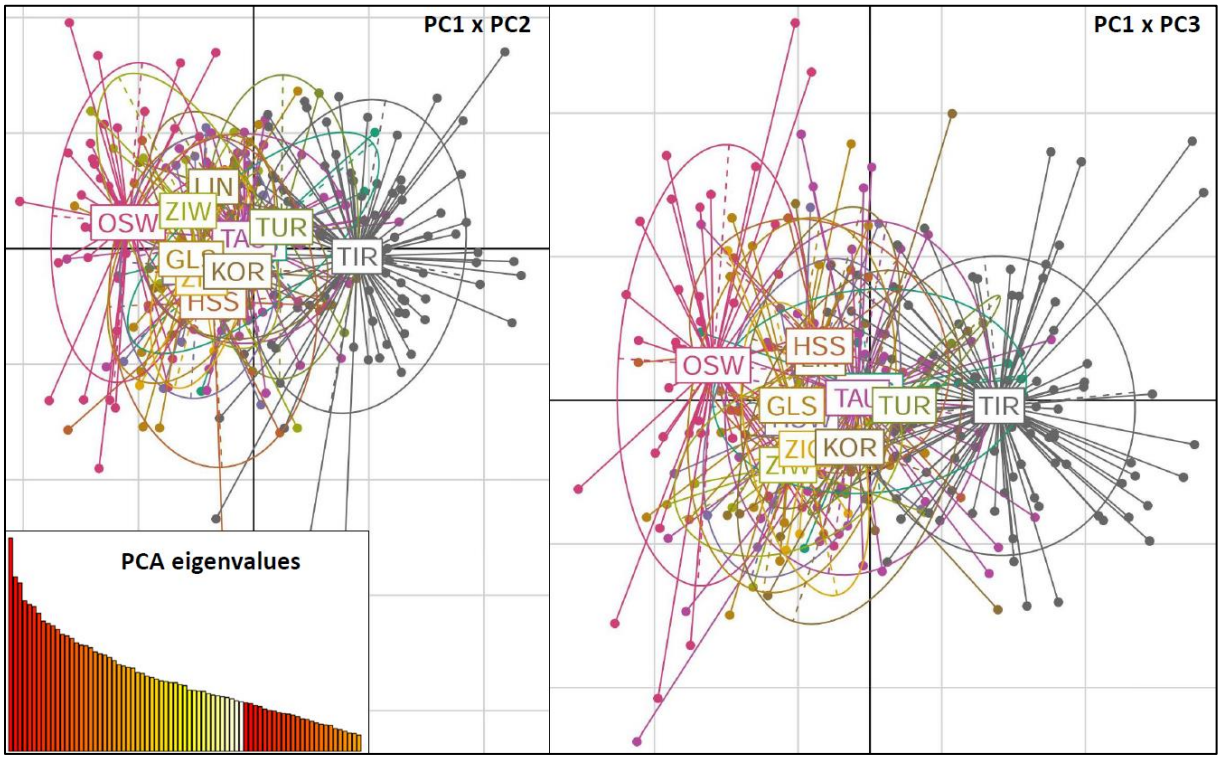


Fig. 8 Scatterplots for principal component analysis displaying the subpopulations, for PC1 against PC2 on the left and PC1 against PC3 on the right. PC1 is both times assigned to the x-axis. The inset displays the eigenvalues of the principal components

Discriminant analysis of principal components

Two discriminant analyses of principal components (DAPC) with different prior group membership assignments were calculated in order to complete the analysis of population structure.

The first DAPC was calculated based on group membership inferred with a K-means clustering approach implemented in adegenet (Jombart 2008; Jombart and Ahmed 2011). The most probable number of clusters, inferred from the lowest Bayesian Information Criterion (BIC), was K = 7. While a cluster (cluster 5) was clearly separated, all other clusters overlapped and no differentiation was possible. However, especially Tyrolean individuals were present in all clusters, with two clusters containing 29 (cluster 1) respectively 27 (cluster 5) individuals. Therefore, K-means clustering did not correspond to predefined subpopulations.

For the second DAPC calculated, the predefined subpopulations were used as groups. A differentiation based on the first principal component was visible between Styria and Tyrol, although both regions overlapped (Fig. 9). Results corresponded to the PCA results, with Oststeiermark/Wechsel (OSW) differentiated the most and Tauern (TAU) situated between all subpopulations. Looking at the assignment of individuals into groups by the DAPC, 93 Tyrolean samples were assigned correctly to the Tyrolean cluster. Additionally, four Styrian individuals from different subpopulations were assigned to the Tyrolean cluster as well, while within Styria, individuals were interchanged between subpopulations resulting in high assignment of individuals to Tauern (TAU).

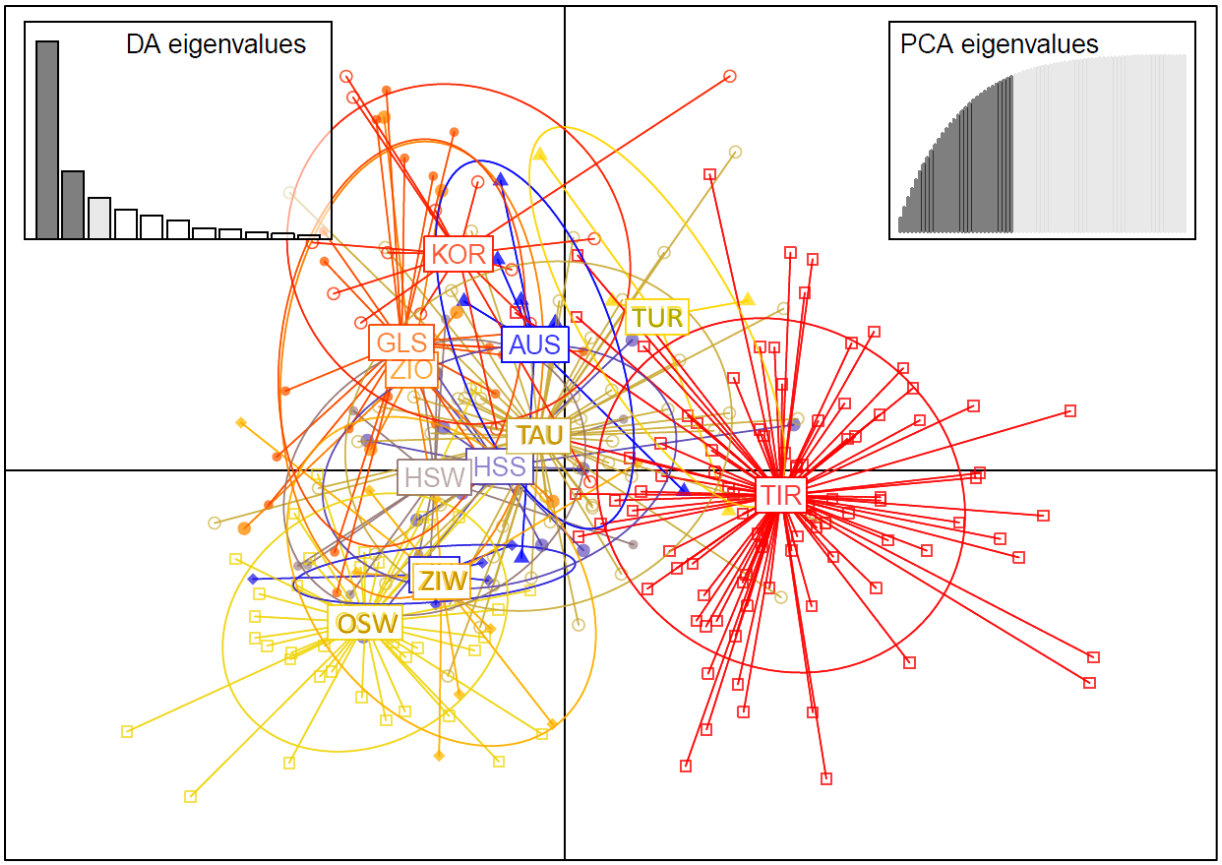


Fig. 9 Scatterplot for discriminant analysis of principal components based on the predefined subpopulations. Insets display the eigenvalues of the discriminant functions (DA) as well as of the principal component analysis (PCA). Discriminant function 1 is displayed on the x-axis

5. Discussion

Black grouse genetic diversity was studied with different approaches. First, genetic diversity was analyzed per region per locus, looking for differences and similarities. Explicitly addressing individual loci allowed for conclusions on a fine scale. However, as genetic diversity at subpopulation level is only accurately represented by averaging over various loci (Frankham et al. 2010), analysis was done additionally by comparing the individual subpopulations.

Afterwards, population structure was inferred from the pooled dataset in order to accomplish a comprehensive view over all study sites.

5.1. Genetic diversity of the regions

Comparing genetic diversity based on the standard summary statistics of the Styrian and the Tyrolean region per locus, only slight differences were detectable. Even a detailed approach looking at specific allelic frequency distributions yielded no differentiation despite of significantly different distributions. Significant results derived from certain alleles exaggerating in frequency compared to expectation under the assumption of independence, but the overall patterns per locus appeared to be similar. A higher genetic diversity was only visible for Tyrol at locus BG19, due to more uneven distributions of allele frequencies in Styria. For locus BG6, Styria showed more than twofold numbers of alleles than Tyrol. However, as these alleles all appeared at frequencies below 0.05 %, the differences were presumably the consequence of the different sample sizes. Apart from BG19, the standard summary statistics of genetic diversity corresponded well and loci specific patterns like low number and strong one-sided distribution of alleles at TUT2 became evident for both regions.

Private alleles, usually taken as reliable sign for differentiation, occurred in both regions. However, these only occurred at the extrema of allelic ranges with frequencies below 0.05 %. Consequently they only allowed for limited interpretation as they presumably derived from stochastic sampling effects as already mentioned for BG19. The only exception was allele 180 at BG15, occurring in the middle of the allelic range. Interestingly, allele 280 at BG6 was proven once in Tyrol, being separated by several mutational steps according to the stepwise mutation model (Ellegren 2004) from the next following allele. Again, as the missing alleles appeared at very low frequencies in

Styria, they could just happen to be missed due to random sampling and the smaller sample size for the Tyrolean region.

The slightly higher effective number of alleles in Tyrol compared to Styrian subpopulations presumably originated from different sampling regimes. While Styrian subpopulations were sampled based on well-known assumptions, Tyrolean sampling regime was not intentionally designed to correspond a predefined subpopulation. Thus individuals from more than one subpopulation or interchanging individuals were presumably included. This pattern also became evident in the separate Tyrolean STRUCTURE analysis, being more diverse than for Styria with a few individuals appearing further separated than the rest. Regarding allelic richness, genetic diversity again appeared identical.

5.2. Population structure of the regions combined

The hierarchical AMOVA showed only 2.5 % of total genetic variation being explained by differences between the two regions, which is less than the extent of variance (3.3 %) explained by differences between subpopulations in Styria only. Therefore, no significant differentiation between Styria and Tyrol based on regional differences could have been proven. Presumably, the AMOVA might have yielded a significant distinction based on differences between pooled Styrian and pooled Tyrolean subpopulations when including more than just one subpopulation for Tyrol. However, compared to other studies (Segelbacher et al. 2003a; Pavlovska 2012), the low F_{CT} value also indicates only small distinction.

F_{ST} values are commonly used to infer gene flow or barriers to gene flow between subpopulations (Holderegger and Segelbacher 2016). Theoretically, F_{ST} values reach from 0 (no differentiation) to 1 (complete differentiation, fixed alleles in subpopulations) and values of 0 to 0.05 are considered to depict little genetic differentiation, while values of 0.05 to 0.15 reflect moderate differentiation. However, as high polymorphism and the use of multilocus genotypes further decrease F_{ST} values, a value of 0.05 could already be interpreted as threshold for important genetic distinction (Balloux and Lugon-Moulin 2002; Bird et al. 2011). Consequently, F_{ST} values being statistically significant might be more meaningful indicators than absolute values (Balloux and Lugon-Moulin 2002). Addressing absolute F_{ST} values, no differentiation was visible. However, focusing on the significance of values yielded a separation within Styria. This

might be explained by the dispersal barrier Mur-Mürz valley and the geographical distance between northern and southern occurrences east of the valley, where no connection seems to exist. Tyrol is significantly different from almost all Styrian subpopulations while still showing relatively low F_{ST} values. Apparently, Tyrol is more differentiated than the subpopulations within Styria. Yet distinction between the two regions is minor compared to pairwise F_{ST} values of black grouse studied in Czech Republic (Svobodová et al. 2011) or Central Europe (Höglund et al. 2007; Segelbacher et al. 2014).

Solely based on genetic information, no clear distinction between Styria and Tyrol was possible using STRUCTURE as individuals of Styria as well as Tyrol showed membership of both clusters. Only by including LOCPRIOR information, STRUCTURE was able to separate Styria and Tyrol, with one cluster per region being most probable. This confirmed a low differentiation between the two regions, however admixture of the regions is visible. Results showed a membership of the westernmost Styrian subpopulation Tauern (TAU) to the Tyrolean cluster, which is even traceable in neighboring subpopulations This indicates a gradient of gene flow especially into northern Styrian subpopulations while the southern Styrian subpopulations appeared separated. Comparing the combined analysis to the distinctive STRUCTURE runs per region, conclusions stayed the same. Differentiation within Styria seems to be independent from Tyrol. The Tauern (TAU) subpopulation indicated exchange with other Styrian subpopulation while still partially sharing the Tyrolean cluster. It is therefore assumed to be a connecting subpopulation.

PCA is a method for addressing differences in genetic diversity between groups (Reich et al. 2008) and thus concluding on population structure. In this study, PCA did not yield any principal component with a high amount of covered variance displaying distinctive subpopulations, as it would have been expected in case of a high separation between the study regions (see Galbusera et al. 2004 for white-starred robin *Pogonocichla stellata* or Spurgin et al. 2014 for Berthelot's pipit *Anthus berthelotii*). Thus, only weak separation visible in principal component 1 fitted the overall result of weak separation between Styria and Tyrol. However, Jombart et al. (2009) emphasize not to discard interpretation of principal components with low proportion of variance, as those components might be biologically relevant as well. On a finer scale, principal

component 1 confirmed previous STRUCTURE results. A gradient from Tyrol into Styria was visible as the differentiation was shown to be weak and subpopulations overlapped. Again, the subpopulation Oststeiermark/Wechsel (OSW) appeared to be the most separated.

A PCA tries to reduce the dimensions of variation, but it does not differentiate variance between groups or subpopulations and variance within such groups (Jombart et al. 2010). Therefore, results of PCA bear some uncertainty as variation within groups can account for a high amount of explained variance of the principal components, especially when differentiation between groups is low. To account for this problem, a DAPC displays differentiation maximizing between group variance while minimizing within group variance (Jombart et al. 2010). The first DAPC was conducted without prior subpopulation definition, therefore a clustering step had to be done. However, cluster assignment of individuals did not represent the different subpopulations or regions and in consequence no differentiation between regions could have been confirmed with the DAPC (see Caizergues et al. 2003a for rock ptarmigan *Lagopus muta*). The second DAPC was calculated based on the predefined subpopulations. Here, the DAPC differentiated between Styria and Tyrol, although no clear distinction was visible. These results corresponded to the PCA, confirming the analysis despite the low explained variance. Oststeiermark/Wechsel (OSW) again appeared to be separated while the remaining Styrian subpopulations were not differentiated.

5.3. Gene flow across the Austrian Alps

Regarding genetic diversity and population structure, Austrian Alpine black grouse populations did not show any differences when compared with continuous Scandinavian populations (Höglund et al. 2007; Corrales and Höglund 2012). In contrast, isolated populations display a lower genetic diversity and higher differentiation between populations (Svobodová et al. 2011; Pavlovska 2012; Segelbacher et al. 2014). As a consequence, Austrian Alpine black grouse populations may not be best described as contiguous populations, as originally assumed from samples of the Southern Alps (Caizergues et al. 2003b). This confirms findings of Larsson et al. (2008), categorizing Austrian occurrences as continuous rather than contiguous alongside Norwegian populations.

However, a metapopulation system in the Austrian Alps is clearly visible (Sittenthaler et al. 2016, Sittenthaler et al. in prep.). Results of pairwise F_{ST}, PCA, DAPC and STRUCTURE analyses between Styria and Tyrol indicate gene flow (Segelbacher et al. 2003a; Frankham et al. 2010), although its extent and occurrence is unclear. However, as effects of fragmentation take some time to produce differences in genetic diversity or population structure, recent isolation processes may be undetectable yet (Höglund et al. 2011; Holderegger and Segelbacher 2016). Consequently no conclusion can be drawn on recent gene flow. Besides the time delay, a one-time analysis of genetic condition is not sufficient to conclude on already existing effects of fragmentation. Comparison with historical data, as done by Larsson et al. (2008), Pavlovska (2012) and Segelbacher et al. (2014) is necessary for a comprehensive understanding and forecasting (Höglund 2009). In accordance, results of this study can serve as a reference for future genetic monitoring, highlighting increasing or decreasing gene flow over the Austrian Alps.

In a species like the black grouse where males are philopatric, female dispersal is responsible for sustaining population connectivity (Lebigre et al. 2008; Corrales and Höglund 2012). Thereby, most migration probably occurs as natal dispersal (Lebigre et al. 2010). Sufficient gene flow might counteract genetic effects of metapopulation systems. Despite of recent fragmentation, metapopulation systems might appear as continuous populations (Frankham et al. 2010). Preservation of genetic diversity due to source-sink-systems within a metapopulation system has been observed for Alpine capercaillie (*Tetrao urogallus*) (Segelbacher et al. 2003b). Austrian Alpine capercaillie populations were assumed to form a metapopulation system such as contiguous populations of black grouse (Höglund et al. 2007), thereby reflecting genetic effects of fragmentation and isolation (Segelbacher and Storch 2002). However, Austrian Alpine black grouse yielded remarkably higher genetic diversity.

5.4. Implications for conservation

Analyses of Austrian Alpine black grouse populations indicate recent gene flow and consequently preserved genetic diversity and less population differentiation. However, black grouse seem to occur in a metapopulation system, which is especially prone to genetic consequences of increasing fragmentation (Frankham et al. 2010). Isolation of

subpopulations due to anthropogenic land use change can ultimately lead to extinction. This has been assumed as major threat for the species (Segelbacher et al. 2014), as observed for Central European populations in the past decades (Höglund et al. 2007). For the Alps, preserving and restoring connectivity and thereby enabling dispersal is crucial. So-called stepping stones, suitable habitats connecting distant black grouse occurrences, are of major importance and can counteract fragmentation even when distances between habitats exceed presumed dispersal distances (Höglund et al. 2003, 2007; Frankham et al. 2010; Corrales et al. 2014). Establishment and protection of both stepping stones and migration corridors, ensuring not only structural but also functional connectivity (Manel and Holderegger 2013), should thus be major target of black grouse conservation.

Evaluating population structure through the Austrian Alps, the Tauern (TAU) subpopulation seems to be substantial, connecting Tyrol with Styria as well as Styrian subpopulations within. Conservation measures need to concentrate on such subpopulations. Regarding differentiation within Styria, exterior subpopulations like Koralm (KOR) and Oststeiermark/Wechsel (OSW) show fragmentation on a low level and are therefore predisposed for further isolation and decline. Conservation measures as stated by Frankham et al. (2010) would be: (1) primary enlargement of habitat area, (2) improvement of habitat quality as well as (3) creating corridors and stepping stones. Furthermore, to support viable populations, enhance genetic diversity and inhibit inbreeding, translocation of bred individuals of captive black grouse has been recently discussed by Höglund (2009), Krzywinski et al. (2009) and Segelbacher et al. (2014). While being the only solution for already extinct subpopulations, reintroduction in general needs further discussions as it involves potential deleterious effects ultimately destroying local adaptations (Höglund 2009; Barbanera et al. 2010).

5.5. Potential of including additional genetic information

While neutral markers such as SSRs are useful tools to study genetic variation and derive conservation measures, genomic information might have further potential. Segelbacher and Höglund (2009) underline, that especially studying local adaption at selected loci and functionally important genes opens up a new perspective on conservation questions. Höglund (2009) emphasizes the selective markers power to

study the consequences of reduced major histocompatibility complex (MHC) gene variation in fitness. Also exploring quantitative trait loci (QTL) might enable additional perceptions onto genetic variability, with the power to detect local adaptations. This could complement and enhance conservation measures (Höglund et al. 2011).

5.6. Methodical considerations of calibration

While interlaboratory calibration of microsatellite data seems to be relatively easy, it often poses some difficulties (Moran et al. 2006; Ellis et al. 2011). These might result from: (1) the electrophoretic mobility depending on multiple factors (de Valk et al. 2009), (2) different laboratory equipment, (3) different chemicals and recipes or (4) even different methodical approaches being conducted. Various errors are possible and result in inconsistencies between laboratories (Ellis et al. 2011). These problems might be enhanced when using non-invasive sampled degraded DNA, originating from e.g. faeces, feathers, or hair.

The applied approach to calibrate datasets via re-analyzing predefined samples is an established procedure, but it is time consuming and costly as a representative number of samples needs to be included for every SSR locus to cover the known allelic range. Additionally, for non-invasively sampled DNA, replications or a multi-tubes approach is required to acquire the necessary certainty. Allelic ladders as described by de Valk et al. (2009) could provide alternatives for efficient interlaboratory calibration when amounts of DNA eluate are limited and needed for the research itself.

6. Conclusion

Austrian Alpine black grouse populations do not show genetic patterns of isolated populations but rather express genetic diversity and population structure comparable to continuous populations. However, Alpine black grouse occurrence is prone to a metapopulation system with migration barriers present. As historical data is lacking, no conclusions about trends of genetic diversity and population structure can be drawn. This study could be a reference for future research monitoring genetic diversity and population structure over the Austrian Alps.

As combining of datasets originating from two laboratories was successful, a basis for further genetic studies of Austrian black grouse is provided. Thereby, use of the same SSR loci, exchange of allelic ladders and in consequence establishment of a database of genotypes would be desirable and could enhance scientific cooperation as well as research quality.

Regarding conservation measures, preservation of connectivity is important to prevent depletion of genetic diversity and extinction events, especially in a metapopulation system. Conservation measures thereby need to be implemented on different spatial scales, addressing connectivity within a metapopulation system like Styria as well as between distant regions.

Gene flow is highly dependent on spatial factors. Functional connectivity of subpopulations is essential for individuals to migrate. As a further approach for inferring gene flow over the Austrian Alps, landscape genetic approaches incorporate population genetics and landscape ecology. Thus, assessment of landscape and environmental features affecting subpopulation connectivity is enabled (Segelbacher et al. 2010). As a consequences, a more detailed look onto connectivity between Austrian black grouse subpopulations would be possible.

On a larger scale, in a phylogeographical study using mitochondrial DNA, Corrales et al. (2014) found black grouse to be divided into a northern clade (Central Europe and the entire northern distribution) and a southern clade (including Italy, Switzerland and France). Corresponding patterns are assumed for capercaillie (Segelbacher and Piertney 2007), with a division between Iberian occurrences and European occurrences. The Alps are situated between those major distributions. While direct exchange of black grouse individuals between the distributions couldn’t have been

shown (Corrales et al. 2014), gene flow between both genetically distinct clades and the Alpine populations is likely. Caizergues et al. (2003b) assumed northern Alpine black grouse populations to be differentiated from southern Alpine populations. Thus, a subsequent study combining northern and southern Alpine populations could result in a comprehensive understanding of the Alpine black grouse populations and their functional role in black grouse conservation.

7. References

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