# Phenotypic and genetic variation in natural populations of *Festuca rubra s. l.* in Europe

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**Abstract**

***Background***: Phenotypic variation within species challenges the identification of meaningful taxonomic units and the quantification of evolutionarily relevant biodiversity.

***Aims***: We studied usefulness of the present taxonomic classification of species and subspecies within *Festuca rubra* complex.

***Methods***: We categorised *F. rubra s. l.* plants collected from natural populations across Europe such as the endemic species of *F. rothmaleri* and *F. rubra* subspecies (subsp. *rubra* and *arctica*) or between-subspecies hybrids (*arctica* × *rubra*). The plants were grown in a common garden, which was followed by an examination of variation in regard to 17 morphological traits, ploidy levels, nuclear ribosomal DNA, and chloroplast DNA.

***Results***: Phenotypic and cpDNA markers demonstrated stronger differentiation between geographic regions than between species or subspecies. Of the morphological traits, only lemma hairiness distinguished between *F. rubra* subspecies. Ploidy level varied within the *F. rothmaleri* and *F. rubra* subspecies. cpDNA and nrDNA markers showed no genetic differentiation among the *F. rubra* subspecies and their hybrids but clustered *F. rubra* taxa and *F. rothmaleri* as separate groups. Several additive polymorphic sites in nrDNA sequences indicated hybridisation in the *F. rubra* taxa.

***Conclusion***: Commonly used traits may not be reliable in determining evolutionary relevant taxonomic entities within *F. rubra* complex.

**Key words:** fungal endophytes, grasses, morphology, phenotypic plasticity, ploidy

**Introduction**

The taxonomic concepts of species, subspecies, and varieties have been used to describe morphologically distinct allopatric groups or incipient species (Mayr 1982). Furthermore, the subclasses of species have traditionally been determined based on morphological characteristics as well as additional information regarding the geographic location of the sampled individuals. However, estimating the importance and taxonomic relevance of extensive and often discontinuous phenotypic variation within species is a major challenge. Recently, molecular methods have revolutionarily broadened the ability to examine phylogenetic relationships. Notwithstanding, phenotypically distinct groups are still often defined as separate species, subspecies, or varieties belonging to a species complex of closely related taxa without information regarding any ecological or genetic differences detected from empirical eco-evolutionary studies (Wilson and Brown 1953; Mayr 1982; Mallet 2008, 2010; Mallet et al. 2015; Bastida et al. 2014, 2015; Dirihan et al. 2016; Shapiro et al. 2016). This is problematic especially because the taxonomic status is commonly used by non-taxonomists (e.g., in conservation legislation), and, as a result, the subspecies and variety concepts have sometimes been particularly criticised (Braby et al. 2012). On the other hand, these concepts enrich the understanding of speciation and biogeography, playing a critical role in certain management strategies to conserve and recognise the bio-economical potential of existing genetic resources (Turesson 1922ab; Clausen et al. 1941, 1942, 1947; Wilson and Brown 1953; Mayr 1982; Ryder 1986; Phillimore and Owens 2006; Ebach and Williams 2009; Patten 2009; Frankham et al. 2012; Kauppinen et al. 2016). However, in order to be evolutionarily meaningful, the taxonomic rank of subspecies should identify differences that are supported by genetic differences.

A largely overlooked challenge in taxonomic classification, especially in regard to plants, arises from phenotypic plasticity (Schlichting 1986; Coleman et al. 1994; Linhart and Grant 1996; Sultan 2000). Moreover, morphological characterisation is based on samples collected in the wild, in which genetically- and environmentally-based EPP induced effects on phenotype are

interconnected. Therefore, the environmental conditions of a sampling site have a large impact on morphology, and, thus, this study argues this can be key in identifying relevant taxonomic units for biodiversity and conservation purposes, particularly in the case of widely distributed species complexes. This study proposes that the establishment of a complete framework of phenotypic taxa obtained from quantifying a large number of traits preferably in a uniform environment might be essential in delimiting taxonomic ranks.

The taxonomic subdivision of species and species complexes into distinct, meaningful, evolutionary entities is further complicated by patterns of neutral genetic variation, for which the molecular-genetic techniques involved do not necessarily reveal the amount of genetic variability in the selected traits nor the evolutionary potential of the examined populations (Kirk and Freeland 2011). The examination of neutral genetic pattern variation has often challenged subspecies classification, e.g. in birds (Zink 2004), reptiles (Torstrom et al. 2014) and butterflies (Joyce et al. 2009). Furthermore, the genetic distinctiveness of subspecies may largely depend on their degree of geographic isolation and resulting genetic divergence, as continental subgroups have often been found genetically less distinct than their island-dwelling counterparts (Phillimore and Owens 2006). The absence of genetic differences in neutral genetic markers does not imply a lack of genetically-based, local adaptation, since heritable differences can arise as a result of natural selection in certain genomic areas (Zink 2004; Braby et al. 2012; Cheng et al. 2016). Nevertheless, studies on the genetic distinction between closely related taxa can help define evolutionary units of conservation, especially when combined with other types of evidence.

This study examines taxonomic classification by focusing on genetics-based morphological variation quantified in a uniform environment as well as the differences among both nuclear internal transcribed spacer (ITS) regions and neutral genetic markers (cpSSR) in the perennial grass *Festuca rubra* L. complex (Poaceae; Pooideae). The idea to examine taxonomic classification arose when *F. rubra sensu lato* plants were collected across Europe by Dirihan et al. (2016) in order to

study the adaptive evolution of the species; however, inconsistencies among the morphological characteristics challenged the ability to distinguish presumed species and subspecies in the wild.

This concept of taxonomic species in this study with regard to classification and speciation is based on the biological species concept (Mayr 1942); however, it is in agreement with the evolutionary species concept (Simpson 1951) (i.e. gradual evolution employing the Darwinian continuum above and below species level) (see e.g. Turesson 1922ab; de Queiroz 2007; Mallet 2008; Gourbière and Mallet 2009; Hausdorf 2011; Shapiro et al. 2016). Within this framework, to ensure the usefulness of the taxonomic subdivision of species and species complexes, it was determined that classification should be based on standardised, objective criteria in terms of recognised taxonomic ranks as well as provide insight into the speciation process.

Thus, *F. rubra* was selected as a model for these eco-evolutionary studies, since members of the genus *Festuca* are globally distributed, taxonomically and phenotypically variable, and display potential in regard to their rapid, adaptive evolution to new or changing environmental conditions (Cheng et al. 2016). In addition, many species of *Festuca* are of great agronomic importance, as grasses are widespread inhabitants of various ecosystems. Indeed, 70% of agricultural regions in the world are comprised of grasslands, with more than half of dietary proteins derived from grasses (Soussana et al. 2013; Kauppinen et al. 2016). *Festuca* is widely distributed throughout arctic, boreal, and temperate zones, with diversity hotspots in eastern Asia, Europe, South America, and North America, containing between 400 and 500 species (Stančík and Peterson 2007). The genus is closely related to *Lolium*, and evidence from phylogenetic studies show that this genus is not monophyletic (Inda et al. 2008).

*Festuca rubra* is a morphologically variable species complex exhibiting extensive hybridisation, polyploidy, and variation in the presence of the seed-transmitted endophytic fungus *Epichloë festucae* Leuchtmann, Schardl & Siegel (Jenkin 1955; Ainscough et al. 1986; Catalán 2006; Dirihan et al. 2016). In general, similar to the taxonomy of the genus *Festuca*, the taxonomy of the *F. rubra* complex has been controversial (see e.g. Catalán et al. 2004, 2007; Soreng et al.

2015; Cheng et al. 2016; The International Plant Names Index (IPNI); Tropicos.org). The species complex has a broad circumarctic-circumboreal distribution and a complex evolutionary history, partially due to glaciations that have repeatedly resulted in the isolation of *F. rubra* populations (Aiken and Fedak 1992; Inda et al. 2008). During temperate periods, these isolated refugial populations migrated northwards as the glaciers retreated, producing today's overlapping distributions and extensive morphological variation (Markgraf-Dannenberg 1980).

Descriptions of taxa included in the *F. rubra* complex have largely been based on its morphological characteristics, especially its leaves and inflorescence (Markgraf-Dannenberg 1980). Two main subspecies can be found in Northern Europe: plants with glabrous lemma are described as subsp. *rubra* and plants with hairy lemma as subsp. *arctica* (Hack.) Govor. Species *F. rothmaleri* (Litard.) Markgr.-Dann. (*F. rubra* L. subsp. *rubra* var. *rothmaleri* Litard; *Festuca rubra* var. *rothmaleri* Litard.) is commonly found in southern Europe (de la Fuente Garcia and Sánchez Mata 1987). The species is thought to be endemic to the western Iberian Peninsula, commonly appearing at high elevations in Spain. It is identified mainly by the size and morphology of its panicles, glabrous ovary, and the anatomy of its leaf blades. Historically, it was first described as a variety, but, at present, it is accepted as a full species that belongs to the *F. rubra* complex (Al-Bermani et al. 1992). Many other subspecies of the *F. rubra* complex have been identified by other morphological traits, such as stolon length (very long in subsp. *arenaria*), plant height (below 45 cm for subsp. *litoralis*), and leaf scabridity (strongly scabrid in subsp. *asperifolia*) (Markgraf-Dannenberg 1980).

To understand the genetic relationships within the *F. rubra* complex, the patterns of genetic variation were examined using biparentally inherited nuclear ribosomal DNA (nrDNA) and maternally inherited haploid chloroplast DNA markers (cpDNA). NrDNA sequences have been commonly used for phylogeny, whereas cpDNA markers have been used extensively as effective tools for evolutionary studies of plants (Jarne and Lagoda 1996; Provan et al. 2001; Inda et al. 2008; Ebert and Peakall 2009; Cheng et al. 2016).

In addition to genetics-based morphological variation and genetic differentiation, ploidy levels of the plants were determined. Polyploidy is common in plants and is thought to promote speciation (Lewis 1979; Otto and Whitton 2000; Comai 2005) and affect size-related morphological characters (Balao et al. 2011). Moreover, polyploidy is assumed to shape phenotypic diversity and geographic distribution as well as division within the *F. rubra* complex, since the fitness of different polyploid cytotypes may vary with environmental conditions (van de Peer et al. 2017). Thus, chromosome number has often been cited as an important taxonomic trait for certain species and subspecies.

As in many other grasses, the haploid chromosome number in the *F. rubra* complex is n = 7, with natural populations consisting of polyploid (tetra-, hexa-, octo-, and decaploid) individuals (Moore 1982; Stace 1989; Catalan et al. 2007; Diaz-Perez et al. 2008; Dirihan et al. 2016). *F. rubra* subsp. *rubra* and *arctica* have been reported to be hexaploids (Tropicos.org 2015, Markgraf-Dannenberg 1980), while *F. rothmaleri* has been reported to be a hexa-, tetra-, and octoploid (Al-Bermani et al. 1992; de la Fuente et al. 2001; Loureiro et al. 2007).

The adaptive evolution of *Festuca* is often assumed to be promoted by systemic *Epichloë* endophytes that form life-long symbioses with their host grasses (Saikkonen et al. 2002; Dirihan et al. 2016). In the symbiosis, the fungus grows intercellularly and asymptomatically in above-ground grass tissues. The growth of the fungus into developing seeds allows for its vertical transmission into all the host's offspring. Furthermore, an increasing body of literature suggests that ecologically and evolutionary relevant plant traits are often modulated by coevolving microbial species, and phenotypic selection often regards coevolving species as a single phenotypic unit (Saikkonen et al. 2004; Tripp et al. 2017). Thus, coevolving microbes can affect the adaptive radiation of the host plant species. *Epichloë* - grass symbiosis is commonly assumed to be mutualistic: while the endophyte benefits from receiving shelter, nutrients, and dispersal from plants, the endophyte increases plant resistance against various abiotic and biotic stressors such as drought, flooding, herbivores, and pathogens (Wilson 1995; Cheplick and Faeth 2009; Saikkonen et al. 2006, 2010a,

2016). Similar to many other biotic interactions, *Epichloë* - host grass interactions are labile and context-dependent, ranging from antagonistic to mutualistic (Ahlholm et al. 2002; Faeth 2002; Saikkonen et al. 2004, 2010b). As the fitness of the endophyte is highly dependent on the fitness of the host grass, it has been suggested that the fungus is then able to retain control over both the functions of the host grass and the morphological traits that benefit the endophytic fungus (Cheplick and Faeth 2009). *F. rubra*, is commonly symbiotic with *E. festucae*; however, the frequencies of endophyte symbiotic plant individuals vary within and among populations and geographic regions (Saikkonen et al. 2000; Wäli et al. 2007; Dirihan et al. 2016).

This study was conducted as part of a research project focusing on the eco-evolutionary dynamics of *F. rubra* and its symbiotic fungal endophyte, *E. festucae*, across a broad geographic scale within Europe. It used field-collected *F. rubra sensu lato* plants to examine the usefulness of the present taxonomical classification of species and subspecies within the morphologically variable species complex. The presented hypotheses were that: (1) morphological characteristics measured in a uniform environment and ploidy level variation correlate with the currently implemented taxonomic species and subspecies classification, (2) other examined factors, such as geographic origin or endophyte status, partly explain the observed genetically-based differences in morphology, and (3) genetic analyses would support the taxonomic classification of the species complex.

## Material and Methods

### *Plant material*

Flowering individuals of *F. rubra s. l.* were collected from natural populations in Spain, Switzerland, southern and northern Finland, the Faroe Islands, Iceland, and Greenland in 2011 and 2012 (documented in Dirihan et al. 2016; Table 1). The plants were sampled at each site by extracting the soil core surrounding the roots, which was then transported in plastic bags to a greenhouse in the Botanical Garden of Turku University. The plants were planted in 250 ml pots

filled with a mix of peat and sand (without disturbing the original soil core), grown over several tillering cycles in an ambient temperature, and photoperiod in the greenhouse.

*Identification of species, subspecies, and between-subspecies hybrids*

After transportation to the greenhouse, the field-collected plants were identified as *F. rubra* subsp. *rubra*, subsp. *arctica*, *arctica* × *rubra* hybrids, and *F. rothmaleri*. The number of plants representing each species and subgroup within the population samples from each geographic region is shown in Table 2.

Ploidy level (2*n* = 28, 3*n* = 42, 4*n* = 56) was determined via flow cytometry as described by Dirihan et al. (2016), specifically using *F. rubra* individuals with known ploidy levels for calibration. Additionally, one to three plants were randomly chosen from each population and cytotyped microscopically. The fungal endophyte (*Epichloë festucae*) status of all plants was determined by monitoring the systemic hyphal growth of three surface-sterilised leaf sheaths from each of the plants in Petri dishes containing 5% potato dextrose agar (Dirihan et al. 2014, 2016). The leaf sheaths were surface-sterilised via incubation for one minute in 90% ethanol, four minutes in 4% sodium hypochlorite, and 30 seconds in 90% ethanol (Dirihan et al. 2014, 2016). Endophyte status was also verified by microscopically examining the stained seeds (Saha et al. 1988).

*Common garden experiment and morphological measurements*

After growing in the greenhouse, the plants were split and vegetative clones were planted in the common garden in two experimental fields based on a fully randomised design at the Ruissalo Botanical Garden, Turku, Finland (60°26’N, 22°10’E). The experimental fields were located ca. 150 m apart and were fenced in order to prevent the access of large vertebrate grazers. For quantifying the morphological differentiation, the common garden experiment included a total of 239 plants, of which 17 morphological characters (Table 3) that have been used throughout the taxonomic literature of *F. rubra* L. (e.g., Markgraf-Dannenberg 1980; Dubé and Morisset 1987; Aiken et al. 2007) were recorded. Samples for the morphological measurements were collected

from flowering plants in 2015. Additionally, micromorphological traits were measured from scaled images obtained from NIS Element Version 3 SP 7 with a microscope (Nikon SMZ745T) using the Image J –program (version 1.42, National Institute of Mental Health, Bethesda, MD, USA).

*Genetic analysis*

We first examined if the presupposed species and subspecies differed based on nrDNA ITS sequence data. The ITS region has been traditionally employed in phylogenetic analysis (Hsiao et al. 1995; López et al. 2018), but the biparentally inherited ITS region is also used to investigate hybridisation processes occurring between related taxa, since polyploid species and hybrids are prone to the presence of additive polymorphic sites (i.e., both parental ribotypes present) in sequence chromatograms (Aguilar and Feliner 2003; Segarra-Moragues et al. 2007).

Fifteen representatives of the *F. rubra* complex were investigated to identify variability in the ITS region (ITS1 spacer - 5.8S gene - ITS2 spacer). The data included the complete ITS sequences of four *F. rubra* subsp. *rubra*, four *F. rubra* subsp. *arctica*, three *rubra* x *arctica* and four *F. rothmaleri* individuals (Table 1 and 2; GenBank accession numbers and further information regarding the samples are provided in Table S1). Genomic DNA was extracted from fresh leaves of the plant samples according to the methods described by von Cräutlein et al. (2014), which was based on the PCR procedures used in the studies by Torrecilla and Catalan (2002) and Torrecilla et al. (2004). The PCR amplifications produced a band of ca. 630 bp corresponding to the *Festuca* ITS DNA. The bands were separated in 1% agarose gel, excised from the gel following electrophoresis, and purified with a Nucleospin®Gel and PCR Clean-up kit (Macherey-Nagel). The purified PCR-products were submitted to Macrogen Inc. (Seoul, South Korea) for sequencing. Both upstream and downstream primers were used for sequencing to minimise the inclusion of incorrect readings as polymorphisms. The ITS sequences were initially visualised and manually corrected from both forward and reverse sequencing directions using Chromas version 2.6.5. (2018) and aligned with ClustalW, which was followed by manual adjustment using MEGA version 10.0.5 (Kumar et al. 2018). The additive nucleotide polymorphisms of the ITS sequences were manually determined and

coded using IUPAC nucleotide ambiguity codes. A site was regarded as polymorphic when double peaks were present in the electrophenogram in the same position on both the direct and reverse strands.

The study additionally examined whether the studied taxa could be distinguished based on the maternal inheritance of cpDNA (Jarne and Lagoda, 1996; Diekmann et al. 2012). The recent extensive survey on the postglacial colonisation history of the *F. rubra* complex in Europe shows that chloroplast microsatellite (cpSSR) markers are effective tools for determining the genetic structure of natural populations of *F. rubra* (von Cräutlein et al. 2019). Highly variable cpSSR markers were used following von Cräutlein et al. (2014, 2019) and included 136 plant samples (n = 19, 60, 30 and 21 for *F. rothmaleri*, *F.rubra* subsp. *arctica*, *F. rubra* subsp. *rubra* and *rubra* × *arctica* hybrids, respectively) (Tables 1 and 2).

*Statistical methods*

The study first examined whether the species, subspecies, and between-subspecies hybrids could be distinguished from each other based on various morphological traits important to taxonomic identification within the *Festuca* genus. Whether or not the different subspecies differed based on the continuous morphological traits presented in Table 3 was then tested via a principal component analysis using the *prcomp* function, with results plotted using the 'ggfortify' package in R 3.3.2 (R Core Team 2016).

Likelihood ratio tests between the linear models in R were then used to test for significant differences in morphology among the species, subspecies, geographic origin, included ploidy level, and endophyte status in all models. Since not all the subspecies were distributed across all geographic areas, subgroups and geographic origin were analysed in separate models. The significance of the morphological differences between the ploidy levels and endophyte statuses were estimated from models with geographic origin. The normality of the residuals and the homoscedasticity requirement were inspected visually using the plot function on the full model (trait ~ subspecies or geographic origin + ploidy level + endophyte status). All explanatory

variables were treated as fixed factors. The likelihood ratio tests were performed by comparing a model in which the factor of interest was moved to the full model. To avoid false positives as a result of repeated testing and between-trait correlation, a Bonferroni-corrected threshold for P values (alpha = 0.0029; 0.05/17 traits) was used. The significance of the categorical variables between the species and subspecies or the geographic origin was tested with an approximative (Monte Carlo) Pearson chi-squared test using the *chisq test* function in the R package 'coin'. The significant differences among the species and subspecies, geographic origin, and ploidy levels were tested using non-parametric Kruskal-Wallis tests as well as the Mann Whitney U test for endophyte status that employs the *kruskal.test* function in R.

Since hybridisation and reticulation are likely to occur within the *F. rubra* complex, evolutionary relationships were visualized based on the 15 ITS sequences with NeighborNet analyses using SplitsTree4 4.14.8 (Huson and Bryant 2006), applying uncorrected P distances and ambiguities handled as average. Bootstrap support values for infernal splits were calculated with 1000 replicates.

To examine the genetic structure of the *F. rubra* complex based on the cpSSR data set, a model-based approach was used in the Bayesian Analysis of Population Structure (BAPS) version 6.0 (Corander et al. 2013), clustering with linked loci options and in accordance with the methods described by von Cräutlein et al. (2019). BAPS identified that the optimal division of populations into clusters occurs with 11 clusters (the highest marginal log-likelihood value = -1092.87) combined with several small clusters (6 clusters with 1-5 individuals each). Therefore, a UPGMA tree based on the Kullback-Leibler divergence matrix was compiled to visualize the uppermost hierarchical levels of the genetic structure (Figure S1; Table S2). Based on this, a fixed K model with 200 iterations for six clusters (K=6 ) was used (see also, von Cräutlein et al. 2019). Moreover, the basic statistics of the 13 chloroplast microsatellite loci, including the number of alleles (Na), the number of effective alleles (Ne), the information index (I), and the unbiased haploid diversity (uh)

for the *F. rubra* subspecies *rubra*, *arctica*, their hybrids, and *F. rothmaleri* were computed using GenAlEx 6.5 software (Peakall and Smouse 2006, 2012) (Supplementary Table S3).

## Results

### *Morphological differentiation*

The species and subspecies were not observed to be morphologically distinct based on the multivariate PCA analysis of continuous traits within the common garden (Figure 1). Greater variation was observed to occur within than among the populations (Figure 2; Table S4). The first and second principal component explained 37% and 18% of the multi-trait variation, respectively.

When separately comparing each trait in the common garden, there were, in general, more morphological differences between plants of different geographic origins than between presupposed species and subspecies (Table 4, Figures 1 and 2). Some traits were significantly different among the various species and subspecies prior to correction for multiple testing (Table 5). The plants categorised as *F. rothmaleri* were the tallest and displayed the longest inflorescences (Table 4). Analysis of the discrete traits revealed significant differences among species and subspecies in terms of traits related to hairiness, plant colour, and number of leaf veins (Table 6). Only lemma hairiness (the main trait used to distinguish between subsp. *rubra* and *arctica*) separated the subspecies within the common garden environment (Figure 3).

All continuous traits except lemma length were found to be significantly different between plants of varying geographic origins after the Bonferroni correction for multiple testing (Table 5). In general, the plants from northern regions were shorter than their southern counterparts (Table 4). For example, the plants from Switzerland displayed very short stolons and long awns, whereas the plants from the Faroe Islands exhibited broader leaves (Table 4). Moreover, the plants showed significant differences in all categorical traits between geographic regions except for number of flowers in a spikelet; however, variation within regions was also observed in regard to most traits (Figure 3).

Plants with different ploidy levels differed in lemma hairiness and number of leaf veins. No significant differences following the Bonferroni correction were found between plants with or without the symbiotic endophyte (Table 5 and 6).

*Species, subspecies, and ploidy level variation*

Three different ploidy levels (tetra-, hexa- and octoploid) were found in the data set. All three ploidy levels were present in the plants presumed to represent *F. rothmaleri* and the subspecies *arctica* (Table 7), indicating that ploidy level is not a suitable trait for taxonomic delimitation in this species complex. Furthermore, all plants of subsp. *rubra* were found to be hexaploids (Table 7). Most of the plants categorised as hybrids between subsp. *rubra* and *arctica* were also hexaploids, but some were tetraploids (Table 7).

*Molecular evolutionary analysis based on its sequences*

In total, nine out of 598 nucleotide positions were polymorphic in the ITS sequences. Two and six sites were polymorphic and additive in *F. rothmaleri* and *F. rubra* taxa, respectively (Figure 4). The NeighborNet analysis of the ITS sequences indicated a clear separation of *F. rothmaleri* and *F. rubra* subspecies and their hybrids (bootstrap support value 95.4; Figure 4). Both clusters were well supported but differed in their internal variability. All four *F. rothmaleri* individuals represented different ribotypes, while seven out of 11 individuals represented different ribotypes among *F. rubra* taxa. The subspecies *arctica* and *rubra* and their hybrid *rubra* × *arctica* share similar ribotypes and clusters without the appearance of any subspecies-based pattern.

*Patterns of neutral genetic differentiation*

The characteristics of the 13 chloroplast microsatellite loci in the *F. rubra* subspecies *rubra* and *arctica* and their hybrids and *F. rothmaleri* are shown in Table S3. The uppermost hierarchical level of the genetic structure with the fixed K model and six clusters (K=6, the highest marginal log-likelihood value = -1145.6) shows that the majority of the individuals were distributed into large,

uniform genetic clusters in three main geographical regions, including the Northern Atlantic, Fennoscandia, and certain Spanish regions, with individuals from two small clusters also present in the northern Atlantic and Fennoscandia regions (Figure 5, Table S2, see also von Cräutlein et al. 2019).

The subspecies *arctica* and *rubra* and their hybrid *rubra* × *arctica* shared genetic clusters without exhibiting any subspecies-based pattern (Figure 5, Table S2). Cluster 1 (n=28) formed a genetically unique, large group in the wide Northern Atlantic region (Faroe Islands, Iceland, Greenland), including samples of species/subspecies *arctica*, *rubra,* and their hybrids, which appeared to be formed as either *F. rubra* ssp. *rubra* or *F. rubra* ssp. *arctica* as a seed parent. Similarly, cluster 6 (n=74) formed genetically unique groups in southern and northern Finland, which included samples of subspecies *arctica*, *rubra,* and their hybrids. Furthermore, two small clusters (2: n=4; 3: n=6) were determined as having mainly northern Finland and Faroe Islands origins without differentiation based on subspecies.

The individuals from Spain identified as *F. rothmaleri* were distributed in two different clusters: 4 (n=10) and 5 (n=14). Individuals within clusters 4 and 5 also occurred in northern populations with differing taxonomic status. Cluster 5 shared two individuals with the Faroe Islands identified as different subspecies (subsp. *rubra* and subsp. *arctica*) as well as an individual of subsp. *rubra* from Southern Finland, whereas cluster 4 shared two individuals identified as subsp. *arctica* from Southern Finland (Figure 5, Table S2).

## Discussion

This large-scale comparison demonstrated a substantial morphological and genetic variation occur within the *F. rubra* complex; however, the patterns of morphological differentiation and cpDNA variation showed more distinct differences among geographic regions than among presupposed taxonomic subgroups. Moreover, nuclear rDNA showed close relatedness mainly for *F. rubra* taxa

in different geographic regions but also for *F. rothmaleri* and *F. rubra* taxa due to the low number of substitutions between the taxa. However, the genetic analysis based on the cpDNA and nrDNA markers distinguished *F. rothmaleri* as separate clusters compared to *F. rubra* taxa. The presupposed species, *F. rothmaleri*, was found only in Spain, and when grown in a uniform common garden location and based on analyses of continuous morphological traits, the species did not differ from the *F. rubra* subspecies expect in terms of plant height and inflorescence length. In the case of *F. rubra*, only one (lemma hairiness) out of 17 morphological traits supported the subspecies delimitation. Based on these findings, the taxonomic status of the *F. rubra* subspecies (subsp. *rubra* and *arctica*) or between-subspecies hybrids (*arctica* × *rubra*) is questionable, since the patterns of variation did not correlate with the subspecies classification. Similar findings have been previously reported in comparative studies regarding morphological and genetic variation between subspecies in other taxa (Braby et al. 2012).

Differences in categorical characteristics are commonly thought to provide reliable diagnostic features used for taxonomic identification, but these results suggest that most of the categorical variables are better explained by the geographic origin of the plant rather than its taxonomic differentiation. It was found that the variation in lemma hairiness used to distinguish between the subspecies *rubra* and *arctica* was also present in the common garden, implying that this taxonomic trait has a genetic basis and is not a phenotype induced in natural environments.

In contrast to previous taxonomic classifications, the species and subspecies were not distinguished by different ploidy levels in this study. The co-occurrence of several ploidy levels within presupposed taxa suggests that the taxonomic rank of commonly adopted subgroups fails to capture the breadth of variability inherent in wild *F. rubra* populations. A recent study by von Cräutlein et al. (2019) have also suggested that with more extensive sampling of the same populations, they found that plants of different ploidy levels can be closely genetically related within different geographic areas, indicating the independent formation of polyploids within different maternal lineages. Since differences in ploidy level could also act as a barrier to sexual

reproduction, these results call for controlled crossing experiments evaluating possible hybridization barriers within the *F. rubra* complex.

*Phenotypic variation*

Even though morphological variation in the *F. rubra* complex does not clearly differ between species and subspecies, it does suggest certain evolutionary patterns for plants originating from different geographic regions. Phenotypic variation observed under common garden conditions results from both local adaptation as well as demographic history. Moreover, patterns of natural selection are likely to differ between geographic origins of the sampled locations, and, thus, differences in selection pressures can arise (e.g., adaptation to growing season timing, moisture availability, intensity of solar radiation, and grazing). In fact, multiple independent and discontinuous selection forces are likely to operate simultaneously on several plant traits (Wiens 1976).

Differences in size within the common garden have often resulted from differences in growth rates, which can be slower for plants originating in harsh environments in locations such as Greenland, the Faroe Islands, and Iceland. In addition, plasticity in the form of clonal growth may facilitate the establishment of plant genotypes within a patchy habitat. A tufted bunch of vegetative propagules enables plants to efficiently compete for resources, while long stolons facilitate foraging for new resource-rich patches. We acknowledge that plant size can reflect patterns of local adaptation and that overall stress levels involved in the growth environment at the common garden planting site can differ between plants from different geographic origins, as recently suggested by Leinonen et al. (2019). In a four-way, multi-year reciprocal transplant experiment with *F. rubra* from northern and southern Finland, the Faroe Islands, and Spain, which was also partly used in this study, they found higher survival rates for plants of local geographic origin compared to nonlocals at three of the four sites. Similarly, plant growth showed evidence of local adaptation, but, in general, the aboveground biomass production of all plants was substantially higher in Spain (Leinonen et al. 2019).

Plant hairs have been found to protect against various stresses, such as UV radiation (Sun et al. 2014) and herbivores, but their production can be associated with a fitness cost (Sletvold et al. 2010). Hairiness of plant inflorescence parts can protect plants from seed predation (War et al. 2012) or facilitate seed dispersal via animals (Howe and Smallwood 1982; Cheplick 1998). Additionally, awn length can mirror the soil type, since awns facilitate seed penetration into the soil and promote seed germination success, especially in dry habitats (Garnier and Dajoz 2001; Elbaum et al. 2007).

Similarly to hairiness, a dark greyish or bluish colour may indicate thicker cuticle anthocyanin production and can contribute to UV light tolerance in plants (Winkel-Shirley 2001; Ramakrishna and Ravishankar 2011; Welch et al. 2008; Markovskaya et al. 2012). Bluish colour may also indicate toxicity and unpalatability to herbivores (Lev-Yadun and Gould 2009). In accordance with this reasoning, this study detected darker colour morphs as more prevalent at northern latitudes. Further estimates of fitness and selection in natural environments are needed for establishing a link between adaptive evolution and the traits studied, and, thus, the heritable variation detected in this study reveals promising avenues for future studies in the field.

*Association of cytological variation with morphology*

Polyploidisation has been suggested to be positively associated with plant fitness (Husband et al. 2013), and differences in ploidy levels can play an important role in speciation (Husband and Sahara 2004; Wood et al. 2009). In the present study, ploidy level variation was associated with differences in certain morphological traits (i.e., lemma hairiness and number of leaf veins). In other studies, polyploidisation has been found to especially be associated with traits affected by cell size (Balao et al. 2011); however, in some cases, no phenotypic differences between plants with different ploidy levels have been found (Achenbach et al. 2012).

*Demographic history of the taxa*

Chloroplast DNA markers were not able to discriminate among the subspecies *rubra* and *arctica* or their putative hybrids. Instead, the data suggests that plants clustered geographically form divergent maternal lineages, three of which mainly comprised of members from Spain, the Northern Atlantic region, and Fennoscandia, as shown by Cräutlein et al. (2019) in their larger sample set. The occurrence of representatives of Spanish cpDNA clusters in south Finland and the Faroe Islands indicates that the *F. rubra* complex has successfully migrated and colonised across Europe, specifically from the Iberian peninsula toward northern Europe, after the last glacial period (von Cräutlein et al. 2019; Inda et al. 2008; see also Hewitt 1996, 1999). Consequently, individuals of the *F. rubra* complex with different origins have accumulated locally and formed genetically mixed populations (von Cräutlein et al. 2019). Furthermore, several polymorphic additive sites (APS) in nrDNA sequences of the *F. rubra* taxa observed in northern populations might imply recent hybridisation events between closely related taxa in the northern region. In contrast, fewer polymorphic additive sites among *F. rothmaleri* individuals may suggest the concerted evolution of the nrDNA sequences toward one of the parental genomes, which is typical among more ancient hybrids. However, further genetic studies across wider geographical areas are required to enhance understanding of the reticulation evolution of and the taxonomic relationships in the *F. rubra* complex.

**Conclusions**

Our results revealed phenotypic and genetic differentiation among geographic regions rather than the current species or subspecies of the *F. rubra* complex. This suggests that commonly used characteristics measured in *in situ* individuals may not be reliable in the classification of morphologically and biogeographically unique species and subspecies. Therefore, it is argued that phenotypic plasticity, especially in plants, must be considered when determining evolutionarily relevant taxonomic units for quantifying patterns of species variation. The detected phenotypic and genetic differentiation among geographic regions is, however, in accordance with the extended

formulation of the evolutionary species concept (Simpson 1951), which argues that species are comprised of 'separately evolving (segments of) metapopulation lineages' (de Queiroz 2007; Hausdorf 2011). It is therefore proposed that, instead of arguing whether the current species or subspecies of *F. rubra* complex are meaningful taxonomic units, future studies should focus on how the evolution of the widely geographically distributed species complex with locally adapted and hybridising ecotypes can ultimately produce reproductively isolated lineages that are no longer capable of crossing.

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**Data availability**

Data will be deposited in the Dryad digital repository. The ITS sequences have been submitted to the National Center for Biotechnology Information database (GenBank).

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**Table 1.** Geographic region, population, latitude, longitude, altitude, and habitat type of the collection site and sample size (n) of natural *Festuca rubra sensu lato* populations used in the morphological (M) and genetic studies (C=cpDNA; N=nrDNA). *=Codes used in the nrDNA study.

| Geographic region | Population | Code* | Latitude | Longitude | Altitude (m a.s.l.) | Habitat type | M n | C n | N n |
|---|---|---|---|---|---|---|---|---|---|
| Spain | Cáceres | SPGD | N 40° 12' 1.12" | W 5° 45' 11.03" | 768 | Xerophytic forest | 9 | 7 | 1 |
| Spain | Salamanca 1 | SPLV | N 40° 56' 20.16" | W 6° 7' 6.6" | 863 | Meadow | 5 | 2 | 2 |
| Spain | Salamanca 2 | SPPOR | N 40° 58' 24.28" | W 5° 57' 33.69" | 812 | Meadow | 14 | 10 | 1 |
| Switzerland | Andermatt | - | N 46° 37' 47.28" | E 8° 35' 25.7" | 1500 | Meadow | 9 | - | - |
| Switzerland | Biez | - | N 46° 53' 56.19" | E 8° 42' 9.47" | 1600 | Meadow | 5 | - | - |
| Switzerland | Piasca | - | N 46° 32' 18.58" | E 8° 40' 31.21" | 1850 | Meadow | 5 | - | - |
| S Finland | Hanko 1 | HA2 | N 59° 50' 27" | E 23° 13' 15" | 0 | Meadow | 9 | 6 | 1 |
| S Finland | Hanko 2 | HA1 | N 59° 50' 23" | E 23° 13' 40" | 0 | Meadow | 18 | 8 | 1 |
| S Finland | Hanko 3 | HA3 | N 59° 53' 0" | E 23° 5' 52" | 0 | Meadow | 16 | 9 | - |
| Faroe Islands | Mykines | FAS1 | N 62° 5' 50.7" | W 7° 40' 55.9" | 125 | Meadow | 16 | 7 | - |
| Faroe Islands | Vidoy | FAS2 | N 62° 22' 3.4" | W 6° 32' 31.8" | 148 | Meadow | 3 | 3 | - |
| Faroe Islands | Sandoy | FAS3 | N 61° 50' 11" | W 6° 51' 21.3" | 69 | Meadow | 6 | 4 | - |
| Faroe Islands | Nolsoy | FAS4 | N 62° 1' 14.9" | W 6° 41' 8.2" | 55 | Meadow | 7 | 5 | 1 |
| Iceland | Iceland 1 | - | N 64° 47' 34.2" | W 21° 32' 0.4" | 390 | Meadow | 2 | - | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Iceland | Iceland 2 | ICE1 | N 64° 48' 52.4" | W 23° 23' 14.4" | 10 | Meadow | 5 | 3 | - |
| Iceland | Iceland 3 | - | N 66° 1' 20.7" | W 20° 23' 39.2" | 38 | Meadow | 2 | - | 1 |
| N Finland | Kevo 1 | MS1K | N 69° 43' 56.4" | E 27° 1' 11.6" | 91 | Meadow | 21 | 21 | 1 |
| N Finland | Kevo 2 | MS2K | N 69° 45' 32.4" | E 26° 59' 18.8" | 85 | Meadow | 14 | 13 | 2 |
| N Finland | Kevo 3 | KS3 | N 69° 38' 5.6" | E 27° 5' 0.9" | 107 | Meadow | 31 | 12 | - |
| N Finland | Kevo 4 | RBS1 | N 69° 54' 35.1" | E 27° 2' 0.15" | 73 | Riverbank | 10 | 8 | 1 |
| N Finland | Kevo 5 | RBS3 | N 69° 56' 10.5" | E 26°27' 45.2" | 106 | Riverbank | 6 | 4 | 1 |
| N Finland | Kevo 6 | RBS2 | N 69° 56' 41.0" | E 26° 43' 21.9" | 85 | Riverbank | 10 | 5 | 1 |
| Greenland | Greenland 1 | GL1 | N 69° 14' 59" | W 53° 31'15" | 0 | Meadow | 12 | 5 | - |
| Greenland | Greenland 2 | GL2 | N 69° 15' 27" | W 53° 31' 15" | 0 | Meadow | 4 | 4 | 1 |

**Table 2.** Number of *Festuca rubra s. l.* individuals from each geographic region, classified as species, subspecies, or a between-subspecies hybrid, used in the morphological (M) and genetic studies (C=cpDNA; N=nrDNA).

| Subspecies | Geographic origin | | | | | | |
|---|---|---|---|---|---|---|---|
| | Greenland | N Finland | Iceland | Faroe | S Finland | Switzerland | Spain |
| | M, C, N | M, C, N | M, C, N | M, C, N | M, C, N | M, C, N | M, C, N |
| *arctica* | 16, 9, 1 | 62, 44, 2 | 4, 2, 0 | 5, 2, 0 | 19, 9, 1 | 0 | 0 |
| *rubra* | 0 | 13, 8, 2 | 4, 1, 1 | 21, 14, 1 | 11, 7, 0 | 19, 0, 0 | 0 |
| *rubra* × *arctica* | 0 | 17, 11, 2 | 1, 0, 0 | 6, 3, 0 | 13, 7, 1 | 0 | 0 |
| *F. rothmaleri* | 0 | 0 | 0 | 0 | 0 | 0 | 28, 19, 4 |
| Total | 16, 9, 1 | 92, 63, 6 | 9, 3, 1 | 32, 19, 2 | 43, 23, 2 | 19, 0, 0 | 28, 19, 4 |

**Table 3.** Morphological measurements of *Festuca rubra s. l.* in the common garden experiment.

| Continuous traits | Discrete traits |
|---|---|
| Plant height | Plant colour (light green, grey, blue) |
| Inflorescence length | Tuft (loose/dense) |
| Spikelet length | Leaf scabrousness (non-scabrid, slightly scabrid, scabrid) |
| Awn length | Glume hairiness (glabrous, scattered-hairy, hairy) |
| Lemma length | Lemma hairiness (glabrous, scattered-hairy, hairy) |
| Stolon length | Rachilla hairiness (glabrous, scattered-hairy, hairy) |
| Lower glume length | Number of leaf veins |
| Upper glume length | Number of flowers in the spikelet |
| Basal leaf width | |

**Table 4.** Descriptive statistics (mean ± standard deviation) for the morphological traits of plants with different geographic origins and species/subspecies measured in the common garden of the *Festuca rubra s. l.* plants.

| | Plant height (cm) | Stolon length (cm) | Inflorescence length (cm) | Spikelet length (mm) | Lemma length (mm) | Awn length (mm) | Lower glume length (mm) | Upper glume length (mm) | Basal leaf width (mm) |
|---|---|---|---|---|---|---|---|---|---|
| *Geographic origin* | | | | | | | | | |
| Greenland | 30.86 ± 7.01 | 12.07 ± 5.04 | 4.59 ± 1.11 | 9.01 ± 1.17 | 5.65 ± 0.78 | 1.00 ± 0.43 | 3.25 ± 0.74 | 4.75 ± 0.63 | 1.28 ± 0.27 |
| N Finland | 47.45 ± 11.51 | 5.15 ± 5.01 | 5.33 ± 1.29 | 7.72 ± 1.51 | 5.05 ± 0.65 | 0.99 ± 0.50 | 2.80 ± 0.59 | 4.09 ± 0.64 | 1.22 ± 0.32 |
| Iceland | 35.80 ± 16.94 | 3.44 ± 3.56 | 4.02 ± 1.32 | 7.03 ± 1.43 | 5.07 ± 0.81 | 0.95 ± 0.52 | 2.90 ± 0.56 | 4.10 ± 0.80 | 1.11 ± 0.21 |
| Faroe Islands | 31.82 ± 11.59 | 7.89 ± 9.70 | 4.48 ± 1.34 | 7.68 ± 1.35 | 5.16 ± 0.59 | 0.94 ± 0.52 | 2.95 ± 0.51 | 4.06 ± 0.55 | 1.53 ± 0.50 |
| S Finland | 55.41 ± 11.32 | 6.34 ± 6.11 | 6.20 ± 1.54 | 7.29 ± 1.25 | 4.81 ± 0.56 | 1.08 ± 0.43 | 2.68 ± 0.43 | 3.92 ± 0.49 | 1.24 ± 0.25 |
| Switzerland | 59.57 ± 18.97 | 0.57 ± 1.71 | 6.01 ± 2.00 | 6.87 ± 1.09 | 4.96 ± 0.78 | 1.68 ± 0.68 | 2.35 ± 0.44 | 3.68 ± 0.42 | 0.98 ± 0.35 |
| Spain | 57.12 ± 19.72 | 5.86 ± 4.27 | 7.94 ± 3.16 | 8.08 ± 1.75 | 4.93 ± 0.93 | 1.33 ± 0.72 | 2.83 ± 0.51 | 3.96 ± 0.66 | 1.20 ± 0.27 |
| Species/subspecies | | | | | | | | | |
| *F. rubra* | | | | | | | | | |
| *ssp. arctica* | 44.85 ± 13.73 | 6.40 ± 5.75 | 5.25 ± 1.36 | 7.78 ± 1.48 | 5.07 ± 0.72 | 0.97 ± 0.44 | 2.82 ± 0.62 | 4.13 ± 0.64 | 1.25 ± 0.33 |
| *ssp. rubra* | 47.63 ± 17.50 | 4.34 ± 6.65 | 5.46 ± 1.83 | 7.44 ± 1.45 | 5.06 ± 0.65 | 1.20 ± 0.65 | 2.73 ± 0.53 | 3.94 ± 0.55 | 1.24 ± 0.40 |
| *ssp. rubra* × *arctica* | 46.50 ± 15.81 | 7.02 ± 7.50 | 5.30 ± 1.49 | 7.46 ± 1.30 | 4.99 ± 0.62 | 1.07 ± 0.52 | 2.86 ± 0.55 | 4.13 ± 0.70 | 1.27 ± 0.39 |
| *F. rothmaleri* | 57.12 ± 19.72 | 5.86 ± 4.27 | 7.94 ± 3.16 | 8.08 ± 1.75 | 4.93 ± 0.93 | 1.33 ± 0.72 | 2.83 ± 0.51 | 3.96 ± 0.66 | 1.20 ± 0.27 |

**Table 5.** Results of the likelihood ratio tests (F- and P-value; d.f.=1) between the linear models in R testing for significant differences in the continuous morphological traits between *Festuca rubra s. l.* plants of different subspecies, ploidy levels (tetra-, hexa, or octoploid), and endophyte status (with or without endophyte). Morphological traits were measured in an outdoor common garden in Southern Finland. Species, subspecies, and geographic origin were tested in separate models with ploidy level and endophyte status as fixed factors. Results for ploidy level and endophyte status in this table are estimated from models with geographic origin. Significance threshold after Bonferroni correction is 0.0029.

| | Species/subspecies | | Geographic origin | | Ploidy level | | Endophyte status | |
|---|---|---|---|---|---|---|---|---|
| | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* |
| Plant height | 3.82 | 0.0107 | **19.54** | **< 0.0001** | 1.29 | 0.2774 | 0.01 | 0.9146 |
| Stolon length | 2.34 | 0.0738 | **7.30** | **< 0.0001** | 1.10 | 0.3334 | 1.20 | 0.2748 |
| Basal leaf width | 0.07 | 0.9743 | **6.31** | **< 0.0001** | 0.78 | 0.4576 | 0.83 | 0.3635 |
| Inflorescence length | 4.11 | 0.0073 | **6.83** | **< 0.0001** | 1.26 | 0.2842 | 3.23 | 0.0738 |
| Lemma length | 0.11 | 0.9556 | 3.09 | 0.0063 | 1.14 | 0.3216 | 0.06 | 0.8111 |
| Awn length | 2.78 | 0.0419 | **5.44** | **< 0.0001** | 0.15 | 0.8571 | 2.81 | 0.0950 |
| Lower glume length | 0.42 | 0.7367 | **4.86** | **0.0001** | 2.55 | 0.0802 | 0.09 | 0.3478 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Upper glume length | 1.32 | 0.2698 | **5.37** | < **0.0001** | 4.26 | 0.0153 | 0.34 | 0.5569 |
| Spikelet length | 1.17 | 0.3214 | **4.26** | **0.0004** | 0.00 | 0.9984 | 0.23 | 0.6328 |

**Table 6.** Results of approximative (Monte Carlo) Pearson chi-squared test or nonparametric *kruskal.test* test in R testing for significant differences in categorical morphological traits between *Festuca rubra s. l.* species/subspecies, geographic origin, ploidy level, and endophyte status. Morphological traits were measured in an outdoor common garden in southern Finland. Significance threshold after Bonferroni correction is 0.0029.

| | Species/subspecies | | Geographic origin | | Ploidy level | | Endophyte status | |
|---|---|---|---|---|---|---|---|---|
| | *X2* | *P* | *X2* | *P* | *X2* | *P* | *X2* | *P* |
| Leaf scabrousness[1] | 16.19 | 0.0114 | **39.96** | **0.0002** | 2.32 | 0.7199 | 5.77 | 0.0553 |
| Lemma hairiness[1] | **447.1** | **<0.0001** | **117.59** | **<0.0001** | **19.62** | **0.0006** | 1.33 | 0.5318 |
| Glume hairiness[1] | **51.54** | **<0.0001** | **69.91** | **<0.0001** | 10.82 | 0.0430 | 6.48 | 0.0416 |
| Rachilla hairiness[1] | **71.58** | **<0.0001** | **40.62** | **0.0003** | 6.00 | 0.2020 | 0.87 | 0.6557 |
| Plant color[1] | **25.40** | **0.0004** | **92.52** | **<0.0001** | 10.04 | 0.0373 | 5.43 | 0.067 |
| Tuft type[1] | 12.84 | 0.0043 | **29.55** | **<0.0001** | 3.12 | 0.2279 | 0.18 | 0.6836 |
| Number of leaf veins[2] | **31.61** | **<0.0001** | **51.53** | **<0.0001** | **24.08** | **<0.0001** | 2.70 | 0.1006 |
| Number of flowers in spikelet[2] | 6.31 | 0.0976 | 18.89 | 0.0043 | 0.56 | 0.7555 | 1.48 | 0.2236 |

[1]approximative (Monte Carlo) Pearson chi-squared test; [2]Kruskal Wallis test

**Table 7.** Ploidy levels of *Festuca rubra s. l.* species/subspecies in this study compared to previous taxonomic literature.

| Subspecies | Previously reported ploidy level | Reference | Our findings | | |
|---|---|---|---|---|---|
| | | | 4x | 6x | 8x |
| *F. rubra* subsp. *rubra* | 6x | Tropicos.org (2014) | - | 100 % | - |
| *F. rubra* subsp. *arctica* | 6x | Markgraf-Dannenberg (1980) | 1 % | 94 % | 5 % |
| subsp. *rubra* × *arctica* hybrids | - | - | 3 % | 97% | - |
| *F. rothmaleri* | 8x | Al-Bermani et al. (1992) | 68 % | 25 % | 7 % |

**FIGURE LEGENDS**

**Figure 1.** The separation of *Festuca rubra s. l.* taxa using principal component analysis of continuous morphological traits. The plants were grown in a common garden, southern Finland.

**Figure 2.** Results of principal component analysis of continuous morphological traits of *Festuca rubra s. l.* species/subspecies in relation to geographic origin. The plants were grown in a common garden, southern Finland.

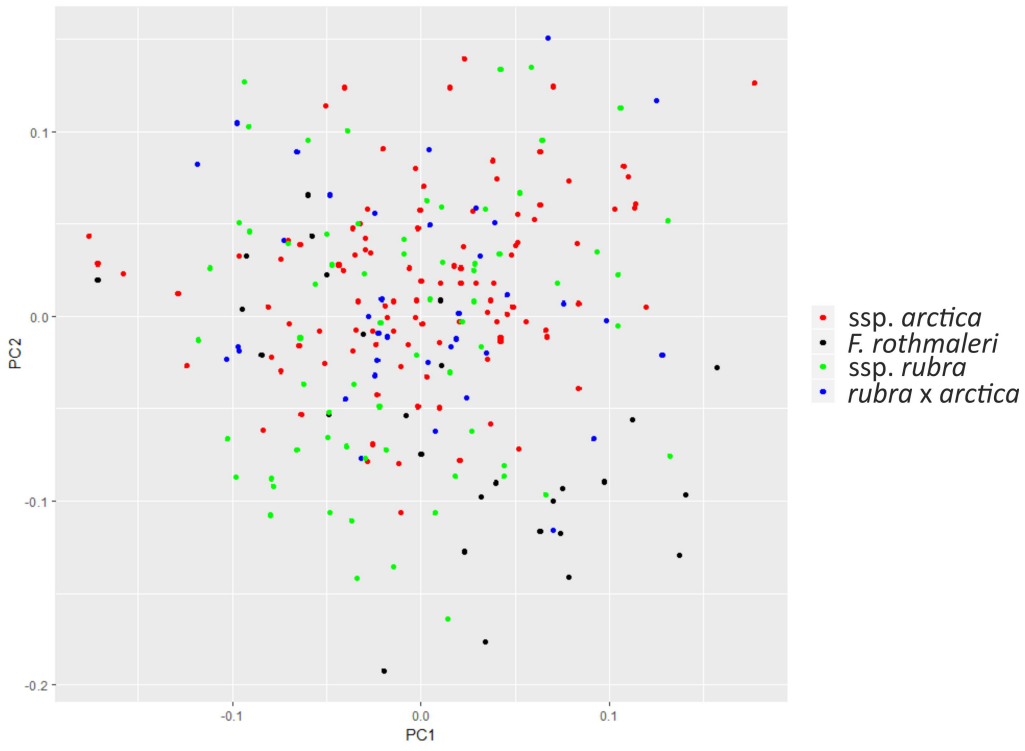
**Figure 3.** Proportion of *Festuca rubra s. l.* plants in each trait category in the common garden. The coloured bars on the left refer to the distribution of plant colour (light-green/bluish/greyish). Bar colours ranging from black to white show distributions of tuft type (loose or dense), leaf scabrousness (scabrid / slightly scabrid / smooth), number of leaf veins (5, 7 or 9), number of flowers in spikelet (2, 3, 4, 5, 6, 7, 8), hairiness of lemma, rachilla, and glumes of inflorescence (hairy / scattered-hairy / glabrous).

**Figure 4.** A. Polymorphisms of ITS (ITS1+5.8S+ITS2) sequences within *Festuca rubra* complex, including four *F. rothmaleri* individuals originated in Spain (SP) and four *F. rubra* subsp. *rubra*, four *F. rubra* subsp. *arctica*, three *rubra* x *arctica* individuals originating in the Faroe Islands (FA), Iceland (IC), southern Finland (HA) and northern Finland (RBS, MS). Ambiquity codes: Y=C/T; K=T/G; R=A/G; S=G/C. **B.** NeighborNet analyses of ITS sequence variability within *F. rubra* complex. Further information of the samples used is given in Table S1.

**Figure 5**. The Bayesian Analysis of Population Structure (BAPS) cluster assignments at K=6 for chloroplast microsatellite data of 136 wild European individuals of two *Festuca rubra* subspecies *arctica* and *rubra*, *rubra* × *arctica* hybrids, and *Festuca rothmaleri* in different geographical

regions. The origin of the samples were: FA, the Faroe Islands; GR, Greenland; IC, Iceland; N-FI, Northern Finland; S-FI, Southern Finland; SP, Spain.

**Figure S1.** A UPGMA tree based on the Kullback-Leibler divergence matrix visualizes the uppermost hierarchical levels of the genetic structure obtained from the software BAPS. The BAPS analyses identified that the optimal division of the populations in the *Festuca rubra* complex into clusters is acquired through 11 clusters (the highest marginal log-likelihood value = -1092.87). The number of individuals across clusters was: Cluster1, N= 28; cluster 2, N=55; cluster 3, N=4; cluster 4, N=3; cluster5, N=3; cluster 6, N=5; cluster 7, N=19; cluster 8, N=7; cluster 9, N=8; cluster 10, N=3; cluster 11, N=1.


0.1
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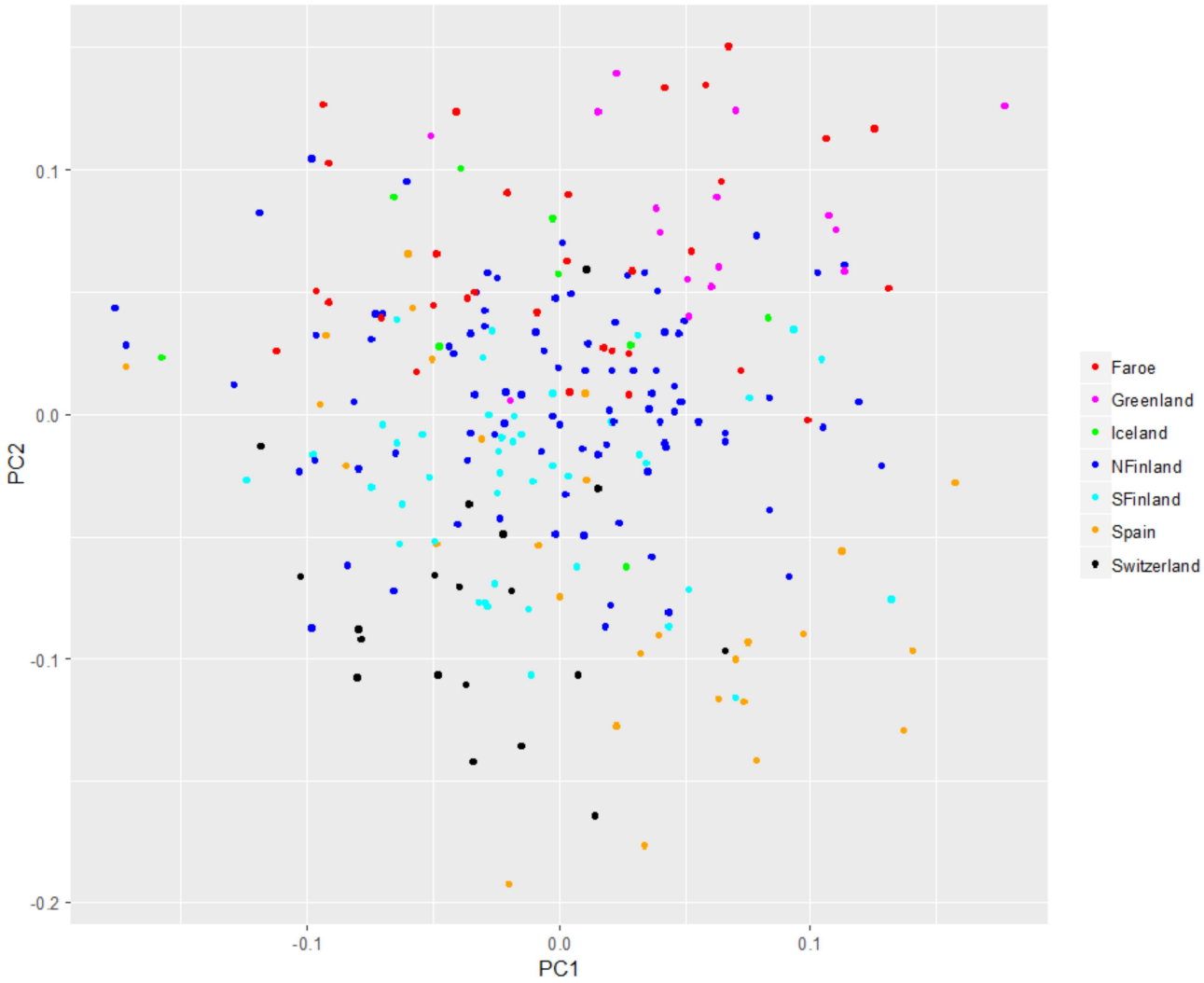
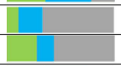
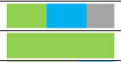
PC2
-0.1
0.0
0.1
PC1
ssp. arctica
F. rothmaleri
ssp. rubra
rubra x arctica

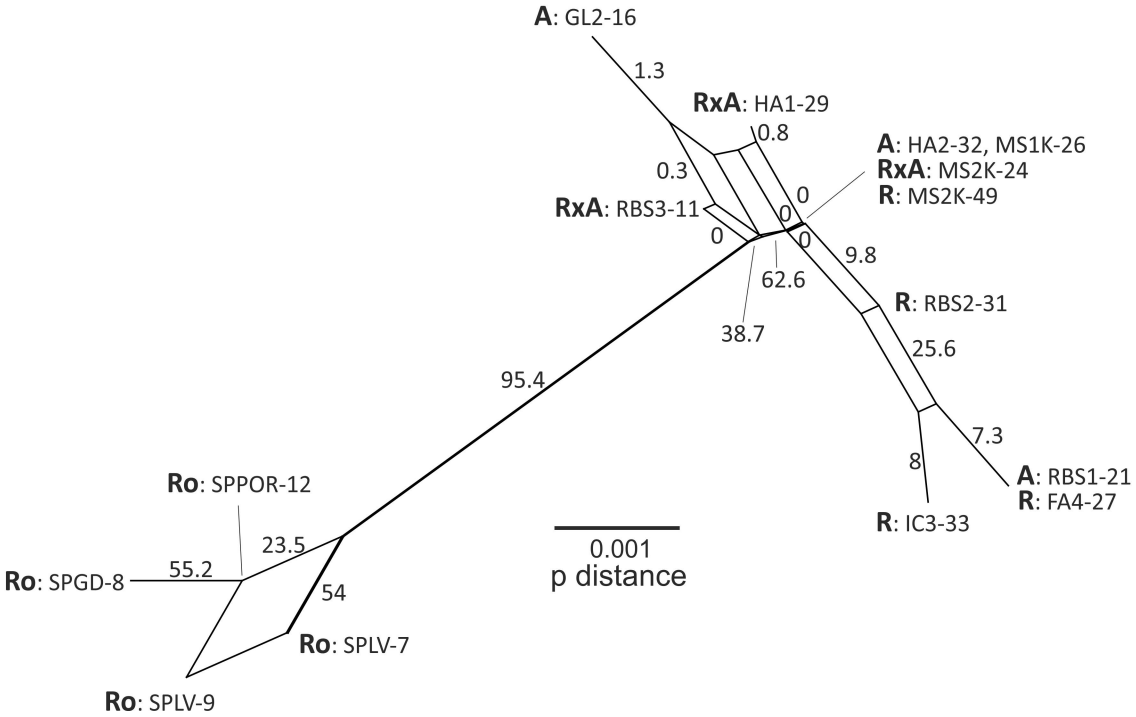

0.1
0.0
-0.1
-0.2
PC2
-0.1
0.0
0.1
PC1
Faroe
Greenland
Iceland
NFinland
SFinland
Spain
Switzerland

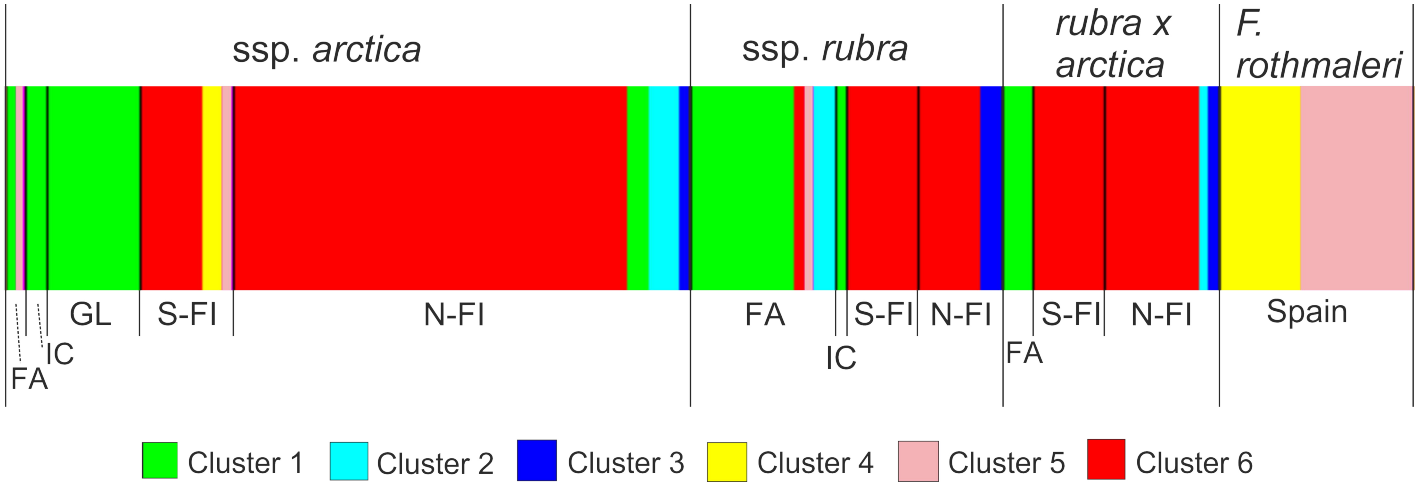
| | Plant colour | Tuft | Leaf scabrousness | Number of leaf veins | Number of flowers in spikelet | Hairiness | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Lemma | Rachilla | Glumes |
| Geographic origin | | | | | | | | |
| Greenland |  |  |  |  |  |  |  |  |
| N Finland |  |  |  |  |  |  |  |  |
| Iceland |  |  |  |  |  |  |  |  |
| Faroe |  |  |  |  |  |  |  |  |
| S Finland |  |  |  |  |  |  |  |  |
| Switzerland |  |  |  |  |  |  |  |  |
| Spain |  |  |  |  |  |  |  |  |
| Species/subspecies | | | | | | | | |
| *F. rubra* ssp. *rubra* |  |  |  |  |  |  |  |  |
| *F. rubra* ssp. *arctica* |  |  |  |  |  |  |  |  |
| *rubra* × *arctica* |  |  |  |  |  |  |  |  |
| *F. rothmaleri* |  |  |  |  |  |  |  |  |

A

| Taxon | Sample code | ITS1 | | | 5.8S | ITS2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 51 | 79 | 199 | 350 | 414 | 547 | 556 | 576 | 580 | nt position |
| *F. rothmaleri* | SPLV-7 | C | A | G | C | T | G | C | A | C | |
| *F. rothmaleri* | SPLV-9 | C | A | G | Y | T | G | C | A | C | |
| *F. rothmaleri* | SPPOR-12 | C | A | G | Y | Y | G | C | A | C | |
| *F. rothmaleri* | SPGD-8 | C | A | G | T | Y | G | C | A | C | |
| *rubra x arctica* | RBS3-11 | C | K | G | C | C | G | C | G | C | |
| *F. rubra* ssp. *arctica* | GL2-16 | Y | K | G | C | C | R | C | G | C | |
| *rubra x arctica* | HA1-29 | C | G | G | C | C | R | C | G | C | |
| *F. rubra* ssp. *arctica* | HA2-32 | C | G | G | C | C | G | C | G | C | |
| *F. rubra* ssp. *arctica* | MSIK-26 | C | G | G | C | C | G | C | G | C | |
| *rubra x arctica* | MS2K-24 | C | G | G | C | C | G | C | G | C | |
| *F. rubra* ssp. *rubra* | MS2K-49 | C | G | G | C | C | G | C | G | C | |
| *F. rubra* ssp. *rubra* | RBS2-31 | C | G | G | C | C | G | C | G | Y | |
| *F. rubra* ssp. *arctica* | RBS1-21 | C | G | K | C | C | G | S | G | Y | |
| *F. rubra* ssp. *rubra* | FAS4-27 | C | G | K | C | C | G | S | G | Y | |
| *F. rubra* ssp. *rubra* | ICE1-33 | Y | G | K | C | C | G | C | G | Y | |

B




ssp. arctica
ssp. rubra
rubra x arctica
F. rothmaleri
GL
S-FI
N-FI
FA
IC
FA
IC
S-FI
N-FI
FA
S-FI
N-FI
Spain
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6