Building knowledge for a forage breeding program on native *Festuca* species in the highlands of Bolivia

KARINA ROSSIO USTARIZ OLIVERA
Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Breeding
Alnarp

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Cover: 1) *Festuca orthophylla*, 2) Seeds of *F. orthophylla*, 3) Panicle of *Festuca* sp., 4) *Festuca dolichophylla*, 5-7) chilliwar grasslands in Potosí, La Paz, and Oruro, Bolivia.
(Photos: Karina Ustariz, Katia Ramirez)

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Abstract

Native grasses constitute the most important source of feed for camelids, sheep and cattle in the highlands of Bolivia, where the genus *Festuca* is one of the major feed components. The two most important species of this region are *F. dolichophylla* J. Presl (known as "chilliwa") and *F. orthophylla* Pilg (known as "iru ichu"). This research aimed to investigate the phylogenetic relationships within the *Festuca* genus from the highlands of Bolivia, to evaluate the genetic diversity using EST-SSR markers and to assess the nutritional value for its use in breeding and conservation. The phylogenetic analysis, which was based on sequences derived from nuclear (ITS, *CEN*, *Acc*1) and chloroplast DNA (*mat*K) regions, revealed that the Bolivian *Festuca* species derived from a common ancestor of the fine-leaved lineage of the *Festuca* sub-genus. In addition, Bolivian fescues where all grouped together, thus indicating that they belong to the American clade II of the Loliinae subtribe. The evaluation of genetic diversity performed using 12 EST-SSR markers in 43 populations indicated absence of clear population structure. However, four primer-pairs (FES 04, FES 13, FES 24 and NFA 142) were at the top in their polymorphism and gene diversity indicating the putative use of them in future population genetic analysis. Moreover, they could be helpful in pursuing the development of effective conservation management strategies for native and endangered *Festuca* species in Bolivia. The assessment of the mineral composition, protein, ash and cellulose contents, which were determined in 11 Bolivian *Festuca* ecotypes and 2 cultivars from Argentina, revealed as candidates for genetic improvement, the accessions 29, 10, 21, 32, 38 and 23 due to their high protein, Ca, Mg and P contents. The result generated in this work represents the starting material to develop a forage breeding program of the *Festuca* species in Bolivia as well as develop strategies for *ex situ* and *in situ* germplasm conservation.

Keywords: EST-SSR, *Festuca*, Genetic diversity, Nutritional value, Phylogeny

Author's address: Karina Ustariz, SLU, Department of Plant Breeding
P.O. Box 101, SE-23053 Alnarp, Sweden.
E-mail: karina.ustariz@slu.se or karinaustariz@hotmail.com

Bygga kunskap för ett foderförädlingsprogram på inhemska *Festuca*-arter på höglandet i Bolivia

Sammanfattning

Inhemska gräs utgör den huvudsakliga foderkällan för kamelider, får och nötkreatur på höglandet i Bolivia, där släktet *Festuca* (svinglar) är en av de viktigaste foderkomponenterna. De två viktigaste arterna av släktet i regionen är *F. dolichophylla* J. Presl (känd som "chilliwa") och *F. orthophylla* Pilg (känd som "iru ichu"). Denna forskning syftar till att undersöka de fylogenetiska släktskapen inom *Festuca* från höglandet i Bolivia, att utvärdera den genetiska mångfalden med hjälp av EST-SSR-markörer och att bedöma näringsvärdet för dess användning i förädlingsarbete och bevarande. Den fylogenetiska analysen, som baserades på sekvenser från regioner i nukleärt DNA (ITS, *CEN*, *Acc*1) och kloroplast-DNA (*mat*K), visade att de bolivianska *Festuca*-arterna härrör från en gemensam förfader till den smalbladiga undergruppen (subg.) *Festuca* . Dessutom grupperar alla bolivianska svinglar tillsammans, vilket indikerar att de tillhör den amerikanska clade II i subtribus Loliinae. Studierna i genetisk mångfald utfördes med 12 EST-SSR-markörer i 43 populationer och indikerade frånvaro av en tydlig populationsstruktur. Fyra primer-par (FES 04, FES 13, FES 24 och NFA 142) visade den högsta polymorfismen och kan användas i framtida populationsgenetiska analyser. Dessutom kan de vara till hjälp vid utvecklingen av effektiva bevarandestrategier för inhemska och hotade *Festuca*-arter i Bolivia. Från studierna i mineralsammansättning, protein, ask och cellulosainnehåll, som bestämdes hos 11 bolivianska *Festuca*-ekotyper och två sorter från Argentina, kunde flera kandidater för genetisk förbättring identifieras. Accessionerna 29, 10, 21, 32, 38 och 23 har ett högt innehåll av protein, Ca, Mg och P. Resultatet från detta arbete kan användas som utgångsmaterial för att utveckla ett förädlingsprogram av nya fodergrödor från släktet *Festuca* i Bolivia samt utveckla strategier för bevarande av ex *situ* och *in situ*.

Key words: EST-SSR, *Festuca*, genetisk mångfald, näringsvärde, filogeny

Author's address: Karina Ustariz, SLU, Institutionen för växtförädling
P.O. Box 101, SE-23053 Alnarp, Sweden
E-mail: karina.ustariz@slu.se or karinaustariz@hotmail.com

Dedication

To my beloved daughter Jasmine

"Whoever would name Festuca must first sharpen their knife"
Renvoize, 1998

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ustariz Karina*, Geleta Mulatu, Hovmalm P. Helena, Gutierrez Franz, Rojas Beltrán Jorge A, Ortiz Rodomiro. Phylogenetic study of *Festuca* species from the highlands of Bolivia based on DNA sequences derived from nuclear and chloroplast DNA regions (manuscript)

II Ustariz Karina*, Geleta Mulatu, Hovmalm P. Helena, Gutierrez Franz, Rojas Beltrán Jorge A, Ortiz Rodomiro. Analysis of genetic diversity in Bolivian fescue populations using EST-SSR markers (submitted)

III Ustariz Karina*, Geleta Mulatu, Hovmalm P. Helena, Gutierrez Franz, Rojas Beltrán Jorge A, Ortiz Rodomiro (2019). Mineral composition and nutritive value of *Festuca* ecotypes originated from the highland region of Bolivia and cultivars from Argentina. *Australian Journal of Crop Science*. doi: 10.21475/ajcs.19.13.10.p1889

* Corresponding author.

The contribution of Karina Ustariz to the papers included in this thesis was as follows:

I Planned the experiment together with the supervisors, performed experimental work, analysed the data and wrote the manuscript with input from co-authors.

II Planned the experiment together with the supervisors, performed experimental work, analysed the data and wrote the manuscript with input from co-authors.

III Planned and performed the experimental work, analysed the data and wrote the manuscript with input from co-authors.

Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of molecular variance
cDNA	Chloroplast DNA
CIF	Centro de Investigación en Forrajes
CEAC	Centro Agropecuario Condoriri
EST-SSR	Expressed Sequence Tags - Simple Sequence Repeats
ITS	Internal Transcribed Spacer
masl	Meters above sea level
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
Sida	Swedish International Development Agency
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
UMSS	Universidad Mayor de San Simón
UPGMA	Unweighted pair group method with arithmetic mean
UTO	Universidad Técnica de Oruro

1 Introduction

Bolivia is located in the heart of South America, which includes lowlands below 300 meters above sea level (masl) as well as high mountains up to almost 7000 masl (Ibisch, 2005). It is considered as one of the most biodiverse countries of the world, containing virtually all tropical vegetations and a great variety of geologic formations (Ibisch *et al*., 2003). With a surface of 1,098.581km^2, it is divided into three distinct eco-zones: (1) a vast semi-arid Altiplano plateau; (2) a semi-tropical Yungas and the temperate valleys of the eastern range; and (3) the eastern lowlands, including de semi-arid Chaco.

Bolivia has a high biodiversity in its ecological regions and ecologically similar smaller units, called native grazing fields, which have facilitated the evolution of a rich native flora. Within this floristic diversity, native forage plants play important functions, such as, provide forage and shelter to wild and domestic animals, soil protection, incorporation of organic matter into the soil, and capture and management of rainwater (Alzérreca, 2004).

The highland region of Bolivia, which is the geographical area considered in this study, is a region where native grasslands are an important source for animal feed specially camelids, ruminants and sheep (Campero, 2004). However, the degradation of the grassland ecosystems in the area is increasing as a consequence of overgrazing, climate change, drought, advance in the agricultural frontier and unsuitable management techniques (e.g. uncontrolled use of fire) causing loss of valuable forage germplasm, proliferation and multiplication of undesirable species with no forage value and reduced vegetation cover.

Among the native grasses, *Festuca* species shows high tolerance to drought and low temperatures in the region. Seventeen *Festuca* species from 41 described in the flora of Bolivia, are considered endemic for the country and five were reported to be in danger of extinction (Jorgensen *et al*., 2014; Mercado *et al*., 2012; Meneses *et al*., 2012). *Festuca dolichophylla* (known as “chilliwa”), *F. orthophylla* (known as “Iru Ichu”), *F. rigescens*, *F. boliviana* and *F. humilior*

have been described so far as a major forage sources in the region (Mercado *et al*., 2013). However, very little is known about other uses and role in the grass ecosystems of the other *Festuca* species (Jorgensen *et al*., 2014).

Phylogenetic approaches that focus on the potential relationships between different *Festuca* species gave some insights about the centre of origin of older *Festuca* lineages as well as tentative migration routes that allowed the more aggressive polyploid lineages colonize other continents (Catalán, 2006). It seems that the *F. rubra* group, which belongs to the sub-genus *Festuca* and section Aulaxyper, is closely related to the majority of the South American taxa (Catalán *et al*., 2004; Inda *et al*., 2008). However, further study is needed in order to confirm this result and to have better insights about the evolution of *Festuca* species in Bolivia in general.

Natural genetic variability within crop species has been used for many years in order to meet subsistence food requirement (Govindaraj *et al*., 2015). Due to the introduction of molecular tools such as simple sequence repeats (SSR) markers, nowadays it is possible to get valuable information for breeding programs with the aim of developing suitable cultivars adapted to different environments and utilization systems (Kölliker *et al*., 1999). Hence, considering that forages are the plant basis for beef and milk production in the highlands, and knowing that the *Festuca* genus is considered by farmers as one of the best native forages in Bolivia (Alzérreca and Cardozo, 1991; Mamani-Linares *et al*., 2013), it is important to generate more knowledge about this genus with the aim of developing a plant breeding program. Forage of high quality and yield will give local farmers the required improved plant germplasm for producing milk, meat and wool for self-consumption and for selling the products at the local markets.

2 Background

2.1 Taxonomy

The classification of the *Festuca* genus proposed by Linné (1753) is as follows:

Order Poales
Family Poaceae
Subfamily Pooideae
Tribe Poeae
Subtribe Loliinae
Genus *Festuca*

However, based on morphological characters, the classification has changed over the last 200 years, as new taxa have been incorporated or segregated. Hackel (1882) divided the European fescues into six sections (Ovinae, Bovinae, Subbulbosae, Variae, Scariosae and Montanae), which were based on characters associated with leaf vernation, leaf sheath, auricles, spikelets, floral bracts (lemma and palea), presence or absence of ovary pubescence, insertion of styles, adherence of caryopsis to palea, and hilum length. Years later, and based on morpho-anatomical characters Piper, 1906; Hackel, 1906; Saint-Yves, 1922; Krechetovich and Bobrov, 1934; Krivotulenko, 1960; Tzvelev, 1971 and Alexeev 1977, 1978, 1980, 1981, 1986 proposed a new classification, which included 11 subgenera, many of which were further divided into sections.

One of the most accepted and widespread classification based on morphological and anatomical characters, was made by Clayton and Renvoize (1986). Both authors recognized nine of the 11 subgenera previously established by Alexeev describing *Festuca* and *Helleria* as fine leaved fescues, and *Drymanthele, Schedonorus*, *Subulatae*, *Subuliflorae*, *Obtusae*, *Hesperochloa* and *Xanthochloa* as broad-leaved fescues.

Despite having carried out different studies to improve the classification of *Festuca* species, the interpretation and identification of many taxa is still problematic and sometimes even nearly impossible when they are based on morphological characters alone (Cheng *et al*., 2016). Therefore, molecular systematic (Müller and Catalán, 2006) and cytotaxonomic studies of this genus (Abyaneh *et al*., 2018) have been proposed to improve the classification at the subgeneric and section levels (Müller and Catalán, 2006; Abyaneh *et al*., 2018).

The two agriculturally most important forage crops of the *Festuca* genus are the hexaploid tall fescue (*F. arundinacea* Schreb.) and the diploid meadow fescue (*F. pratensis* Huds.). Other *Festuca* species with some importance as forage and turf species are red fescue, *F. rubra* L. and sheep fescue, *F. ovina* L., which are much better adapted to abiotic stresses as heat, drought, and low temperature, but show poor establishment and low quality in animal forage production (Yamada, 2011).

2.2 Botanical description of the genus *Festuca*

Habit, vegetative morphology. Perennial; rhizomatous, or stoloniferous, or caespitose, or decumbent. Culms 2–200 cm high; herbaceous; unbranched above; tuberous, or not tuberous. Culm nodes glabrous. Culm internodes solid, or hollow. Young shoots extravaginal or intravaginal. Leaves mostly basal, or not basally aggregated; auriculate, or non-auriculate. Sheath margins joined, or free. Leaf blades linear to linear-lanceolate; narrow; 0.2–15 mm wide; setaceous, or not setaceous; flat, or folded, or rolled (convolute or involute); without cross venation; persistent; rolled in bud, or once-folded in bud. Ligule an unfringed membrane (sometimes ciliolate); truncate; 0.1–1.5 (–5.5) mm long (usually less than 1mm) (Watson and Dalwitz, 1992).

Reproductive organization. Plants bisexual, with bisexual spikelets (*Leucopoa* being excluded); with hermaphrodite florets. The spikelets all alike in sexuality. Plants outbreeding and inbreeding (by cleistogamy). Exposed-cleistogamous, or chasmogamous. Viviparous (sometimes), or not viviparous.

Inflorescence. Inflorescence paniculate; open (usually), or contracted (rarely); when contracted spicate, or more or less irregular; with capillary branchlets, or without capillary branchlets; espatheate; not comprising 'partial inflorescence' and foliar organs. Spikelet-bearing axes persistent. Spikelets not second; pedicellate.

Female-fertile spikelets. Spikelets 3–20 mm long; compressed laterally; disarticulating above the glumes; disarticulating between the florets. Rachilla prolonged beyond the uppermost female-fertile floret; hairy, or hairless. The rachilla extension with incomplete florets. Hairy callus absent. Glumes 2; very

unequal; shorter than the spikelets; shorter than the adjacent lemmas; pointed; awnless; carinate to non-carinate; similar (usually narrow to ovate-lanceolate). Lower glume 1–3 nerved. Upper glume (1–)3–5 nerved. Spikelets with incomplete florets. The incomplete florets distal to the female-fertile florets. The distal incomplete florets merely underdeveloped.

Female-fertile florets 2–14 (rarely 1). Lemmas similar in texture to the glumes to decidedly firmer than the glumes; not becoming indurated; entire, or incised; when entire pointed, or blunt; when incised, not deeply cleft; awnless, or mucronate, or awned. Awns when present, 1; from a sinus, or apical; non-geniculate; much shorter than the body of the lemma (usually), or about as long as the body of the lemma (sometimes, rarely somewhat longer); entered by one vein. Lemmas hairy (rarely), or hairless; non-carinate; 3–7 nerved. Palea present; relatively long; tightly clasped by the lemma; apically notched; awnless, without apical setae; textured like the lemma; not indurated (submembranous); 2-nerved; 2-keeled. Lodicules present; 2; free; membranous; ciliate or glabrous; toothed. Stamens 3. Anthers 0.4–6 mm long; not penicillate. Ovary glabrous, or hairy; without a conspicuous apical appendage. Styles free to their bases. Stigmas 2; white.

Fruit, embryo and seedling. Fruit adhering to lemma and/or palea, or free from both lemma and palea; small, or medium sized, or large; fusiform, or ellipsoid; longitudinally grooved; compressed dorsiventrally; with hairs confined to a terminal tuft. Hilum long-linear (usually about as long as the grain, but sometimes elliptical and only half as long). Embryo small; not waisted. Endosperm hard; without lipid; containing compound starch grains. Embryo with an epiblast; without a scutellar tail; with a negligible meso-cotyl internode. Embryonic leaf margins meeting. Seedlings with a long mesocotyl; with a loose coleoptile, or with a tight coleptile. First seedling leaf with a well-developed lamina. The lamina narrow; erect; 3–5 veined (Watson and Dalwitz, 1992).

The ploidy levels of this genus vary from diploid ($2n = 2x = 14$) to dodecaploid ($2n = 12x = 84$) where most of the species are allopolyploid. The reproduction is in general outbreeding and rarely inbreeding by cleistogamy (Watson and Dalwitz, 1992; Stammers *et al*., 1995; Loureiro *et al*., 2007; Hand *et al*. 2010).

2.3 Geographical distribution

The genus *Festuca* comprises around 500 species. It is widely spread in the holartic region but also inhabit in cool and temperate areas in the southern hemisphere. The species are found in a large variety of different habitats

including, wetlands, drylands, mountains and Arctic/sub-Antarctic areas (Inda *et al*., 2008).

In Bolivia, *Festuca* L. is distributed in the Andean tropical region, which includes the biogeographic provinces Yungueño Peruano-Boliviano, Mesophytic Puna, Xerophytic Puna and the Boliviano-Tucumano, in altitudes varying from 1500 to 4800 masl (Fig.1). Most of the *Festuca* species described in the country have been found in the highlands of La Paz, Potosi and Oruro but they can also be found in Cochabamba, Chuquisaca, Tarija and Santa Cruz (Table 1).

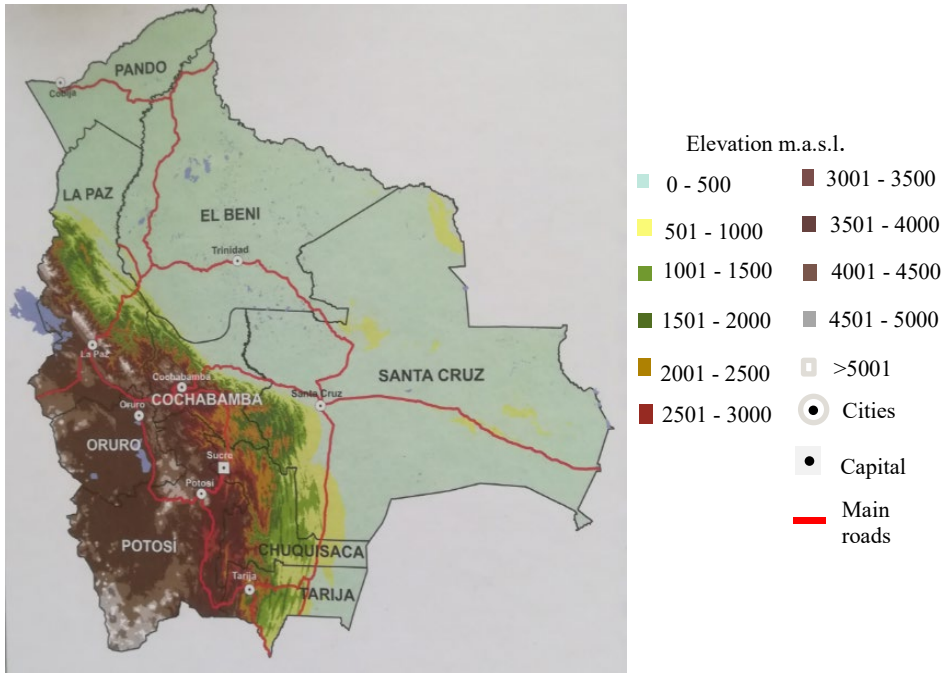

Figure 1. A map of Bolivia showing its altitudinal distribution

Table 1. *Festuca species described in the Bolivian flora*

Taxa	Department	Altitude
Festuca argentinensis (St.-Yves) Türpe	PO	3500–5000
Festuca arundinacea Schreb.	LP	3500–4000
Festuca asplundii E.B. Alexeev	LP	3500–4000
Festuca boliviana E.B. Alexeev*	CO	2000–2500
Festuca carrascana Stancik & Renvoize*	CO	2000–2500
Festuca chrysophylla Phil.	PO	3500–5000
Festuca chuquisacae Stancik & Renvoize*	CH	2500–3000
Festuca cochabambana E.B. Alexeev*	CO,SC	2000–3000
Festuca copei Renvoize*	CH,CO,LP,PO	2500–4000
*Festuca cuzcoensis Stancik &*P.M. Peterson	LP	3000–4000
Festuca dolichophylla J. Presl	CH,CO,LP,OR,PO	3000–5000
Festuca fiebrigii Pilg*	CH,CO,LP,OR,SC,TA	2500–4500
Festuca hieronymi Hack	CH,CO,PO,TA	1500–4500
Festuca humilior Nees & Meyen	CH,CO,LP,TA	3000–4500
Festuca hypsophila Phil.	PO	4000–4500
Festuca laeteviridis Pilg*	PO	2500–3000
Festuca lanifera E.B. Alexeev*	CO	2000–2500
Festuca lasiorrhachis Pilg.	N/D	3500–4000
Festuca lilloi Hack	TA	2000–3000
Festuca nemoralis Türpe	CO,LP	3000–4000
Festuca orthophylla Pilg.	LP,OR,PO,TA	3000–5000
Festuca parvipaniculata Hitchc.	CO,LP	2500-4500
Festuca parodiana (St.-Yves) Nicora	LP,SC,TA	2000–3500
Festuca peruviana Infantes	LP,OR	3500–5000
Festuca petersonii Renvoize*	PO	4000–4500
Festuca procera Kunth	CO,LP,PO,TA	3000–4500
Festuca potosiana Renvoize*	LP,PO,TA	3500–5000
Festuca rigescens (J.Presl) Kunth	CO,LP,PO,TA	3500–5000
Festuca rubra L.	CO	2500–3000
Festuca samensis Joch. Müll.*	CH,SC,TA	2500–3000
Festuca scabrifolia Renvoize*	LP	3500–4000
Festuca scabriuscula Phil.	LP	3000–4500
Festuca soratana E.B. Alexeev*	LP	3500–5000
Festuca stebeckii Renvoize*	CO	3000–3500
Festuca steinbachii E.B. Alexeev*	CO	2500–3000
Festuca stuebelii Pilg.*	CO,LP,PO	3000–5000
Festuca subulifolia Benth.	N/D	N/D
Festuca tovariensis Stancik & P.M. Peterson	LP	2500–3500

Taxa	Department	Altitude
Festuca trollii E.B. Alexeev*	CO	2500–4000
Festuca ulochaeta Nees ex Steud.	N/D	N/D
Festuca villipalea (St.-Yves) Alexeev	LP,OR,PO	3500–5000

ALT = Altitude in meters above the sea level; CO = Cochabamba; CH = Chuquisaca, LP = La Paz; OR = Oruro; PO = Potosí; SC = Santa Cruz; TA = Tarija; N/D = No data; * = endemic for the country.

2.4 Importance of *Festuca* species in the highlands of Bolivia

Native vegetation results from a short rainy season and low temperatures. But it can be also influenced by other factors such as solar radiation, wide variation in temperatures, low humidity, low oxygen pressure, geomorphology and type of soils (Alzérreca and Lara, 1988; Campero, 2004).

Different natural vegetation types have been described for the highlands of Bolivia where *Festuca* species have been always recognized as part of native grasslands. Alzérreca and Lara (1988), divided the region into five types of plant associations, which included tolars, grasslands, tolar-grasslands, wetlands and gramadals. On the other hand, Moraes and Beck (1992), divided the region into three types of plant associations, which was based only on changes in humidity from the north to the south of the country. Those three types were the following: 1) humid Altiplano, with vegetation characterized by the presence of *Festuca dolichophylla*, commonly known as chilliwar grassland, 2) dry Altiplano, where the tola schrubs (*Parastrepia lepidophylla*) and the ichu (*F. orthophylla*) were the most dominant species and 3) desert region, which included different xerophytic plants. Years later, Genin and Alzérreca (2006) updated the information adding more plant associations in the region. *F. dolichophylla* was found to be predominant in the chilliwar grassland and together with *F. rigescens* it was also found in bofedals. *F. orthophylla* was described as the most dominant and with the major importance in livestock feed in the iru ichu-grassland (Table 2).

Table 2. *Main plant associations, dominant species and forage production of native grazing fields in the highlands of Bolivia*

Plant association	Dominant species	Forage production (dry matter in kg ha^{-1})
Tolar-*Parastrepia*	*Parastrepia lepidophylla*	200–700
	Erodium cicutarium	
	Nasella pubiflora	
Tolar-*Baccharis*	*Baccharis incarum*	150–650
	Tetraglochin cristatum	
Other tolars	*Fabiana densa*	150–600
	Lampaya castellani	
Iru ichu-grassland	***Festuca orthophylla***	100–500
	Deyeuxia spp.	
Ichu-grassland	*Stipa ichu*	100–500
	Bouteloua simplex	
Gramadal	*Distichlis humilis*	700–1000
	Mulhenbergia fastigiata	
Hordeum-grassland	*Hordeum muticum*	900–3300
	Distichlis humilis	
Chilliwar-grassland	***Festuca dolichophylla***	550–2000
	Trifolium amabile	
Bofedals	*Distichia muscoides*	750–6000
	Oxychloe andina	
	Plantago tubulosa	
	Festuca dolichophylla	
	Festuca rigescens	
Meadows	*Azorella compacta*	220–300
	Pycnophyllum spp.	
	Calamagrostis vicunarum	
Totoral	*Schoenoplectus tatora*	2000–15000
	Myriophyllom spp.	
Kemparal	*Bacharis juncea*	2000–4000
	Distichlis humilis	
Churquiales, palquiales	*Prosopis ferox*	500–700
	Acacia feddeana	

Source: Genin and Alzérreca, 2006

The chilliwar grassland, which is usually found in deep soils, is considered as a native grazing field of high forage potential (Fig.2). It is intensively used for grazing of camelids and ruminants (Genin *et al*., 1994; Campero, 2004). Even though the nutritive value of *F. dolichophylla* is not very high when compared

with other forages, the nutritional value of this grassland is high due to the presence of increases thanks to associated plant species such as: Layu (*Trifolium amabile*), Sillu sillu (*Lachemilla pinnata*), Siqui (*Hypochoeris spp*.), Cebadilla (*Bromus catarthicus*), cola de ratón (*Hordeum muticum*), chiji blanco (*Distichlis humilis*), kemallu (*Eleocharis spp*.), poita (*Poa annua*) and chiji negro (*Muhlenbergia fastigiata*) (Genin and Alzérreca, 2006).

Figure 2. Chilliwar grassland (*Festuca dolichophylla*) (Photos: Karina Ustariz, SLU)

Festuca orthophylla, is the most dominant grass species in the iru-ichu grassland. Owing to the massive root-stock, compact clonal growth and great longevity, this species plays a key role in the erosion control and has been considered as a 'landscape engineer' (Monteiro *et al*., 2010). However, camelids (especially llamas), feed on fresh/regrown leaves and inflorescences reducing its reproductive potential. This perennial plant presents hard and erect leaves and it is usually found in soils of poor quality with high percentage of sand. It is generally associated with shrubs of the genera *Baccharis* and *Parastrepia* and other species that appear during the wet season such as *Distichlis humilis*, *Mulhenbergia fastigiata* and *Deyeuxia* species (Genin and Alzérreca, 2006). Despite the low nutritional value, it constitutes an important feed source for camelids and ruminants in the highlands, especially during the dry and cold season (Genin *et al*., 1994) (Fig. 3).

Figure 3. Iru-ichu grassland (*Festuca orthophylla*) (Photos: Karina Ustariz, SLU)

Bofedal is a humid meadow where natural vegetation is always green providing high quality of forage. It is found in all the biogeographic provinces from the Andean Tropical region. Even though most representative species belong to the genera *Distichia*, *Oxychloe* and *Plantago*, *F. dolichophylla* and *F. rigescens* have been described as part of this wetland and constitute an important feed resource for cattle and sheep, contributing to the production of milk, meet and wool in the highlands (Genin and Alzérreca, 2006) (Fig. 4).

Figure 4. Bofedal (Photos: Franz Gutiérrez, UMSS and Karina Ustariz, SLU)

2.5 Phylogenetic studies of *Festuca* L.

Darbyshire and Warwick (1992) were the pioneers in conducting a phylogenetic study in North American fescues and related genera using chloroplast DNA restriction site variation, where two main groups were found. The first one included the majority of the *Festuca* species, belonging to the subgenera *Drymanthele*, *Subulatae*, *Subuliflorae*, *Obtusae* and *Festuca*, as well as *Vulpia*, and the sub-genus *Leucopoa* sect. *breviaristatae*. The second group included sub-genus *Schedonorus*, sub-genus *Leocopoa* sect. *Leocopoa* and the genus *Lolium*. Further phylogenetic analysis using Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Internal Transcribed Spacer (ITS) sequences of ribosomal DNA by representing the genus with larger number of species, confirmed the presence of those two major groups: 'fine-leaved' and 'broad-leaved' *Festuca*. Among the fine-leaved, the red fescue (*F. rubra*) and the sheep fescue (*F. ovina)* groups were found to be close to the genus *Vulpia*, whereas within the broad-leaved, meadow fescue (*F. pratensis)* and tall fescue (*F. arundinacea*) were grouped together with the *Lolium* group (Charmet *et al*, 1997; Torrecilla and Catalán, 2002).

An extensive phylogenetic study based on separated and combined analyses of ITS and chloroplast *trn*L-F sequences using representatives from Europe, Asia, North America and few samples of Africa, revealed some broad-leaved taxa placed either in intermediate positions between the two main groups or within the clade of fine-leaved fescues. These results were explained as a result of high mutation rates, which were observed in most of the annual lineages of the fine-leaved group (Torrecilla *et al*., 2004). Using the same combined DNA sequences (ITS and *trn*L-F) and adding more taxa within the Loliinae subtribe, Catalán *et al*. (2004) and Catalán *et al*. (2007) confirmed the monophyly of the

subtribe. Moreover, a basal paraphyly of the broad-leaved fescue group with respect to the more recently evolved fine-leaved fescue group FEVRE (*Festuca* + *Vulpia* + Related Ephemerals) was observed. The broad-leaved group was weakly supported and included samples of the subgenera *Schenodorus*, *Drymanthele*, *Leucopoa*, *Subulatae* and the genus *Lolium*, while the fine-leaved group was highly supported and included samples of the subgenera *Festuca* and the genus *Vulpia*.

The most exhaustive phylogenetic study of *Festuca* and its closest relatives was perfomed one year later by Inda *et al*. (2008). The study included new sequence data from representatives of the almost unexplored New World, New Zeland and Eastern Asia centres as well as sequence data previously published by Catalán, *et al*. (2004) and Torrecilla *et al*. (2004). The divergence of the subtribe Loliinae into broad-leaved and fine-leaved group was maintained, as well as the intermediate position of some broad-leaved within the fine-leaved group. Within the *Festuca* genus, the fine-leaved *Festuca* clade included the *F. rubra* group, *Vulpia* (2*x*), American II, *F. ovina* group, *Vulpia* (4*x*–6*x*), American I (Andean and Patagonian) and Neozeylandic I/American sub-clades. The intermediate position included the *Subulatae* and *Subuliflorae* subgenera. However, some representatives of the *Drymanthele* and *Leucopoa* subgenera were also included. The broad-leaved *Festuca* clade included the *Schedonorus-Lolium* group, *Drymanthele*, *Leucopoa* and the Asian American sub-clade. Within this study, species of the genus *Festuca* from South America were mostly located within the American II and American I clade. In addition, unexpected relationships among South American and New Zealand lineages were found.

Despite that the ITS and *trn*L-F based study have provided interesting results to help understand the phylogenetic relationships among the Loliinae subtribe, with emphasis on the *Festuca* genus, there is still uncertainty in the resolution of their phylogeny (Inda *et al.* 2008; Ospina, 2016). There is still lack of taxonomical studies at the sub-generic, infra-generic and sectional assignments in *Festuca* species of South America (Ospina, 2016). The unexpected placement of some broad-leaved lineages within the fine-leaved clade, suggests biological and historical events. It could be that the traits related to the broad-leaved fescues, evolved secondarily within the fine-leaved clade, as those attributes seem to be rather plesiomorphic (Catalán *et al*., 2007). A history of colonization and hybridization might have also been involved in the origin of polyploid *Festuca* species from South America (Inda *et al*., 2008).

2.6 Genetic diversity

Genetic diversity in plants provides opportunity for plant breeders to develop new and improved cultivars with desirable traits, which include both farmer-preferred traits (e.g. yield potential and large seeds) and breeder-preferred traits (e.g. host plant resistance to pathogens and pests). From the very beginning of agriculture, natural genetic variability has been exploited within crop species to meet subsistence food requirement in growing populations. Moreover, conserved plant genetic resources are important for crop improvement in order to meet future global challenges in relation to food and nutritional security (Govindaraj *et al*., 2015).

The assessment of genetic diversity within and among plant populations has been performed along the years using morphological characteristics, biochemical characterization or evaluation and molecular techniques. The first one is based on visually accessible traits such as flower colour, seed shape, growth habits and pigmentation. Even though they are often prone to phenotypic plasticity, this type of marker is still used in the field. The biochemical marker includes the allelic variants of enzymes called isozymes, which are detected by electrophoresis and are codominant in nature. They detect diversity at the functional gene level and have simple inheritance, but the resolution of genetic diversity is limited to explore. The third technique constitutes the molecular markers, which have been employed for analysis of genetic and molecular variation. These markers are divided into (i) hybridization-based markers (e.g. RFLP)); (ii) PCR-based markers (e.g. RAPD, AFLP, microsatellites or simple sequence repeats (SSR)) and; (iii) sequence-based markers (e.g. Single Nucleotide Polymorphism (SNP)) (Jiang, 2017).

One of the most widely used molecular markers for genetic diversity studies in fescues are SSR (also called microsatellites), which have been developed *in silico* due to the availability of large-scale gene sequences or expressed sequence tag (EST) information (Thiel *et al*., 2003). Microsatellites have become the marker class of choice because of their hypervariability, reproducibility, codominant nature, locus specificity, random genome distribution and high rates of transferability across species (Gaitán-Solís *et al*., 2002; Thiel *et al*., 2003; Saha *et al*., 2004).

Thanks to an ETS project on tall fescue that started at the Samuel Roberts Noble Foundation (*Mian et al*., 2002), a large set of SSR primer-pairs from tall fescue EST were developed, as well as primer pairs that amplify reliable PCR products in a range of grass species. These tall fescue EST-SSR markers did not only demonstrate to be useful in the evaluation of genetic relationships among the *Festuc*a and *Lolium* species, but also proved to be highly transferable to other

plant species such as rice (*Oryza sativa*) and bread wheat (*Triticum aestivum*) (*Saha et al.*, 2004). Moreover, EST-SSR markers have been described to be useful for comparative genomics of tall fescue with major cereal, forage and turf grass species e.g. rice, wheat, maize (*Zea mays*), barley (*Hordeum vulgare*), ryegrass (*Lolium* spp.) and meadow fescue (Saha *et al*., 2005).

2.7 Nutritional and mineral content

Livestock production is the dominant activity of smallholders living in the arid highlands of Bolivia where native grasslands constitute the basis for camelid, cattle and sheep feeding (Genin and Alzérreca, 2006). Mixed herds of camelids and sheep have the capacity to use properly the overall available forage in the region due to different grazing behaviour (Genin and Tichit, 1997; Campero, 2004). Camelids consume the highest proportions of dominant coarse bunchgrasses such as *F. orthophylla* and *Stipa ichu*, while sheep seek for more fine herbaceous plants, which grow mostly under the shrubs (Alzérreca, 2004).

The tall and coarse bunchgrasses of *Stipa ichu* (paja brava) and *F. orthophylla* (iru ichu) are the dominant species in the highlands of Bolivia. *F. orthophylla* covers around 30% of the central highland (Alzérreca and Lara, 1988; Monteiro *et al*., 2010) and it is considered by smallholders, as a poor forage, because of its low nutritive value and its roughness (Genin *et al*., 1994; Zapata, 2005; Monteiro and Körner, 2013). The crude protein reported for this species varies from 2.3 to 7.6 % dry matter and has a mean ash content of 7.4% (Alzérreca and Cardozo, 1991; Genin *et al*. 1994; Genin and Alzérreca, 2006; Mamani-Linares et al., 2013). *F. orthophylla* has a high importance in agriculture, as it is sometimes the only available forage for the herds in the region especially during the dry season (Genin *et al*. 1994).

F. dolichophylla (chilliwa) is the second important species of the *Festuca* genus in the highlands. The chilliwar grassland is highly used for grazing cattle, sheep and camelids (Villca and Genin, 1995). The nutritional value of this species is considered moderate with a crude protein content ranging from 3.7 to 7.7% dry matter and 7.2% of ash content (Mamani, 2003; Alzérreca and Cardozo, 1991; Genin and Alzérreca, 2006; Mamani-Linares *et al*., 2013).

The nutritional value of native grasses ensures an optimal production, health and fertility in camelids, cattle and sheep (National Research Council, 2007). The concentration of those nutrients, however, is not only influenced by genetic differences, but also depend on the soil pH and fertility, forage species, maturity stage of the plant, season and climate, irrigation, and atmospheric inputs (McDowell *et al*., 1996; Givens *et al*., 2000).

3 Aim and objectives

The overall aim of this thesis was to generate scientific knowledge about the *Festuca* genus from the highlands of Bolivia, in order to stablish strategies for conservation and sustainable utilization in forage breeding programs. To achieve this aim, the specific objectives of this work were to:

- Establish the phylogenetic relationship between *Festuca* species from the highlands of Bolivia based on DNA sequences derived from nuclear and chloroplast DNA regions.
- Evaluate the genetic diversity of Bolivian fescue populations using EST-SSR markers.
- Assess the mineral composition and nutritional value of *Festuca* ecotypes originated from the highland region of Bolivia and cultivars from Argentina.

4 Material and methods

4.1 Plant material

Fifty-seven samples representing different *Festuca* species and other species from closely related genera were used for the phylogenetic study (Paper I). The germplasm included twenty-three genotypes representing *Festuca* species collected in the highlands of the departments of Oruro, La Paz, Cochabamba and Potosi, three *Festuca* species from the National Herbarium of La Paz-Bolivia, one *Festuca* species from the Herbarium Martín Cárdenas of Cochabamba-Bolivia, five accessions from the Nordic Genetic Resource Center (NordGen), Sweden comprising *Festuca rubra, Phalaris arundinacea, Phleum pratense, Poa pratensis* and *Lolium perenne.* In addition, sequences derived from nuclear and chloroplast DNA regions of 22 genotypes of *Festuca* species and three species of *Lolium* generated in previous studies were downloaded from the GenBank and used together with the aforementioned samples.

To assess the genetic diversity of Bolivian fescue populations (Paper II), germplasm of 43 populations of *Festuca* especies collected in the highlands of the departments of Oruro, La Paz, Cochabamba and Potosi were used. Each population was represented by 12 individuals.

Germplasm of eleven *Festuca* ecotypes collected in the highlands of the departments Oruro, La Paz, Cochabamba and Potosi as well as two cultivars from Argentina (*Festuca arundinacea* cv. 'Taita' and Festulolium) were used for the analysis of mineral composition and nutritive value (Paper III).

4.2 Field sampling

Seeds of 43 populations of *Festuca* species were collected, by representing each population with 15 randomly chosen plants in the highlands of the departments Oruro, La Paz, Potosi and Cochabamba from March to May 2015. The collection sites were located within 4312 to 3217 masl., which belong to the geographic xerophytic and mesophytic Puna provinces (Fig.5).

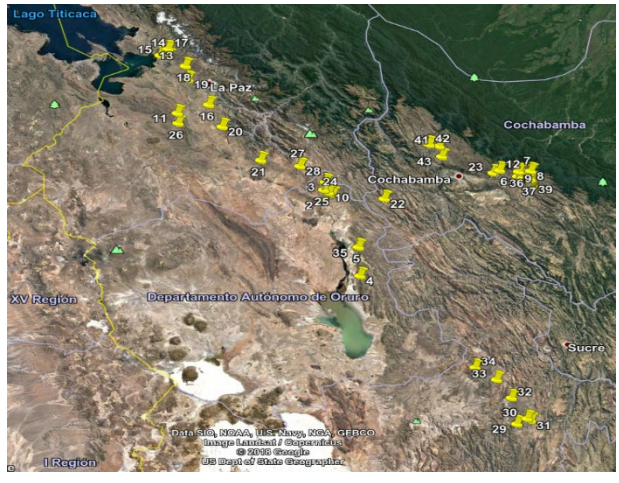

Figure 5.A map showing Geographic localizations of the seed collection sites in Bolivia

The collected seeds were grown in the greenhouse of the Centro de Investigación en Forrajes (CIF) – La Violeta, Universidad Mayor de San Simón, Cochabamba, Bolivia, to obtain seedlings for DNA extraction and field experiment. Young leaves from seedlings were used for DNA extraction for papers I and II. Plants of 5 ½ months old were transported to the Centro Experimental Agropecuario Condoriri, Oruro for field trial (Paper III).

4.3 Field experiments

Field trials were conducted from December 2015 to January 2017 at the Centro Experimental Agropecuario Condoriri (Fig. 6), which is located approximately 50 km from the capital city, Oruro, and 12 km from Caracollo municipality at an altitude of 3830 masl. Besides the 43 *Festuca* populations mentioned above, two more cultivars from Argentina, *Festuca arundinacea* var. 'Taita' and

Festulolium were added to the field experiment. A randomized complete block design (RCBD) with two replications of 15 plants per ecotype or cultivar was used. The plants were spaced at 0.5 m within the rows and 1 m between rows and any fertilizer was applied during the experiment. Plots were hand-weeded and watered sufficiently when necessary (Paper III).

Figure 6. Field trial at the Centro Experimental Agropecuario, Oruro (Photo: Karina Ustariz, SLU)

4.4 DNA extraction

Fresh, freeze-dried leaf tissue and herbarium specimens from one individual per species/cultivar/accession was used for DNA extraction. Leaves (approximately 10 cm in length) were placed into 2 ml Eppendorf tubes containing two steel beads and frozen in liquid nitrogen. Samples were then homogenized in a mixer Mill MM400 (Retsch GmbH, Haan, Germany) and the DNA was isolated in a QIAcube HT extraction robot using the QIAamp DNA mini Kit (Qiagen, Hilden, Germany). The quantity and quality of DNA were measured using spectrometry (NanoDrop ND-1000, Saveen Werner, Sweden) and evaluated by agarose gel (1.5%) electrophoresis (Papers I and II).

4.5 PCR amplification and sequencing

4.5.1 Phylogenetic studies

Target DNA regions were amplified using the primer-pairs described in Hand *et al*., (2010). The *Acc*1 was amplified using primers *Acc*1f1 and *Acc*1r1; *CEN* was amplified using primers *CEN*f1 and *CEN*r3, ITS was amplified using primers ITSL and ITS4, and *mat*K was amplified using primers S5-1f and *trn*K-2R. All amplified products were purified using the PureLink TM PCR purification Kit (Thermo Fischer, Germany) and mixed with forward and reverse primers prior sending to Eurofins (www.eurofins.com) for sequencing.

4.5.2 Genetic diversity analysis

Twelve EST-SSR primer-pairs that consistently amplified their targets were selected after screening and optimization of the PCR conditions. The forward primer-pairs were 5'-labeled with either 6-FAM™ or HEX™ fluorescent dyes. PCR reactions were prepared in 96-well thin wall PCR plates and amplifications were run in a S1000TM Thermal Cycler (Bio-Rad, Hercules, USA) at conditions optimized for each primer-pair.

The PCR products were multiplexed into five panels based on their differences in fragment sizes and/or fluorescent labels of the forward primers, with each panel containing PCR products of two or three SSR loci. The multiplexed PCR products were diluted 25× using Millipore water and the fragment analysis was performed on a 3500 Genetic analyser (Thermo Fisher Scientific, Waltham, USA).

4.6 Nutritional analysis

4.6.1 Protein, ash and cellulose determination

Leaves samples were dried and ground to a fine powder prior to total nitrogen analysis. Two technical replicates using the Kjeldahl method and the organic elemental analyser (Thermo Scientific Flash 2000) were used to estimate the protein concentration (%), which was estimated as: Protein (%) = Nitrogen (%) × 6.25. For the determination of ash and cellulose, method 14.006 and Kurschner method were used, respectively (William, 1984).

4.6.2 Mineral content determination

Mineral elements, Al, B, Ca, Cd, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, S, Si and Zn were analysed in two replicates using Inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES + SS028311; Perkin-Elmer, OPTIMA 3000 DV) at the ICP laboratory of AB Lennart MANSSON International, Helsingborg, Sweden. The content of each element was calculated as absolute content in mg kg^{-1}.

4.7 Data analysis

4.7.1 Phylogenetic analysis

BIOEDIT version 7.2.5 (Hall, 1999) was used to edit 32 DNA sequences generated within this study and twenty-five DNA sequences derived of nuclear and chloroplast regions published by Hand *et al*. (2010). The alignment was performed using ClustalX version 2.1 (Larkin *et al*., 2007) and determination of variable sites, parsimony informative sites and singleton sites within the aligned sequences were calculated using MEGA version 7.0.26 (Kumar *et al*., 2016).

The phylogenetic analysis of the DNA sequence data was performed using two datasets: 1) combined sequences of ITS and *mat*K, and 2) combined sequences of ITS, *mat*K, *CEN* and *Acc*1. In both cases, the maximum parsimony method was performed using MEGA version 7.0.26 (Kumar et al., 2016). The first dataset comprised nucleotide sequences of 57 samples of *Festuca* species and species from closely related genera. The second data set contained DNA sequences published by Hand *et al*. (2010) as well as DNA sequences from the four genomic regions generated in this study from nine *Festuca* genotypes from Bolivia and three representatives of the *Lolium* genus.

4.7.2 Genetic diversity analysis

Gene marker V2.7.0 software (Soft Genetics, LLC State College) was used for SSR peak identification and fragment sizing. Each peak was considered as an allele and the genotype of each individual at each locus was determined and exported to excel for statistical analysis. Observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity of each locus (h), Shannon information index (I), total gene diversity (Ht), within-population gene diversity (Hs) and coefficient of gene differentiation (Gst) were calculated using POPGENE ver 1.32 (Yeh *et al*., 1999).

Nei's measure of genetic distance was used to examine the genetic relationships among the 43 populations and a dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) using MEGA 7 software (Kumar *et al*., 2016). The evaluation of differentiation among groups of populations, among populations and variation within populations was performed using the analysis of molecular variance (AMOVA) with the Arlequin 3.5 software (Excoffier and Lischer, 2010).

4.7.3 Nutritional analysis

Principal component analysis (PCA) was used to characterize the variation among the accessions and clustering. The unweighted pair group method with arithmetic mean (UPGMA) was used to examine the grouping of the accessions from Bolivia and the two cultivars from Argentina. In addition, Spearman correlation coefficients were calculated using all the variables of protein, ash, cellulose, moisture and mineral content.

5 Summary of results

5.1 Phylogenetic relationship between genotypes of *Festuca* species (Paper I)

The maximum parsimony based phylogenetic analysis using dataset that combined ITS and *mat*K DNA sequences from 57 individual plants was conducted. The consensus tree resulted in seven major clades. The first clade (I) included 23 *Festuca* genotypes from the highlands of Cochabamba, Potosi, La Paz and Oruro as well as genotypes representing *F. stebeckii*, *F. rigescens*, *F. orthophylla* and *F. scabrifolia* species. The second clade (II) had the fine-leaved species from the *Festuca* sub-genus, represented by *F. rubra*, *F. tatrae*, *F. circummediterranea*, *F. ovina*, *F. pallens* and *F. valesiaca*. The third clade (III) comprised *Phalaris arundinacea*, *Poa pratensis* and *Phleum pratense*. The fouth clade (IV) included *F. altissima*, *F. lasto* and *F. drymeja*, representing the *Drymanthele* sub-genus. The fifth clade (V) had the Mediterranean tall fescue morphotype represented by *F. arundinacea* var. PF4012 and *F. arundinacea* var. Resolute. The sixth clade (VI) comprised F. *arundinacea* subsp. *atlantigena*, *F. arundinacea* subsp. *Letourneuxiana*, *F. marei*, *F. arundinacea* var. *glaucescens* and two rhizomatous tall fescue morphotypes, *F. arundinacea* var. CT2093 and *F. arundinacea* var. Torpedo II). The seventh clade (VII) included *F. arundinacea* var. Quantum, *F. arundinacea* var. KY31 and *F. arundinacea* var. Jesup (sub-clade VIIA) and *F. pratensis* subp. *apennina*, *F. gigantea*, *F. pratensis* as well as all *Lolium* species as sub-clade VIIB.

The second parsimonious tree, where sequences of all four genomic regions were combined (ITS-*mat*K-*CEN*-*Acc*1) grouped all samples into seven clades. The first clade (I) included the Bolivian genotypes CO-07, CO-38, CO-08 and CO-41 from Cochabamba; PO-30 from Potosi; LP-17 from La Paz; OR-04, OR-

24 and OR-25 from Oruro and genotypes representing *F. tatrae*, *F. circummediterranea*, *F. ovina* and *F. valesiaca* species, which belong to the *Festuca* sub-genus. The second clade (II) had the species *F. altissima*, *F. lasso* and *F. drymeja* from the *Drymanthele* sub-genus. The third clade (III) comprised *F. arundinacea* var. Torpedo II, *F. arundinacea* var. CT2093R, *F. arundinacea* var. *glaucescens*, *F. arundinacea* subsp. *atlantigena*, *F. arundinacea* subsp. *letournexiana* and *F. marei*. The fourth clade (IV) included *F. arundinacea* var. Jesup and *F. arundinacea* var. KY31, which belong to the *Schedonorus* sub-genus. The fifth clade (V) had the two Mediterranean morphotypes *F. arundinacea* var. PG4012 and *F. arundinacea* var. Resolute. The sixth clade (VII) comprised *F. gigantea*, *F. pratensis* and *F. pratensis* subsp. *apennina*. The seventh clade (VII) included the three *Lolium* species.

The phylogenetic analysis using the two data sets, suggests that the Bolivian *Festuca* species belong to the American II clade, which belongs to the fine-leaved lineage of the *Festuca* sub-genus. This suggestion is based on the fact that in previous research by Inda *et al*. (2008) and Ospina (2016), the *Festuca* species from Bolivia (i.e. *F. orthophylla, F. rigescens)* were located all within the American clade II.

5.2 Genetic diversity of Bolivian fescue populations (Paper II)

Among the 12 SSR loci targeted in this study (NFA 150, NFA 147, NFA 142, NFA 136, NFA 126, NFA 094, NFA 036, FES 24, FES 14, FES 13, FES 04 and FES 09), two of them (NFA 126 and FES 09) were monomorphic. Sixty-four alleles (Na) were recorded across the 10 polymorphic loci. The effective number of alleles (Ne) per locus varied from 1.03 (NFA 036) to 3.29 (NFA 142). The Nei's gene diversity (h) of each polymorphic locus varied from 0.03 (NFA 036) to 0.70 (NFA 142) and the Shannon diversity index (I) per locus ranged from 0.08 (NFA 036) to 1.41 (NFA 142) (Table 3).

Table 3. *Genetic diversity indices of the 10 polymorphic SSR loci estimated based on 43 Bolivian fescue populations*

Locus	Na	Ne	h	I	Ht	Hs	Gst
NFA 147	3	1.04	0.04	0.12	0.04	0.04	0.11
NFA 036	3	1.03	0.03	0.08	0.03	0.02	0.10
NFA 150	4	1.94	0.49	0.72	0.49	0.03	0.94
FES 14	4	1.15	0.13	0.31	0.13	0.11	0.16
FES 04	6	2.13	0.53	0.95	0.53	0.25	0.53
FES 24	8	2.83	0.65	1.33	0.65	0.32	0.51
FES 13	8	2.94	0.66	1.32	0.66	0.63	0.05
NFA 136	8	2.06	0.51	0.89	0.52	0.09	0.83
NFA 094	8	1.64	0.39	0.92	0.39	0.37	0.05
NFA 142	12	3.29	0.70	1.41	0.70	0.60	0.14
Mean	6.4	2.00	0.41	0.80	0.41	0.25	0.41
St. Dev	2.9	0.82	0.26	0.49	0.07	0.05	

Na = observed number of alleles, Ne = effective number of alleles, h = Nei's gene diversity, I = Shannon information index, Ht = total gene diversity, Hs = within-population gene diversity, Gst = population differentiation

The allele frequency distribution across the 10 loci ranged from 0.001 (least frequent alleles) recorded in NFA 150, NFA 136 and NFA 142 loci to 0.987 (most frequent allele) recorded in NFA 036 locus (Table 4).

Table 4. *Frequency distribution of the 64 alleles revealed across the 10 polymorphic EST-SSR loci*

	Loci									
Allele	NFA 147	NFA 036	NFA 150	FES 14	FES 04	FES 24	FES 13	NFA 136	NFA 094	NFA 142
A	0.010	0.002	0.011	0.010	0.304	0.327	0.029	0.019	0.007	0.003
B	0.979	0.987	0.380	0.032	0.004	0.036	0.046	0.015	0.040	0.002
C	0.011	0.012	0.609	0.933	0.612	0.014	0.431	0.011	0.051	0.029
D			0.001	0.024	0.021	0.047	0.084	0.345	0.059	0.187
E					0.051	0.489	0.022	0.604	0.041	0.049
F					0.009	0.026	0.005	0.002	0.024	0.432
G						0.054	0.004	0.001	0.775	0.007
H						0.007	0.379	0.002	0.003	0.281
I										0.003
J										0.001
K										0.003
L										0.004

The analysis of molecular variance (AMOVA) using present/absent allele data of the 43 populations revealed no significant genetic variation among populations (P = 1). Likewise, there was no significant genetic differentiation among groups of populations that were grouped according to their biogeographic provinces (FCT = 0.00022; P = 0.24047). However, when populations were grouped according to their departments, a very low but significant genetic differentiation (FCT = 0.00082; P = 0.01369) was found among the groups. When AMOVA was calculated by grouping populations into two altitudinal ranges (populations from altitude < 3800 masl and populations from altitude ≥ 3800 masl), no significant differentiation was found (P = 0.08).

The UPGMA clustering conducted based on Nei's genetic distance at population level, revealed two major clusters. Cluster I included populations 8, 7, 9, 12, 6, 23 and 22 from Cochabamba, populations 21, 13, 17, 14, 15, 18, 19, 20, 11, 26 and 16 from La Paz and populations 2, 1, 24, 25, 5, 3, 10, and 4 from Oruro. Cluster II included populations 36, 37, 38, 43, 40, 39, 41 and 42 from Cochabamba, populations 33, 30, 31, 29, 34, 32 from Potosi, populations 27 and 28 from La Paz and population 35 from Oruro (Fig. 7).

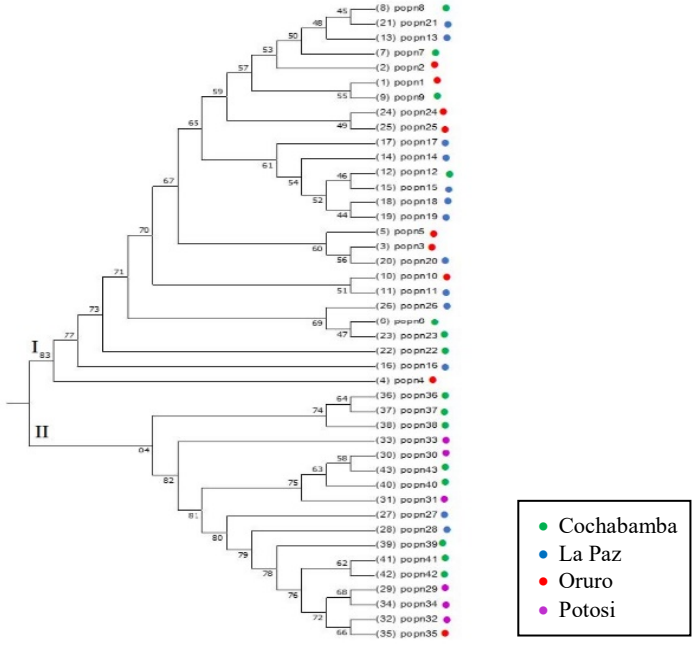

Figure 7. Unweighted pair-group method with arithmetic mean (UPGMA) dendrogram showing genetic relationships among the 43 populations based on Nei's measure of genetic distance

Among the 12 EST-SSR markers, FES 04, FES 13, FES 24 and NFA 142 revealed to be the most polymorphic. On the other hand, populations 16 and 17 were found to be the least diverse, whereas populations 5 and 34 were the most diverse populations. No significant differentiation among the populations were found using AMOVA, indicating absence of clear population structure.

5.3 Nutritional content of *Festuca* ecotypes from the highlands of Bolivia and Argentina (Paper III)

Significant differences in ash and cellulose content were found among *Festuca* ecotypes from Bolivia and cultivars from Argentina. Ash content ranged from 7.3% (Accession 27) to 16.5% (Accession 44). The two Argentinian cultivars, *F. arundinacea* cv. 'Taita' and Festulolium had the highest ash content with 16.5% and 14.3% respectively, followed by the Bolivian accessions 19 (9.7%) and 38 (9.5%). Cellulose content ranged from 18.7 % (accession 44) to 33.6 % (Accession 27). Protein content in accessions 29, 10, 21 and 32 were similar to Festulolium and higher than *F. arundinacea* cv. 'Taita'. Moreover, they also showed high Ca and Mg content. On the other hand, accession 38, 29, 23 and 21 showed high P content. Hence, accessions 29, 10, 21, 32, 38 and 23 could be good candidates for genetic improvement of these traits.

6 Conclusions and future perspectives

The phylogenetic reconstruction using the two DNA sequence data sets (ITS-*mat*K) and (ITS-*mat*K-*CEN*-*Acc*I) indicates that the Bolivian *Festuca* genotypes derived from a common ancestor of the fine-leaved lineage of the *Festuca* sub-genus.

All the Bolivian genotypes of *Festuca* used in this study were grouped together despite being morphologically different and seem to be part of the American clade II.

A widespread geographic representation of *Festuca* species from Bolivia and South America should be considered in future research, to facilitate a better understanding of the phylogenetic relationships of South American fescues.

Among the 12 EST-SSR markers used for the analysis of genetic diversity in Bolivian fescue populations, FES 04, FES 13, FES 24 and NFA 142 were at the top in terms of their polymorphism and gene diversity and hence could be valuable tools for future population genetic analysis.

A two-fold difference between the least diverse populations (populations 16 and 17) and the most diverse populations (populations 5 and 34) of Bolivian fescues was revealed through EST-SSR based genetic diversity analysis. Such significant differences have direct implications in setting conservation strategies, including identification of genetic diversity hotspot for Bolivian fescues for *in-situ* conservation.

Analysis of molecular variance (AMOVA) showed no significant differentiation among the 43 Bolivian fescue populations indicating absence of clear population structure. Therefore, further research including more fescue populations from other locations in Bolivia using the primer-pairs FES 04, FES 13, FES 24 and NFA 142 as well as other highly polymorphic molecular markers should be pursued, to determine the genetic diversity and population structure in more details, so that it can concretely contribute to the development of effective management strategies for native and endangered *Festuca* species in Bolivia.

Even though the Bolivian *Festuca* ecotypes did not show results comparable to Argentinian cultivars in terms of nutritional value, this study contributes to the scientific knowledge about the mineral composition, protein, cellulose and ash content of native fescues for selection of appropriate germplasm for use in the breeding program of fescues for the highlands. Accessions 29, 10, 21, 32, 38 and 23 are good candidates for genetic improvement due to their high protein, Ca, Mg and P contents.

Apart from the three research undertakings included in this thesis (Papers I, II and III), other research activities on the 43 *Festuca* populations were pursued. The first is related to the identification of *Festuca* species. However, due to incomplete descriptions of morphological and anatomical characters for *Festuca* species in the taxonomical key (Renvoize, 1998), it was impossible to identify most genotypes with certainty at a species level. Hence, foliar anatomy and micromorphology (Fig. 8) are being investigated to generate more data that will contribute to accurate identification of the genotypes of Bolivian fescues at species level.

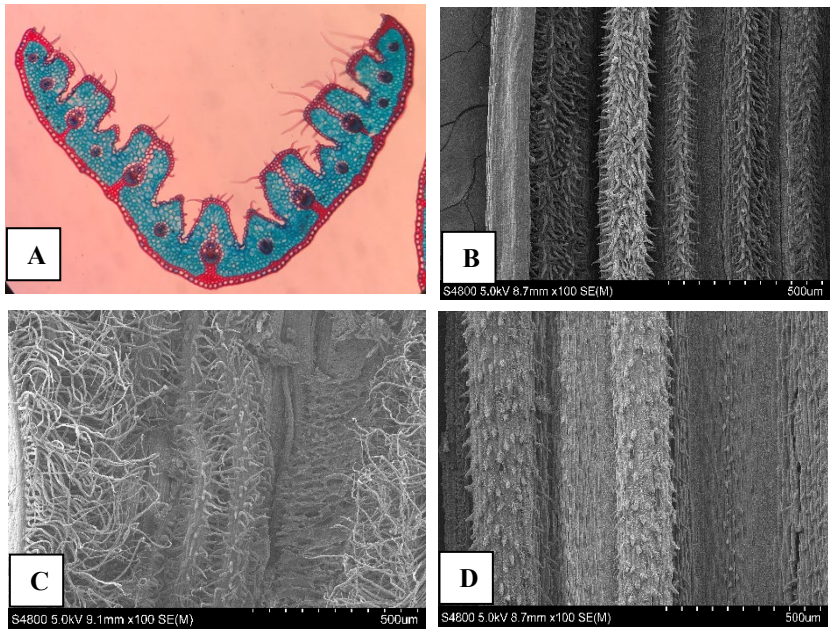

Figure 8. (A) Leaf cross section of *Festuca* genotype sampled from Accession 2; (B, C and D) Scanning electron microscopy images of adaxial leaf-blade epidermis of fine-leaved and broad-leaved *Festuca* species represented by genotypes from Accessions 8, 27 and 23. Scale bars = 500 µm

Another study conducted during two consecutive years (2017 and 2018) at the experimental field site (CEAC, Oruro) was to describe the morphological characters of the 43 Bolivian fescue populations and the two Argentinian cultivars (research still ongoing with data analysis). Once the research undertakings indicated above are completed, there will be enough knowledge to start a breeding program to be led by the Centro de Investigación en Forrajes (CIF)-La Violeta, UMSS, Cochabamba, Bolivia.

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Popular science summary

Bolivia is a country located at the centre of South America. It is geographically diverse containing a very high biological diversity. The highland region (also called Andean region), covers around the 28 % of the national territory and consists of a mountainous zone formed by the Western Cordillera and the Eastern or Royal cordillera together with a flat highland area. The average altitude of the highland region reaches 3750 meters above sea level; it has a relatively dry and cold weather with an average annual temperature of 8.6°C. In this region, native grasslands represent a main food supply for camelids and ruminants, especially in areas where the altitude is over 4000 metres above sea level and the annual rainfall is below 350mm.

Within the native grasslands, the genus *Festuca* constitutes a very important forage, playing a critical role for the livestock systems especially during the winter season, where most of the vegetation disappears and only few forage plants are available for animals. The two most important species of the *Festuca* genus present in the highland region are denominated by farmers as “iru ichu” (*F. orthophylla*) and “chilliwa” (*F. dolichophylla*).

In Bolivia scant research has been conducted on *Festuca* and there is a need to investigate more about this genus in order to stablish a plant breeding program that will contribute to a sustainable use and germplasm conservation. In this research, we want to contribute with scientific knowledge about this genus with the aim to start a breeding program that will allow us to develop a forage grass of high quality and yield for the use of farmers in the highland region.

Populärvetenskaplig sammanfattning

Bolivia ligger i centrala Sydamerika och har en varierande geografi med en mycket hög biologisk mångfald. Höglandsregionen täcker cirka 28% av det nationella territoriet och består av ett bergsområde som bildas av östra och västra kordiljärerna med ett mellanliggande platt högland. Höglandsregionens genomsnittliga höjd når 3750 meter över havet; det har ett relativt torrt och kallt väder med en genomsnittlig årstemperatur på 8,6 ℃. I denna region utgör de naturligt förekommande gräsmarkerna en viktig matförsörjning för kamelider och idisslare, särskilt i områden där höjden är över 4000 meter över havet och den årliga nederbörden är under 350 mm.

På de inhemska gräsmarkerna utgör arter inom släktet *Festuca* ett mycket viktigt foder, och spelar en avgörande roll för djurhållningssystemen, särskilt under vintersäsongen, då större delen av vegetationen försvinner och endast få växter finns tillgängliga som foder till djuren. De två viktigaste arterna i släktet *Festuca* på höglandet benämns av jordbrukare som "iru ichu" (*F. orthophylla*) och "chilliwa" (*F.dolichophylla*).

I Bolivia har så gott som ingen forskning genomförts på *Festuca* och det finns ett behov av större kunskap om detta släkte för att kunna etablera ett förädlingsprogram som kan bidra till en hållbart nyttjande och bevarande av det genetiska materialet. Genom denna forskning vill vi bidra med vetenskaplig kunskap om detta släkte med syftet att starta ett förädlingsprogram som gör att vi kan utveckla ett fodergräs av hög kvalitet och avkastning för användning av jordbrukare i höglandsregionen.

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