Turun yliopisto
University of Turku

GRASS-ENDOPHYTE COEVOLUTION AND PLOIDY LEVELS IN FESCUES

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TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. A II osa - tom. 315 | Biologica - Geographica - Geologica | Turku 2016

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The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6465-9 (PRINT)
ISBN 978-951-29-6466-6 (PDF)
ISSN 0082-6979 (PRINT)
ISSN 2343-3183 (ONLINE)
Painosalama Oy - Turku, Finland 2016

To Roda

This thesis is based on the following publications and manuscripts, referred to in the text by their Roman numerals:

I. Dirihan, S., Terho, P., Helander, M. and Saikkonen, K. (2013). Efficient analysis of ploidy levels in plant evolutionary ecology. *Caryologia International Journal of Cytology, Cytosystematics and Cytogenetics* 66, 251-256.

II. Dirihan, S., Helander, M., Väre, H., Gundel, P.E., Garibaldi, L.A., Irisarri, J.G.N., Saloniemi, I. and Saikkonen, K. Geographic variation in *Festuca rubra* L. ploidy levels and systemic fungal endophyte frequencies. *Manuscript*.

III. Gundel, P.E., Dirihan, S., Helander, M., Zabalgogeazcoa, I., Väre, H. and Saikkonen, K. (2014). Systemic fungal endophytes and ploidy level in *Festuca vivipara* populations in North European Islands. *Plant Systematics and Evolution* 300, 1683-1691.

IV. Dirihan, S., Helander, M., Saloniemi, I., Gundel, P.E. and Saikkonen, K. (2014). Effects of systemic fungal endophyte on the performance of meadow and tall fescues in mixtures with red clover. *Grass and Forage Science* 70, 465-473.

V. Dirihan, S., Väre, H., Helander, M., Saloniemi, I. and Saikkonen, K. Morphological characters, ploidy and subspecies of *Festuca rubra* L. across Europe. *Manuscript*.

CONTENTS

ABSTRACT 8

TIIVISTELMÄ 9

1. INTRODUCTION 10
   - 1.1. Polyploidy in grass species 11
     - 1.1.1. The impact of polyploidy 12
     - 1.1.2. Plant ploidy level determination 13
   - 1.2. Grass-endophyte symbiosis 14
     - 1.2.1. Abiotic factors 14
     - 1.2.2. Biotic factors 15
     - 1.2.3. Genetic factors and endophyte-grass interactions in an evolutionary context 16
   - 1.3. Aims of the thesis 17

2. MATERIALS AND METHODS 19
   - 2.1. Plant material 19
   - 2.2. Study sites and environmental conditions of natural populations 20
   - 2.3. Study system 21
     - 2.3.1. Endophyte detection 21
     - 2.3.2. Ploidy level determinations 22
     - 2.3.3. Plant morphology, polyploidy and endophyte infection status of European red fescue 23
     - 2.3.4. Effects of endophytes on grass-legume mixtures 23
     - 2.3.5. Statistical analysis 24

3. RESULTS 26
   - 3.1. Endophytes of wild fescues 26
   - 3.2. Ploidy level determinations in plants 26
     - 3.2.1. An efficient ploidy level determination for plant evolutionary ecology 26
     - 3.2.2. Ploidy level of wild fescue populations 26
   - 3.3. Effects of endophytes on grass-legume mixtures 27
   - 3.4. Taxonomy, plant morphology, polyploidy and endophyte infection status of European red fescue 27

4. DISCUSSION 29
   - 4.1. Endophytes of wild fescues 29
   - 4.2. Ploidy levels of plants 31
     - 4.2.1. An efficient ploidy level determination for plant evolutionary ecology 31
     - 4.2.2. Ploidy levels of wild fescue populations 31
   - 4.3. Endophyte mediation of grass-legume mixtures 32

4.4. Taxonomy, plant morphology, polyploidy and endophyte infection status of European red fescue 33

5. CONCLUSIONS 34

ACKNOWLEDGEMENTS 35

REFERENCES 37

ORIGINAL PUBLICATIONS 43

ABSTRACT

Fescues consist of wild and cultivated grasses that have adapted to a wide range of environmental conditions. They are an excellent model species for evolutionary ecology studies that investigate symbiosis and polyploidization and their effects on plant performance. First, they are frequently infected with symbiotic endophytic fungi known to affect a plant's ability to cope with biotic and abiotic environmental factors. Second, fescue species have been reported to have substantial intraspecific variation in their ploidy level and morphology. In my thesis, I examined large-scale generalizations for frequency of polyploidy and endophyte infections and their effects on plant morphology.

As a model species, I selected red (*Festuca rubra*) and viviparous sheep's (*F. vivipara*) fescues. They are closely related, but they differ in terms of distribution and endophyte infection frequency. I investigated the biogeographic pattern and population biology of 29 red and 12 viviparous sheep's fescue populations across ≈30^0 latitudes in Europe (40^0-69^0 N).

To examine plant ploidy levels, I implemented time- and cost-efficient plate-based high throughput flow cytometric analysis. This efficient procedure enabled me to analyze over 1000 red fescue individuals. I found three ploidy levels among them: overall 84 %, 9 % and 7 % of the red fescue plants were hexaploid, tetraploid and octoploid, respectively. However, all viviparous sheep's fescue plants were tetraploid. Ploidy level of red fescue appeared to some extent follow gradients in latitude and primary production as suggested by previous studies, but these results could be explained better by taking the sampling design and local adaptation into account. Three Spanish populations were mostly tetraploids and one high elevation population in northernmost Finland (Halti) was octoploid, while most other populations (25 sites) were dominated by hexaploids.

Endophyte infection frequencies of wild fescue populations varied from 0 to 81 % in red fescue populations and from 0 to 30 % in viviparous sheep's fescue populations. No gradients with latitude or primary production of the sites were detected.

As taxonomy of red fescues is somewhat unclear, I also studied morphology, ploidy variation and endophyte status of proposed subspecies of European red fescues. Contrary to previous literature, different ploidy levels occurred in the same subspecies.

In addition to wild fescues, I also used two agronomically important cultivars of meadow and tall fescue (*Schedonorus phoenix* and *S. pratensis*). As grass-legume mixtures have an agronomic advantage over monocultures in meadows, I carried out a mixture/competition experiment with fescues and red clover to find that species composition, nutrient availability and endophyte status together determined the total biomass yield that was higher in mixtures compared to monocultures.

The results of this thesis demonstrate the importance of local biotic and abiotic factors such as grazing gradients and habitat types, rather than suggested general global geographical or environmental factors on grass polyploidization or its association with symbiotic endophytic fungi. I conclude that variation in endophyte infection frequencies and ploidy levels of wild fescues support the geographic mosaic theory of coevolution. Historical incidents, e.g., glaciation and present local factors, rather than ploidy or endophyte status, determine fescue morphology.

TIIVISTELMÄ

Nadat ovat monenlaisiin ympäristöolosuhteisiin sopeutuneita luonnossa kasvavia ja viljeltäviä heiniä. Ne ovat erinomaisia evoluutiobiologian mallilajeja evoluutioekologisiin tutkimuksiin joissa tutkitaan symbioosia, polyploidiaa ja niiden vaikutusta kasviin. Ensinnäkin ne ovat usein infektoituneita symbionttisilla endofyyttisillä sienillä, joiden tiedetään vaikuttavan kasvin kykyyn selviytyä erilaisissa bioottisissa ja abioottisissa ympäristöissä. Toiseksi nadoilla on havaittu runsaasti lajinsisäistä vaihtelua ploidiatasoissa ja morfologiassa. Väitöskirjatyössäni tutkin endofyytti-infektio- ja polyploidiafrekvenssejä, niihin vaikuttavia syitä ja niiden vaikutuksia natojen morfologiaan.

Mallilajiksi valitsin punanadan (*Festuca rubra*) ja itunadan (*F. vivipara*). Ne ovat lähisukuisia lajeja, mutta eroavat levinneisyydeltään ja endofyytti-infektiofrekvenssiltään. Tutkin 29 punanata- ja 12 itunatapopulaation biogeografista esiintymistä ja populaatiobiologiaa ≈30 leveysastetta kattavalla alueella Euroopassa (40^0-69^0 N).

Tarkastellakseni kasvien ploidia asteita käytin kustannustehokasta kuoppalevy-virtaussytometria menetelmää. Tämä aikaa ja materiaaleja säästävän analyysitavan avulla pystyin määrittämään luonnosta keräämieni yli 1000 kasviyksilön ploidia-asteen. Löysin näytteistäni kolme ploidia-astetta: punanadoista 84 % oli heksaploideja, 9 % tetraploideja ja 7 % oktoploideja. Sen sijaan kaikki itunadat olivat tetraploideja. Punanatojen ploidia-aste näyttää jossain määrin korreloivan leveysaste- ja tuottavuusindeksien maantieteellisten etelä-pohjoissuuntaisten gradienttien kanssa, kuten on esitetty aikaisemmissa tutkimuksissa. Omat tulokseni voidaan kuitenkin paremmin selittää, lajien sopeutumislevittäytymisellä aluemaantieteellisesti laajalla otanta-alueella sekä paikallisella sopeutumisella vallitseviin olosuhteisiin. Kolme espanjalaista populaatiota olivat lähinnä tetraploideja, yksi korkealla sijaitseva populaatio Pohjois-Suomessa (Halti) oli oktoploidi, kun taas kaikki muut 25 populaatiota, olivat suurimmaksi osaksi heksaploideja.

Endofyytti-infektiot luonnosta kerätyissä punanatapopulaatioissa vaihtelivat 0 % ja 81 % välillä ja itunatapopulaatioissa 0 % ja 30 % välillä. Endofyytti-infektion, leveysasteiden ja perustuotannon suhteen ei havaittu yhteyttä.

Koska punanatojen taksonomia on jossain määrin epäselvä, tutkin myös eurooppalaisten punanatojen alalajien morfologian, ploidia-asteen vaihtelun ja endofyytti-infektion välistä suhdetta. Toisin kuin aikaisemmassa kirjallisuudessa on esitetty, samassa alalajissa esiintyi eri ploidia-asteita.

Luonnosta kerättyjen natojen lisäksi tutkin myös kahta maataloudessa tärkeää nurminata- ja ruokonatalajiketta (*Schedonorus phoenix* ja *S. pratensis*). Koska heinä-apilaseosten on esitetty olevan parempia kasvustoja niityillä kuin monokulttuurien, toteutin nata- ja puna-apila-kilpailukokeen, jossa havaittiin että heinälaji, ravinteiden saatavuus ja endofyytti-infektio yhdessä määrittivät totaalibiomassan tuotannon, joka oli korkeampi seoksissa kuin monokulttuureissa.

Tämän väitöskirjatyön tulokset painottavat paikallisten bioottisten ja abioottisten tekijöiden merkitystä verrattuna suurempiin globaaleihin maantieteellisiin tai ympäristötekijöiden vaikutuksiin heinien polyploidian määräytymisessä tai endofyyti-infektiofrekvensseissä. Luonnonnatojen endofyytti-infektioiden ja ploidia-asteen vaihtelu tukevat koevoluution maantieteellistä mosaiikkiteoriaa. Historialliset tapahtumat, kuten jääkausi, ja nykyiset paikalliset tekijät ennemmin kuin ploidia tai endofyyttistatus määräävät natojen morfologiaa.

1. INTRODUCTION

Darwin (1859) used the concept of an “entangled bank” to describe the important role of species interactions in the evolutionary process. He emphasized the central role of species interactions in diversity as well as the impact of species diversity in evolution of biological communities. From cooperation to competition, interactions enhance the diversity of life via specialization and coevolution under local selective forces. Multispecies interactions create mosaics among the same species at different locations due to their different spatial interaction history (Thompson 1994). As primary producers, terrestrial plants have initial roles for food webs energy cycles in ecosystems. Co-evolutionary processes of plants include herbivory, plant-plant interactions, nectar-pollination reciprocity, and ecto- and endo-symbiosis with soil- and aboveground-microbiota. For instance, plant-mycorrhizae symbiosis has played a major role for colonization of terrestrial plants. In this ancient relationship, soil nutrients become more acquirable for plants, while mycorrhizal fungi obtain photosynthetically fixed nutrients from the plant (Corradi and Bonfante 2012).

Selection and specialization can favor certain types of symbiont life histories (Thompson 2005). For example, the type of transmission of endophytes may be modified by symbiotic interactions. Some grass-endophyte interactions have evolved from horizontal- to vertical-transmission (see Fig. 1). This alteration is assumed to be a transition from parasitic to mutualistic symbiosis leading to a host-endosymbiont fitness dependency in the vertical transmission of endosymbiont (Lipstch 1995). Vertically-transmitted endophytes receive shelter, nutrients and transmission from host grasses, while grasses obtain alkaloids from endophytes for protection against herbivores. Endophytic interactions can improve also other traits that benefit host-plants, e.g., drought- and flood-resistance and through competitive strength (Clay 1990, Wilson 1995). However, long-term empirical studies have shown that symbiotic interactions are labile varying from antagonistic to mutualistic depending on biotic, abiotic and genetic factors (Saikkonen et al. 1998).

Polyploidy, mutational whole genome duplication is common in plants and especially in grasses (Otto and Whitton 2000). Polyploidy may be an initial stage for an adaptive divergence process (Otto and Whitton 2000) and a significant determiner of species geographic and ecological range (e.g., Brochmann et al. 2004, Ramsey and Ramsey 2014).

Grasses are excellent models to investigate endophyte infection and polyploidy in the same study system to elicit ecological and geographic patterns in an evolutionary context. Variation in endophyte infection and polyploidy of grasses have been well-documented separately. In earlier studies, similar explanatory concepts and response variables have been used in both study systems, including latitudinal/altitudinal/ environmental gradients, life history traits of plants, vigourness, resistance and an adaptation ability to biotic and abiotic conditions. Both phenomena have been studied in terms of glaciation periods. Glaciation may have limited the time-scale of evolution and thus constrained the number of species interactions in these regions (e.g., Stebbins 1984, Brochman et al. 2004, Borer et al. 2012).

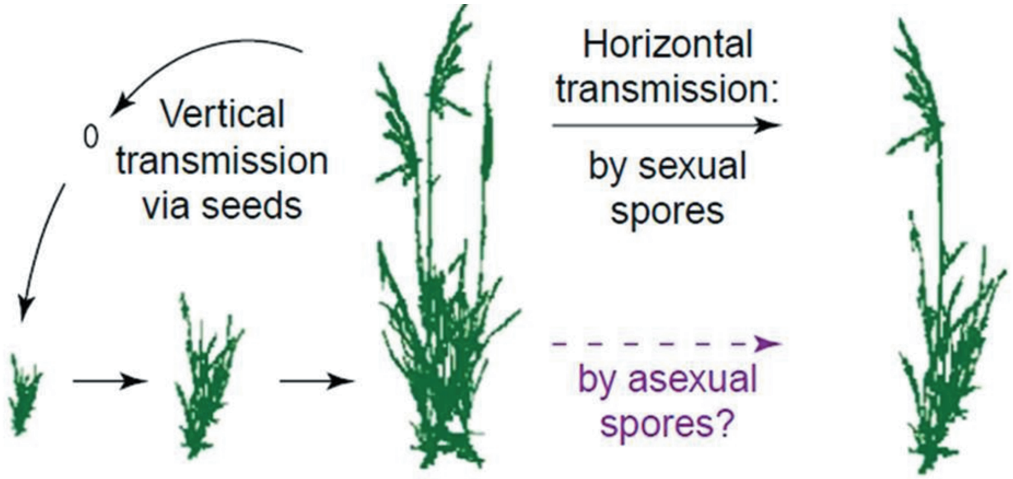

Figure 1. Transmission types of grass endophytes. Vertically-transmitted endophytes are transmitted via host seeds from parental plant to offspring, while horizontally transmitted endophytes reproduce by spores. Reused from Saikkonen et al. (2004) with the permission of Elsevier.

In this thesis I integrated the investigation of endophyte infection and polyploidy of grasses using fescue grass species as models. Beside ecological and taxonomical frameworks, the findings of this thesis are applicable to agricultural and other man-made purposes where fescue grass species are widely used. The plant breeding industry has frequently used ploidy in inter- and intra-specific hybrid production. Similarly plant breeders have been interested in endophytes as a biological control agent and recently “symbiotically modified organisms” (SMO) have been suggested to have great potential in plant production (Gundel et al. 2013). SMO applications are based on the manipulation of endophyte infection by infecting naturally- or manipulatively-uninfected plants with known endophyte strains which can increase yield-related features such as plant growth, reproduction, resistance against diseases and pests and plant adaptability to different abiotic conditions. Since these agricultural exploitations of endophytes could decrease the use of chemical fertilizers and pesticides, SMO applications could be used in sustainable, environmentally friendly and organic farming practices (Kauppinen et al. 2016).

1.1. Polyploidy in grass species

Grasses, “Graminae”, are a large family in the plant kingdom with approximately 10,000 species. Grasses are mostly perennial or annual herbs (Rendle 1953) including economically important cereals and forage plant species. Beside their economic importance, grasses display a wide range of adaptation to extremely different environmental conditions from aquatics to desert with high variation both among and within species (Cheplick 1998). Polyploidy has been detected in most of the grass species (Otto and Whitton 2000). The wide adaptation potential of grasses might be partly related to polyploidy via an impact on physiological and life-history traits, which may even cause differences in geographical and environmental range of species (Ramsey and Ramsey 2014).

Different types of polyploidy are observable in nature. Autopolyploidy is duplication of a single genome (AA➔AAAA, duplication of the same chromosome set), and their meiotic appearance is multivalent. Allopolyploids have two distinctive genomes that arise from most likely a result of hybridization between two species. Their meiotic appearance is bivalent (AB➔AABB). In the third type, segmental allopolyploidy, the genomes are partially differentiated, thus both bivalents and multivalents can be seen in meiosis (AA'➔AAA'A') (Briggs and Walters 1997, Levin 2002, Comai 2005).

1.1.1. The impact of polyploidy

Polyploidization increases the number of alleles, which has been suggested to enhance adaptation potential to different environmental conditions (Comai 2005) including environmental extremes in high latitudes and altitudes (Stebbins 1985, Otto and Whitton 2000). Polyploidization causes genome redundancy where extra gene copies may gain new functions (neofunctionalization) (Comai 2005). Plants with a higher ploidy level have been suggested to promote competitiveness/invasiveness (Pandit et al. 2014), to be more tolerant of unfavorable conditions such as cold (Hagerup 1933), drought (Stebbins 1972) and salinity and have better nutrient uptake (Chao et al. 2013). Since polyploidization causes an increase in cell size and number of alleles, variation in ploidy level might be reflected in morphological characters and taxonomy (Stace 1989, Husband et al. 2013).

Correlations between environmental conditions and the level of ploidy are detected in many studies (Stebbins 1971, Adams and Wendel 2005, Yang et al. 2011, Ramsey 2011). Polyploidy has been found to be rare in subtropics and temperate areas, whereas it becomes more common towards higher latitudes (Löve and Löve 1957, Hair 1966, Stebbins 1971). Polyploids are believed to be more fit in harsh climatic conditions and at higher latitudes (e.g., Brochmann et al. 2004, Ramsey 2011). However, more detailed studies have found an inconsistency in the association between polyploidy and latitude. According to Stebbins (1984), glaciation history of the regions increases polyploidization, and Brochman et al. (2004) found that the association between latitude and polyploidy only in arctic specialist taxa. Neiman et al. (2013) has suggested that the high availability of phosphorous compared to nitrogen at high latitudes may be a key factor for polyploids. More phosphorus may be needed for producing higher DNA content of cells.

Polyploidy may affect plant colonization (Sampoux and Huyghe 2009), and it may cause changes in ecological requirements (Levin 2002). These may promote wider distribution and speciation (Briggs and Walters 1997). Coexistence of different ploidy levels in a population has been regarded as an early phase of polyploid speciation (Husband et al. 2013). Mixed-ploidy populations are expected to be evolutionarily unstable, as mating between different ploidies (Husband 2000) may produce unfit hybrids (Felber 1991). However, in some cases, habitat segregation within the population and phenological asynchrony of different ploidy levels may allow the persistence of different ploidy levels in the same populations (Rodriguez 1996). Polyploidization may also affect species interactions that may cause reproductive segregation and hybridization barriers among plants with different ploidies (Husband and Sahara 2004). For instance, differences in insect visitor composition in pollination (Segraves and Thompson 1999) and insect foraging patterns may correspond to ploidy associated morphological variation (König et al. 2014). Reproductive segregation can be strengthened by flowering asynchrony among different ploidy levels (Jersakova et al. 2010).

Polyploidy may cause changes in morphology and/or physiology of plants (Levin 2002) like altered plant leaf (e.g., Byrne et al. 1981) and flower morphology (e.g., Padoan et al. 2013). Chemically-induced polyploids have revealed ploidy-based morphological alterations in many plant species (e.g., Liu et al. 2007), and to some extent, these alterations have been found also in natural populations (Sampoux and Huyghe 2009). A higher ploidy level may enhance the vigourness of plants (Sampoux and Huyghe 2009). Variation in ploidy level can thus cause variation relevant for taxonomy (Stace 1989, Levin 2002).

1.1.2. Plant ploidy level determination

Ploidy level determination studies started in the early 20th century after the realization that chromosome number is species specific (Stace 1989). During the 1920 to 1930's, karyological studies on plants considerably increased with the technological advances (e.g., Belling 1921, Hillart 1938, O'Mara 1939, Schmuck and Kostoff 1939). Enzyme-included procedures allowing breakage of plant cell-walls and staining the nucleus, e.g., using snail stomach cytase (Faberge 1945), significantly enhanced the efficiency of plant-ploidy research. However, all these methodological innovations required time-consuming microscopic studies, which limited the sample sizes of the studies.

In 1934 the first primitive flow cytometer (FCM) was introduced, and today this technique is commonly used in plant ploidy level estimations due to its considerable advantage over microscopic ploidy determination. This method has recently been improved to allow detailed analysis, e.g., chromosome sorting (Vrána et al. 2012). FCM is a method which is based on measuring optical features such as fluorescence of flowing isolated particles (e.g., cells, nuclei) in a narrow liquid stream with a strong beam of light (Suda 2004). Although FCM has been applied in medicine since 1949 (Doležel 1991), the widespread use of FCM in plant polyploidy studies started only during the 1980s. This delay was mainly caused by the difficulties in obtaining single-particle suspension of nuclei.

To date, a tube-based FCM procedure has been commonly used for plant ploidy level determination. However novel and higher throughput FCM devices provide acceleration especially for large sample-sized research. These devices allow the use of well-plates with automated tools. However, adequate automated devices were not applicable for plants with lysis difficulties due to their leaf/stem tissue compounds (e.g., silica (Si) on grasses). Cytological FCM studies commonly suggest that the number of nuclei should be more than 5000 and coefficient variation (CV) of each measurement should be less than 5 % (e.g., Bennett and Leitch 1995, Doležel et al. 2007, Greilhuber et al. 2007). Recent studies have shown that plate-based FCM ploidy analysis could meet resolution requirements in some plant species, e.g., in Brassica spp. (Cousin et al. 2009), while Poaceae plants remained difficult to analyze. In addition, FCM-based ploidy research requires proper gating in the running process and practical software for analysis of cytometric data.

1.2. Grass-endophyte symbiosis

The concept of symbiosis is a long-term and adjacent interaction between different species (de Barry 1887). It can be classified as mutualistic (where both sides benefit), parasitic (where one side benefits, while the other is harmed) and commensalistic (where one species benefits, but the other neither benefits nor is harmed) (Begon et al. 1996). Symbiotic interactions can be either obligate or facultative. In obligate symbiosis, host survival is necessary for the partner, and in facultative symbiosis, the partner can continue its life cycle without the host (Sapp 1994). Systemic fungal endophyte associations of grasses are often obligate (Isaac 1992).

Grass endophytes can be either systemic or non-systemic. While systemic endophytes are vertically transmitted via host seeds, non-systemic endophytes are horizontally transmitted via spores by wind or vectors such as insects (Fig. 1). Serpentine hyphae of systemic grass endophytes grow intercellularly in the leaf and stem of plant (Christensen and Voisey 2007). Until recently, systemic grass endophytes were named *Neotyhphodium*, and non-systemic endophytes, *Epichloë*. Now both of them are classified in the genus *Epichloë* (Leuchtmann et al. 2014).

Many studies have tested different host and systemic endophyte genotype combinations in various environments. Especially in natural ecosystems, they have demonstrated different outcomes in terms of biomass, competitive ability of plants, reproduction and resistance to stress factors of the symbiosis (Saikkonen et al. 2006, Cheplick and Faeth 2009) depending on abiotic, biotic and genetic factors (Muller and Krauss 2005).

1.2.1. Abiotic factors

Abiotic factors such as nutrient level (Saikkonen et al. 2006, Newman et al. 2003), temperature (Marks and Clay 1996), water availability (Morse et al. 2007, Nagabhyru et al. 2013), soil pH (Cheplick 1993), heavy metal pollution (Soleimani et al. 2010) and light quality (Newsham et al. 1998) have been shown to affect grass-endophyte interactions. Mutualist systemic fungal endophytes are considered as augmenters for a species range of grasses in variable environments (Clay 1990, Cheplick and Faeth 2009, Afkhami et al. 2014, Wani et al. 2015).

Host plants may benefit from endophyte infection more in a nutrient-rich environment than in resource-limited conditions (Cheplick et al. 1989, Saikkonen et al.

2006). Some studies have shown that better nutrient uptake and accumulation depend on the host genotype and soil nutrient composition (Richardson et al. 1999, Sullivan et al. 2007). Nitrogen requirement of endophyte infected (E+) plants can be higher than that of uninfected (E-) plants due to nitrogen based alkaloid production of endophytes. The presence of endophytes can alter the resource allocation strategy of plants from tolerance to defense depending on the nitrogen availability of the soil (Sullivan et al. 2007). Endophyte infection has been shown to improve metal uptake and metal transportation in grasses (Soleimeni et al. 2010, Ren et al. 2007). E+ grass fitness is reduced during higher levels of UV radiation compared to E- plants suggesting that ozone depletion may have a more negative impact on E+ host species than E- counterparts (Newsham et al. 1998).

Biotic interactions have been shown to decrease by latitude (e.g., Schemske et al. 2009) or by environmental severity (Malinowski and Belesky 2006). In addition, some other factors such as species evolutionary history, environmental conditions and their interactions may also affect endophyte infection frequencies. For example, an altitudinal gradient has been observed to be negatively correlated with infection frequency of red fescue but not that of other fescues (Bazely et al. 2007, Granath et al. 2007), and Saikkonen et al. (2000) found that endophyte frequencies may be very low in southern Finland but high in some populations in northern Finland.

1.2.2. Biotic factors

Any living organism in the environment such as a herbivore, a plant competitor, a soil microbe or a plant parasite may interact with the grass-systemic fungal endophyte symbiosis. Among these, herbivory is the most intensively studied topic (Cheplick and Faeth 2009).

The endophyte-produced alkaloids (e.g., ergots, lolines, lolitrems and pyrroloprazines) are considered defensive chemicals against herbivores (Cheplick and Faeth 2009, Saikkonen et al. 2010). Some studies have suggested that grazing can affect the endophyte frequency of grasses. For example, Granath et al. (2007) showed that endophyte infection frequencies of red fescue populations positively correlated with grazing pressure. While endophyte infection often negatively affects herbivore abundance, in some cases it may positively affect herbivory in multitrophic complexity of natural grasslands, e.g., if endophyte-produced alkaloids deter the natural enemies of herbivores rather than herbivores (Omacini et al. 2001, Cheplick and Faeth 2009). Infected plants may even attract herbivores to escape from their predators via creating chemical shelter/space. Herbivore abundance might be correlated with plant genotype and local abiotic factors rather than endophyte produced alkaloids (Jani 2005). Different endophyte strains can cause alkaloid variation in the plant population thus altering relative abundance of herbivore species (Cheplick and Faeth 2009).

Systemic fungal endophytes of grasses can enhance competition of their host grass via positively affecting growth and reproduction (Hill et al. 1991, Marks et al. 1991, Malinowski et al. 1997, Clay and Holah 1999, Brem and Leuchtmann 2002, Rudgers et al. 2005) or causing allelopathy (chemical inhibition of organisms) for the neighboring plants (Hoveland et al. 1999, Malinowski et al. 1999, Vázquez de Aldana et al. 2012). However, different outcomes can be expected for intra- or inter-specific competition ability of E+ plants (Cheplick and Faeth 2009). Indeed, several studies have shown neutral and even negative effects of the endophytes on competitive ability of host grasses (Hoveland et al. 1997, Brem and Leuchtmann 2002, Faeth et al. 2004, Saari et al. 2010).

Despite that, endophytes are present in aboveground tissues of plants, their effects can be seen on soil microbiota, decomposition rates and in nutrient cycling. Some studies have shown that endophytes can inhibit mutualistic mycorrhizae and decrease the biomass of the host plant (e.g., Omacini et al. 2012). Endophyte infections are often found as a retarder for decomposition rates (Omacini et al. 2004, Siegrist et al. 2010).

Plant succession could be driven by endophyte infection due to its effects on competitive strength of host grass, herbivory, soil microbiota and ecosystem functions which are major determiners of plant composition (Rudgers et al. 2007).

1.2.3. Genetic factors and endophyte-grass interactions in an evolutionary context

Beside the direct effects of host and endophyte genotype, their mutual interactions and interactions with the environment determine the outcome of the grass-endophyte symbiosis (Cheplick and Faeth 2009, Saikkonen et al. 2010). Many studies have shown significant host genotype x endophyte interactions in variety of traits (e.g., Cheplick and Cho 2003, Hesse et al. 2004, Spiering et al. 2006). Moreover, a recent study showed that endophyte effectiveness on grass competitive ability depends on host genotype (Cheplick et al. 2014). The outcrossing sexual system of grasses causes genetic mismatches between endophytes and hosts causing variation in selection pressures in next generations (Saikkonen et al. 2004). Some systemic infections may drive the plants to vegetative reproduction to sustain suitable endophyte-host combinations (Pan and Clay 2002).

A symbiotic association can shift from mutualism to antagonism or vice versa in an evolutionary time-scale (Thompson 1994). Phylogenetic studies (Sachs and Simms 2006) have shown that a host plant may abandon the endophyte symbiosis (Cheplick and Faeth 2009), as this symbiosis is obligatory only for the fungus. Grass-endophyte symbiosis may lead to a neutral association called commensalism (Clay 1988, White 1988). For example, since E+ grasses allocate more nitrogen to defense than growth, weaker nitrogen availability in soil may favor E- plants (Sullivan et al. 2007).

The endophyte-grass interaction is considered an “arms race” evolutionary processes (Thompson 1994, Thompson 2005). Although systemic fungal endophyte-grass interactions have features of pathogenic, parasitic and saprophytic associations besides being mutualistic, this interaction is often treated as a mutualistic interaction. Instead of benefits to the symbionts, the interactions often consist of “mutual exploitations”, or the symbionts are asymmetric (Saikkonen et al. 2004). Growth of the fungal endophyte is regulated by hormonal activity and chitinase production of the host plant suggesting that plants can set sanctions against endophytes (Marshall 1990). Moreover, a loss of sexuality in systemic fungal endophytes of grasses is suggested to be a result of a coevolutionary process. The asexual endophyte is also able to increase asexual reproduction of the host plant by increasing vegetative propagation, inducing vivipary or pseudovivipary (Faeth and Sullivan 2003) or vegetative propagation (tillering) (Pan and Clay 2002). Mycotoxin production of endophyte for host defense is well-documented for agronomic grasses, but this mechanism is rare for natural grass and tree species (Faeth and Sullivan 2003). In natural populations, selective forces are more variable than that in agricultural systems. Thus conflicting effects of selection forces are more likely to occur in natural populations than agroecosystems. Moreover, the integration and specialization level of symbionts are higher in agroecosystems because of human-driven selection. The evolution of systemic fungal endophytes has multi-species and multi-directional biotic and abiotic influences (Saikkonen et al. 2004).

Genetic mismatch between grass and endophytes is an important destabilizing force in the evolution of symbiosis (Saikkonen et al. 2004, Saikkonen et al. 2010). For example, in a new habitat, an outcrossing of plants increases mismatches and incompatibilities between host and endophytes, whereas in established grass populations, successful grass endophyte combinations can persist. Low genetic variability and fertilization cause cultivars in agroecosystems to maintain their matched endophytes (Saikkonen et al. 2004).

Still E- and E+ plants can be found in the same natural grass population. There are several explanations for this phenomenon: (1) genetic mismatches between endophytes and grasses, (2) seed immigration among different grass populations (gene-flow) (Gundel et al. 2008), and (3) imperfect vertical transmission of endophytes (failure of endophyte colonization to plant seeds or vegetative reproductive parts of plants) (Ravel et al. 1997, Saikkonen et al. 2002, Gundel et al. 2009). Interactions among migration, local selection and genotype combinations shape geographical mosaics where some populations represent hot spots (reciprocal local selection) and the others display non-reciprocal local selection (cold spots) for the symbiosis. Reciprocal local selection denotes an “arms race” in species interactions as evolutionary drivers for both species (Thompson 2005).

1.3. Aims of the thesis

In this thesis I focus on ecological interactions between endophyte infection and polyploidy in fescue grass species. The aims of the thesis were to answer the following questions:

1) How do endophyte infection frequencies and ploidy levels of wild grasses vary across latitudes and environmental conditions?

Previous studies suggest endophyte infection to be related with environmental conditions (Bazely et al. 1997, Saikkonen et al. 2000, Wäli et al. 2007, Bazely et al. 2007, Granath et al. 2007), and a recent study suggested that primary production can affect endophyte infection frequencies of grasses in a global scale (Semmartin et al. 2015). The ploidy level of plants is expected to increase by environmental severity and latitudes (Löve and Löve 1957, Hair 1966, Stebbins 1971, Ramsey 2011, Ramsey and Ramsey 2014).

To study the connection between environmental factors, endophyte infection frequencies and ploidy levels, 29 wild red fescue populations were studied across Europe from Spain to northernmost Finland and Greenland (Study **II**), and in 12 wild viviparous sheep's fescue populations from northern European Atlantic islands (Study **III**). Also the phylogeny tree of endophytes of viviparous sheep's fescue were determined in northern European islands (Study **III**).

2) How to determine ploidy level of individual plants efficiently?

I used large plant sample sizes in the ploidy level determination studies of this thesis. Since microscopic chromosome number detection is time-consuming, tube-based FCM devices are used for ploidy level estimation in plant research (Bennett and Leitch 1995, Doležel et al. 2007, Greilhuber et al. 2007). I applied a recently developed fully automated high throughput FCM devices in study **I**.

3) Do endophytes influence the performance of grass-legume mixtures in an agricultural arena?

I studied performance of fescues with or without endophytes in competition with red clover (Study **IV**). Species mixtures, instead of monocultures, have been suggested to produce better forage quality and enhance yield and biodiversity in agricultural areas (Ledgard 1991, Bulson et al. 1997, Malézieux et al. 2009). Previous studies showed that systemic grass endophytes can enhance host plant's growth either directly (Hill et al. 1991, Malinowski et al. 1997, Clay and Holah 1999, Rudgers et al. 2005) or via allelopathic effects on competitors (Vázquez de Aldana et al. 2012). Beside these positive effects, several studies have shown neutral to negative effects of endophytes on the competitive ability of the host plants (Hoveland et al. 1997, Brem and Leuchtmann 2002, Faeth et al. 2004, Saari et al. 2010).

4) What are the taxonomic features of the studied *Festuca rubra* plants? How do ploidy and morphology vary among the studied plants?

Morphological characters are used to determine subspecies of *F. rubra* plants, e.g., inflorescence parts especially lemma hairiness could be used to distinguish subsp. *rubra* and subsp. *arctica*: while plants with hairy-lemma are described as subsp. *arctica*, plants with glabrous-lemma are determined as subsp. *rubra* in some middle and north European red fescues. In southern Europe, especially in the mountains of Spain, the subsp. *rothmaleri* is common. Specific ploidy levels were reported for some *F. rubra* subspecies in the literature. For example, *F. rubra* subsp. *rubra* (Tropicos.org 2015) and subsp. *arctica* (Markgraf-Dannenberg 1980) were reported as hexaploid, while *F. rubra* subsp. *rothmaleri* (Al-Bermani et al. 1992) and subsp. *arenaria* (Hämet-Ahti et al. 1998) were reported as octoploid.

I studied ploidy level and morphological characters of E+ and E- red fescues in a broad geographic scale (Study **V**). Since polyploidization is considered as a cause of adaptive divergence and speciation, chromosome numbers and the karyotype of plant species can be used for taxonomic identification (Stace 1989, Briggs and Walters 1997).

2. MATERIALS AND METHODS

2.1. Plant material

I used perennial wild fescue species to study endophyte infection frequencies and ploidy levels and cultivar fescue species with one agricultural variety of a clover species in competition experiments (Table 1). Fescues are well-adapted to temperate and cold regions and globally distributed both in natural and agricultural areas. Ploidy levels of fescues are reported to vary from 2n = 2x = 14 to 2n = 10x = 70 (Stace 1989, Aiken and Darbyshire 1990). Both wild plants and cultivars of fescue species are commonly infected with systemic endophytic fungus (*Epichloë*). The frequency of endophyte infection can vary from 0 to 100 % in natural and agricultural populations (Saikkonen et al. 2000).

Red fescue (*F. rubra* L.) plants are 10-120 cm in height, and leaves are usually reddish, but turn brown in old age (Aiken and Darbyshire 1990). Red fescue is phenotypically highly variable, and variation in morphological characters reflects ecological factors rather than geographical gradients (e.g., Dube and Morisset 1987).

Viviparous sheep's fescue (*F. vivipara* Sm.) is a member of the fine fescue complex. Plants are 5-45 cm in height. This species' reproduction type is vegetative proliferation which is based on spikelet conversion to a leafy shoot above the glume (vivipary) (Beetle 1980). While *F. vivipara* is genetically undistinguishable from *F. ovina*'s seminiferous and pseudoviviparous ecotypes, it is considered a morpho-species based on ecological and morphological differences. *F. vivipara* is a common arctic species particularly distributed in northwestern Europe and the north Atlantic area. It grows in mountainous, dry, rocky, sandy or peat soil environments, and due to its competitive advantage, it replaces the close relative *F. ovina* in high altitudes (Wycherly 1953, Harmer and Lee 1978).

Red fescue and viviparous sheep's fescue are native to holarctic regions of the world, both of them are classified under fine fescues and fine fescues may be infected with the same systemic fungal endophyte, *Epichloë festucae* (Leuchtm., Schardl & M.R. Siegel) (Leuchtmann et al. 2014).

Tall and meadow fescues [*Schedonorus phoenix* Scop. and *Schedonorus pratensis* (Huds.) P. Beauv] are important forage crops worldwide. Tall fescue plants are 60-200 cm high and native through Eurasia, Transcaucaisa, Asia-minor (Anatolia) and North Africa (Aiken and Darbyshire 1990). Tall fescue is commonly infected with the systemic fungal endophyte *Epichloë coenophiala* [(Morgan-Jones & W. Gams) C. W. Bacon & Schardl, comb. nov.]. Meadow fescue plants grow 30-120 cm high, and they are native to non-Arctic Eurasia and Canada. They are frequently infected with the systemic fungal endophyte *Epichloë uncinata* [(W. Gams, Petrini & D. Schmidt) Leuchtm. & Schardl, comb. nov.] (Leuchtmann et al. 2014).

Red clover (*Trifolium pratense* L., Leguminosae) is a perennial herb, native to Europe, western Asia and northwest Africa, but introduced and cultivated in many parts of the world mainly as a forage species. Red clover is a good partner of grasses in crop mixtures due to its nitrogen fixation ability with the *Rhizobium leguminasorum* biovar *trifolii* in the rhizosphere. Grass-legume mixtures improve forage quality and economic profitability (e.g., Ledgard 1991, Malézieux et al. 2009). Under grazing conditions, a monoculture can be harmful for livestock due to, for example, herbivore-induced chemicals in forage plants. Mixing crops by, for example, cultivating grasses with legumes, can help to overcome those problems.

Table 1. Plant material, their endophytes and studied features in the thesis.

Plant materials	**Wild / Agricultural**	**Endophyte**	**Studied features and study number in the thesis**
Red fescue (*Festuca rubra*)	Wild	*Epichloë festucae*	- Infection frequencies (II, V) - Polyploidy (I, II, V) - Morphology (V)
Viviparous sheep's fescue (*Festuca vivipara*)	Wild	*Epichloë festucae*	- Infection frequencies (III) - Polyploidy (I, III) - Endophyte phylogeny (III)
Meadow fescue (*Schedonorus pratensis*)	Agricultural (cv. Kasper)	*Epichloë uncinata*	Effects of endophytes on grass-legume mixtures (IV)
Tall fescue (*Schedonorus phoenix*)	Agricultural (cv. Kentucky 31)	*Epichloë coenophiala*	
Red clover (*Trifolium pratense*)	Agricultural (cv. Global)	-	

2.2. *Study sites and environmental conditions of natural populations*

Red fescue and viviparous sheep's fescue plants were collected from their natural environment (see sites in Fig. 2 a,b). Considering the broad geographic scale of red fescue collection sites, population structures can be expected to have latitudinal gradients. Many climatic features have been under alteration especially in the current rapid climate-change era, but day-length differences are stable across latitudes. Stable latitudinal day-length variation limits climate change-induced species' range shifts (Saikkonen et al. 2012). Beyond latitudinal differences, the collection sites of red fescue plants were highly variable in terms of the environmental vegetation season and altitudinal gradients among and within latitudes (Fig. 2).

The Spanish red fescue plants came from the lowest latitude of the study area. Two of the three populations were in grasslands with a Mediterranean climate and low soil fertility (Olea and Ayanz 2006), and one population was in a xerophytic forest area. All Swiss populations were in the subalpine zone below the tree-line and at the highest elevational gradient of the study. Three populations from southern Finland (Hanko) were in a cold climate. At the similar latitude, we sampled plants from six populations existing in the Faroe Islands, which has mild winters due to the Gulf Stream. Summers in the Faroe Islands are cool and rainy, the islands are treeless and sheep grazing is prevalent. Three populations were sampled from Iceland just below the Arctic Circle in an oceanic environment. Totally, nine populations were from Northernmost Finland, six from the Kevo area and three from the Halti area. Three of the Kevo populations were meadows, and three populations were on riverbanks, which are annually disturbed by flood and ice cover and have lower soil fertility compared to meadow sites. Also, the reindeer density is high in the Kevo area (Colpaert et al. 2003). The other northernmost populations in Finland were in Halti, which is situated above treeline in the northwest corner of Finland. This area was at the highest elevation of the Finnish sites (up to 1328 m a.s.l.). Greenland populations (Disko Island) were at the same latitudes with the northernmost Finnish sites. The vegetation cover of Disko Island consisted of herbs and small bushy plants. The island lies between high and low arctic zones with a short growing season and low summer temperatures (Fig. 2-a). Among those, the Faroe Islands and Iceland locations were also viviparous sheep's fescue collection sites. Additionally, viviparous sheep's fescues were sampled from three mountainous Great Britain sites [Ben Lawers (Mid Perthshire, Scotland), Snowdon (Caernarvonshire, North Wales) and Craig Cerrig Gleisiad (Breconshire, South Wales)] (Fig. 2-b).

To obtain inter- and intra-annual general vegetation cover estimation for red fescue collection sites (Fig. 2), normalized difference vegetation index (NDVI) values were used (Tucker et al. 1985, Prince 1991, Paruelo et al. 1997). This estimation was based on aboveground net primary production (ANPP) of study sites from February 2000 to December 2012 (see Study **II** Fig. 1). NDVI values were obtained from the NASA-MODIS project using the MODIS global subsets tool (http://daac.ornl.gov/cgibin/MODIS/GLBVZ1Glb/modissubsetorderglobalcol5.pl). The MOD 13 Vegetation Indices product was used via gridded 16-day composite images with 250-m pixel size resolution.

2.3. Study system

2.3.1. Endophyte detection

Endophyte infection frequencies of red fescues were detected from 29 wild populations (10-70 plants / each population, 1019 plants in total) from Spain to northernmost Finland and Greenland (Study **II**) and *F. vivipara* from 12 populations from North European islands (3-13 plants / each population, 117 plants in total) (Study **III**) (Fig. 2). Three green leaves were detached from each plant. Detached leaves were immersed in 75 % ethanol (30 s), 4 % NaOCl (3 min.) and again in 75 % ethanol (15 s) in order to surface sterilization of the samples. Each surface sterilized leaf was aseptically cut to five pieces and placed in a Petri dish containing potato dextrose agar (5 % PDA). The samples were kept at room temperature approximately for a month. When systemic endophyte mycelium grew out from several leaf pieces, the plant was considered as endophyte infected (Wäli et al. 2007).

Beside infection frequencies, the phylogeny tree of viviparous sheep's fescue endophytes were determined using molecular identification based on nucleotide sequence of the ITS1-5.8S rDNA-ITS2 (ITS) region, and 5' region of the ß-tubulin (tub2) gene consisting part of its 1st intron, and the complete 2nd and 3rd introns sequence (Byrd et al. 1990) (Study **III**).

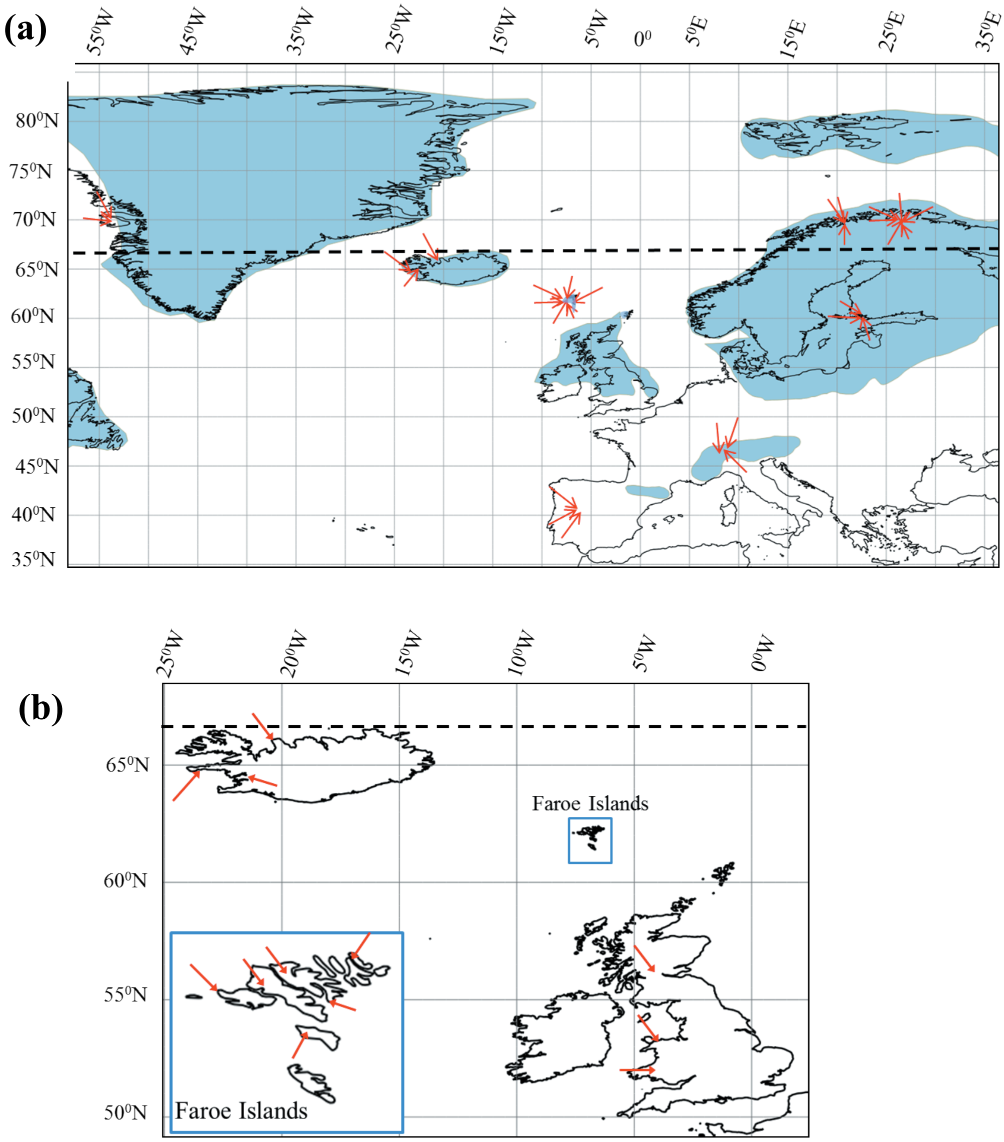

Figure 2. Sampled red fescue (a) and viviparous sheep's fescue (b) populations (red arrows). The Arctic Circle is represented by a dashed line (in a and b). Blue regions in map (a) indicate permanent ice cover during the last glacial period (25,000-15,000 years ago) (Ray and Adams 2001).

2.3.2. Ploidy level determinations

Ploidy levels of the fescues were determined using flow cytometry (FCM) (Study **I**, **II** and **III**). Plants were randomly chosen as reference for FCM. Their meristematic root-tips were stained with aceto-orcein and chromosomes in mitotic metaphase were counted under the microscope (Darlington and La Cour 1969).

FCM was performed using tube-based (FACS Calibur Becton Dickinson San Jose, USA) and plate-based (LSR II, Becton Dickinson San Jose, USA) procedures. A nuclei suspension was prepared using a small amount of leaf tissue for each plant (Doležel et al. 2007). FCM data was analyzed with Flowing Software (Perttu Terho, Turku Centre for Biotechnology, Finland, www. flowingsoftware.com).

Study scale, research questions, resolution requirements and time- and cost-efficiency determine which procedure suits best for each individual study. To compare tube- and plate-based FCM procedures in terms of reliability and time- and cost-efficiency, I applied the two FCM procedures to the same plant individuals, and in addition, counted chromosomes of two red fescue individuals microscopically for confirmation (Study **I**). In another experiment, DNA content of four viviparous sheep's fescue and two red fescue individuals were determined using tube- and plate-based procedures to compare their time- and cost-efficiency (Study **I**).

Ploidy levels of 1019 red fescue individuals from 29 populations (Study **II**), and 117 viviparous sheep's fescue individuals from 12 populations (Study **III**) were determined using plate-based and tube-based FCM, respectively, at the Turku Centre for Biotechnology, Finland.

2.3.3. Plant morphology, polyploidy and endophyte infection status of European red fescue

I conducted a study based on polyploidy of European E+ and E- red fescue plants and some taxonomically important morphological characters (e.g., Markgraf-Dannenberg 1980, Dube and Morisset 1987, Aiken et al. 2007) (Study **V**). Red fescues (167 individuals) were collected from 17 sites in Europe from Spain to northernmost Finland (see Fig. 2). Grasses with known ploidy level and endophyte infection status were planted in the fields of Ruissalo Botanical Garden (60^0 26' N, 22^0 10' E). They were grown in two experimental fields (Field 1 and Field 2) 150 m apart from each other. Numerical (plant height, basal leaf width, inflorescence length, spikelet length, awn length, lemma length, stolon length, lower and upper glume length) and categorical [color, tuft (loose/dense), leaf scabridity, number of veins in leaf, stolon (intra/extra vaginal), number of flowers in spikelet, rachilla-, lemma- and glumes-hairiness] morphological characters of the plants were determined in the flowering time during the summer of 2013 and 2014 (Study **V**). The plant parts were visualized using NIS Element Version 3 SP 7 with the microscope (Nikon SMZ745T), and measured with the Image J program (Image J 1.42, National Institute of Mental Health, Bethesda, MD, USA) (Appendix 1 in study **V**).

2.3.4. Effects of endophytes on grass-legume mixtures

I performed identical competition experiments for two agronomic grass species (meadow fescue c.v. Kasper and tall fescue cv. Kentucky-31) in a mixture with red clover (cv. Global) in the greenhouse of Ruissalo Botanical Garden (60°26' N, 22°10' E), Finland (Study **IV**). Red clover seeds were inoculated with *Rhizobium leguminasorum* biovar *trifolii* before sowing because growth success requires microbial symbiont. E+ and E- grasses were separately grown in a mixture with red clover (legume) in either low- or high-nutrient soil. Each pot had four plants, but proportion of clover varied: 4 grasses, 3 grasses + 1 legume, 2 grasses + 2 legumes, 1 grass + 3 legumes, or 4 legumes per pot. The total number of pots for grass species was 216 with 12 replications [2*2*5*12=240, the same 4-legume pots were used for both experiments 240-(2*12) = 216 pots]. The pots were placed in the greenhouse in randomized block design. Plants were subjected to natural daylight, and air temperature of the greenhouse was adjusted to 25ºC and 20ºC for daytime and nighttime, respectively. After 48 days, the plants were harvested, tiller numbers of fescues were counted, plant heights were measured, the above ground biomass was oven-dried (60º C, 48 hours) and the dry-matter of plants was weighed.

2.3.5. Statistical analysis

Categorical variables (plant color, number of leaf vein, hairiness of inflorescence parts) were studied using $\chi2$ tests in contingency tables (Study **V**). Continuous morphological characters (e.g., basal leaf width, length of inflorescence parts, plant height, stolon length) were compared by ploidy level and endophyte infection status. Ploidy level, presence/absence of endophyte infection, collection sites and their interactions were used as fixed predictors in a three-way analysis of variance (SAS PROC GLM).

Linear regression analyses were used to test the effects of latitudinal and altitudinal gradients on endophyte infection frequencies of viviparous sheep's fescue populations (Study **III**) and to analyze the effects of latitudinal gradients and primary production (mean-NDVI) on endophyte infection frequencies and mean-ploidy level of red fescue populations (Study **II**). Model assumptions were checked graphically and by using the Kolmogorov-Smirnov normality test.

I used a logistic regression model to estimate the effects of ploidy level, latitude, altitude and their interactions on endophyte status (E+ and E-) with the binomial error distribution (logit link). The Akaike Information Criterion (AIC) was used to select best-fitting model (Study **II**).

In study **III**, sequenced endophyte strains data of viviparous sheep's fescues was aligned using Clustal X software. The genetic distance between strains was estimated using Kimura-2 parameter model, and the obtained matrix was used to draw phylogenetic tree according to neighbor-joining method. The robustness of phylogenetic tree was estimated by 1000 bootstrap replicates. These phylogenetic analyses were done using MEGA version 5 software.

In the competition experiments of fescue-red clover mixtures (Study **IV**), the nutrient level of the soil (low and high) and endophyte infection status of the fescue (E+ and E-) were considered to be categorical fixed factors, the proportion of red clover per pot was a continuous fixed factor and the block was a random factor. Continuous response variables (dry matter and plant height of fescues and red clovers) were analyzed in general linear models (SAS mixed model), and logistic models (SAS GENMOD procedure) were applied in analyzing presence or absence of tillering. All two level interactions between factors were estimated, but three-level interactions were reported only when they were statistically significant. Model assumptions including normality and homogeneity were checked and studied graphically, and response variables were transformed to meet assumptions of statistical tests.

3. RESULTS

3.1. *Endophytes of wild fescues*

Endophyte infection frequencies varied among sites for both red fescue and viviparous sheep’s fescue populations. In red fescue study, some geographical variables (e.g., altitude, latitude and their interaction) differed for endophyte infection frequencies. Infection frequencies varied from 0 % to 81 % in the whole study area. All plants from southern Finland and Greenland were uninfected, and 29 % of the grasses were infected in the whole area. Infection frequencies from the Kevo area in northernmost Finland and Faroe Islands sites were as high (up to 75-68 %, respectively) as in the Spanish sites (Fig. 3 in Study **II**). Infection frequencies of red fescue populations were not related with latitudinal gradient, primary production and ploidy level of the plants. Altitude alone, and interactively with latitude, appeared to be significant for infection frequencies of red fescue populations (Table 2, Fig. 4 and Fig. 5 in Study **II**).

The average infection level in viviparous sheep’s fescue was 14 %. Infections were detected in four out of six Faroe Islands populations, two out of three Iceland populations and one out of three Great Britain populations. Endophyte infections of viviparous sheep’s fescue did not vary with latitude or altitude. The ITS and tub2-based phylogenetic analysis confirmed that the endophytic species of viviparous sheep’s fescue was *Epichloë festucae*, the same species as the endophyte of the other fine fescues (Study **III**).

3.2. *Ploidy level determinations in plants*

3.2.1. An efficient ploidy level determination for plant evolutionary ecology

I implemented a plate-based FCM procedure which provides an equally accurate ratio of cell-specific DNA contents compared with a tube-based procedure. I compared plate-based and tube-based procedures in terms of time- and cost-efficiency. While the plate-based FCM procedure generated higher deviation than tube-based procedure, the median values of fluorochrome absorption and the estimated DNA contents for the two procedures were comparable. Moreover, a plate-based FCM procedure in fescues provided 3 times reagent- and 20 times time-consuming advantages over tube-based procedure (Study **I**).

3.2.2. Ploidy level of wild fescue populations

I found ploidy level variation among and within red fescue populations across latitudes. Hexaploids ($2n = 42$; 84 %) were most common, but also tetraploid ($2n = 28$; 9 %) and octoploid ($2n = 56$; 7 %) plants were detected. While most of the lowest ploidy level plants were from Spain (the lowest latitude of the study area), plants at a higher latitude had a higher ploidy level. One of the northernmost populations in Finland (Halti 2) had only octoploids (the highest ploidy level) and one of the lowest NDVI-mean value of the study. The lowest latitude site (Spain, Cáceres, Garganta del

Infierno), which had the highest NDVI, had only tetraploid plants. The other sites including mid- and high-latitude locations were mostly hexaploids (Study **II**). Viviparous sheep's fescue plants from 12 populations (Fig. 2) were all tetraploids ($2n = 4x = 28$) (Study **III**).

3.3. Effects of endophytes on grass-legume mixtures

Total yields of the fescues and red clovers were positively affected by higher nutrient level of soils. Red clover performed better (76 % - 79 % of the total yield) compared to the fescues. Total yields increased from fescue monocultures to red clover monocultures. Meadow fescue biomass was affected by endophyte infection interactively with nutrient level of soils and proportion of red clover. E+ monoculture fescues had 89 % higher biomass than E- monocultures in a high nutrient environment, and this difference gradually decreased with increasing levels of red clovers in the mixture. The favorable effects of endophytes were detected only in high nutrient environment suggesting that endophytes did not have positive effects in resource-limited conditions. Soil nutrients enhanced plant height and tillering in meadow fescue. In the high nutrient conditions, endophyte-induced plant biomass decreased with increasing proportion of red clover. E- meadow fescues appeared to be irresponsive to the presence of red clover. On the other hand, neither endophyte infection nor presence of red clover affected meadow fescue biomass in the low nutrient environment (Fig. 2 in Study **IV**).

Unlike meadow fescue, E- biomass of tall fescue plants was similar to E+ monoculture biomass, and both of them gradually decreased with increasing proportion of red clover. Endophytes had no effects on total biomass, height or tillering of tall fescue. In a high nutrient environment, E- tall fescue biomass and plant height decreased rapidly with the increasing inter-specific competition with red clover. Endophyte infection appeared to enhance the resistance of tall fescue against a competitor only in high nutrient conditions (Fig. 2 in Study **IV**).

Red clover produced 75 % more biomass in high nutrient monocultures compared to low nutrient monocultures. In high nutrient environment and when the partner was tall fescue, red clover biomass was negatively affected by the number of clovers in the same pot. The presence of endophytes in the grass enhanced clover biomass when the competing partner was meadow fescue in high nutrient environment. Clover biomass did not change in low nutrient conditions (Fig. 2 in Study **IV**).

3.4. Taxonomy, plant morphology, polyploidy and endophyte infection status of European red fescue

Spanish plants were mostly tetraploids and all of them were determined as *F. rubra* subsp. *rothmaleri*, Swiss plants mostly belonged to *F. rubra* subsp. *rubra* and Finnish plants were determined as both *F. rubra* subsp. *arctica* and *F. rubra* subsp. *rubra*. Intermediates between *F. rubra* subsp. *rubra* and *F. rubra* subsp. *arctica* were found also, and they were hexaploids and octoploids. Hexaploid plants occurred in all subspecies (In study **V** Fig. 1 and Table 3, see also Table 4 for some features of examined *F.rubra* plants).

Some ploidy-specific associations were found in the study, e.g., the correlation between plant height and basal leaf width was strong for tetraploids and octoploids, r=0.68, p<.0001; r=0.75, p=0.02, respectively, but no correlation was found for hexaploids: r=-0.16, p=0.08. However, these ploidy specific associations vanished when within-population level data was analyzed in the mixed-ploidic populations (Appendix 5, 6, 7 in study **V**).

Ploidy levels of red fescues were not associated with morphology of plants. However, the geographical location of the populations was correlated with the plant morphology. In addition, morphological differences may be reflected by different karyotypes. Thinner leaves, denser tuft, shorter awn-, inflorescence-, spikelet-, glume-length and lower number of leaf-veins were observed in the presence of B-chromosomes of Spanish tetraploids, which was based on the karyotype comparison of two individuals (Appendix 2 in study **V**)

Awns were longer in E+ plants than in E- plants, while other morphological traits were not correlated with endophyte infection status of the red fescue plants.

4. DISCUSSION

This thesis is based on integrative studies of endophyte infection and polyploidy of the fescues. It demonstrates the importance of the local environment and the last glacial period rather than global-scale geographic factors (e.g., latitudinal gradient) on species interactions and population structures. While some researchers have emphasized the importance of latitude and/or primary production in interactions like plant-endophytic fungi symbiosis (e.g., Schemske et al. 2009, Malinowski and Belesky 2006, Semmartin et al. 2015), others have not detected these connections (e.g., Saikkonen et al. 2000). Post-glacial founder effects and effects of partly human-mediated later migration could configurate current population structures overrunning suggested global geographic and productivity-related patterns.

I used two closely related fescue species (red fescue and viviparous sheep's fescue) in the study of wild grasses' endophyte frequencies and ploidy level (Study **II** and **III**). Despite their closeness, they highly differed in terms of their distributional and infection frequencies pattern. For instance, in a previous study conducted in Sweden, while viviparous sheep's fescue was found only in high elevations, red fescue could be found at all elevational gradients (Granath et al. 2007). The infection frequency range of red fescue populations is reported to be high (0 % to 100 %) (Saikkonen et al. 2000) and negatively correlated with the altitude, whereas infection frequency was in the low range and showed no altitudinal gradients for viviparous sheep's fescue populations (infection frequencies could be up to 37 %) (Granath et al. 2007).

Many results in this thesis challenge previous ideas explaining variation in polyploidy or endophyte infection frequencies of grasses. I demonstrated the lack of a latitudinal gradient for endophyte infection in wild populations of red and viviparous sheep's fescues, and the lack of a primary production effect on infection frequencies of red fescue populations. Unlike infection frequency, ploidy level appears, to some extent, to be affected by latitude and primary production of the red fescue sites: higher ploidy level is more common at a higher latitude and at lower mean-NDVI sites (Study **II**). However, this result is based on an extreme case of the study area: three tetraploid-dominated Spanish populations in southernmost and one octoploid Finnish population in northernmost of the study area. I found no association between ploidy level and endophyte status of the wild fescue grass species (Study **II** and **III**) that has been suggested for some mycorrhiza-plant interactions previously (e.g., Tesiteova et al. 2013).

4.1. Endophytes of wild fescues

Altitude and latitude partly explained endophyte infection frequencies in red fescue populations (Table 2 in study **II**), and these results remain inconclusive because my study was not designed to test it. Altitude is a good predictor only when Spanish and Swiss populations are compared (Table 1 in study **II**). Overall infection frequencies were not associated with altitudinal gradient as has been reported in previous studies (Granath et al. 2007, Bazely et al. 2007).

Habitat type might affect infection frequencies at similar latitudinal and altitudinal gradients (Study **II**). For instance, in the highly endophyte infected northern Kevo region, infection frequencies depend on habitat type of populations (e.g., meadow or riverbank). In the whole red fescue data, infection frequencies in meadows are on average two fold higher compared to river banks. Kevo river banks are low nutrient environments: soils are sandy and annually disturbed by ice and flooding (Zabalgogeazcoa et al. 2013). Low nutrient conditions and annual spring flooding might lead to lower endophyte infection frequencies in Kevo riverbanks compared to meadow sites.

A recent global-scale study suggested a positive correlation between primary production and endophyte infection frequencies of grasses (Semmartin et al. 2015), but my study does not support this connection in red fescue populations (Fig. 5 in study **II**). Even if the northernmost region of the red fescue study, Halti (860-920 m a.s.l.), is at higher altitude compared to Kevo (73-107 m a.s.l.). All three populations at the Halti site are endophyte-free, while infection frequencies of Kevo populations ranged from 20 % to 75 %. This difference could partly be connected with altitude as suggested in the previous studies (Bazely et al. 2007, Granath et al. 2007). However, contrary to this, also the highly productive southern Finland populations (Hanko) and the sea level populations in Greenland were endophyte-free.

Strong grazing pressure is common to all populations with high endophyte frequencies (Table 1 in study **II**). Thus grazing may be relevant for endophyte infection frequencies in red fescue populations. Reindeer density and endophyte infection frequencies at Kevo are high, while in the Halti area, red fescues are endophyte-free and reindeer density is low (Colpaert et al. 2003). Higher reindeer grazing in the Kevo area might be an important selective force for the presence of endophytes (Granath et al. 2007). Likewise, high sheep grazing in the Faroe Islands and Iceland may cause higher endophyte infection frequencies of these sites compared to lower altitude Greenland and southern Finland sites (Table 1 in study **II**). It seems that present and historical factors like altitude or grazing pressure might be as important as primary production in determining the present endophyte infection level of a local red fescue population.

I predicted that endophyte infection frequencies of viviparous sheep's fescue (*Festuca vivipara*) would increase by latitude and altitude in an oceanic environment (Study **III**). As an alpine-arctic species, viviparous sheep's fescue populations are decreasing close to sea level in northern oceanic climate environments (Rønning 1996). For instance, in Finland, this species is most commonly found between 800-1100 m a.s.l., and it is not detected lower than 450 m a.s.l. (Väre and Partanen 2012), and in Sweden, it is growing above 750 m a.s.l. (Granath et al. 2007). As the range of altitudinal gradients in my study was large, ranging from 10 to 725 m a.s.l., I expected plants at higher altitudes to be positively correlated with endophyte infection, but our results did not support this connection in altitudinal gradients of North European islands. The average endophyte infection level of viviparous sheep's fescues was 15 %. The infection frequency was not connected either with altitude or latitude of the populations (Study **III**).

4.2. Ploidy levels of plants

One of the important results of this thesis was the implementation of a rapid and cost-efficient procedure for ploidy level estimation of plants. A 96-well plate-based higher throughput FCM was successfully combined with fully automated software allowing analyses of large sample-sizes in plant evolutionary ecology studies (study **I**).

4.2.1. An efficient ploidy level determination for plant evolutionary ecology

Determination of ploidy level of fescues was based on a combination of improved and accelerated high throughput flow cytometry (FCM) devices and a time-saving one-step preparation procedure using the fully automated software (Flowing Software). Use of a tube-based FCM procedure gives less variation (lower CV value) in DNA content measurements and more stained nuclei compared to a plate-based procedure (Bennett and Leitch 1995, Doležel et al. 2007, Greilhuber et al. 2007). However, the plate-based procedure was sufficient and applicable for measuring whole genome duplications in plants. My study species (red fescue and viviparous sheep's fescue) were likely to show lysis problems because grasses have high concentrations of silica compounds in their tissues. However, I succeeded to solve this problem and apply a one-step time-efficient lysis procedure to determine fescue ploidy levels (Study **I**).

4.2.2. Ploidy levels of wild fescue populations

As previous authors (Löve and Löve 1957, Hair 1966, Stebbins 1971) have suggested, the ploidy level of red fescue populations rose with latitude (Study **II**). Ploidy also decreased with primary productivity, which is in accordance with the previous predictions (Stebbins 1971, Adams and Wendel 2005, Yang et al. 2011, Ramsey 2011). However, the latitudinal gradient of ploidy level was mostly based on three tetraploid Spanish populations in the lowest latitude and one octoploid population from northernmost Finland. Moreover, these two extremes were reflected by minimum and maximum primary productivity (mean-NDVI value) of the whole study area: while tetraploids dominate the Spanish sites with the highest mean-NDVI, one of the least productive study site was octoploid. To some extent, these findings support the common opinion that severity of an environmental gradient is associated with ploidy level (Stebbins 1971, Ramsey 2011, Ramsey and Ramsey 2014). The population with a large number of the highest ploidy level plants (Halti 2) is at a high altitude (900 m a.s.l.) and has less grazing pressure compared to the similar latitude populations at the Kevo site (Colpaert et al. 2003). Previous literature (Tropicos 2015) reports different ploidy level of viviparous sheep's fescue populations from diploid to octoploid. Interestingly, I did not find any variation in ploidy level of viviparous sheep's fescue and all examined plants had the same ploidy level in our study area ($2n = 4x = 28$).

Variation in ploidy level may be related with polytopic origin and/or post-glacial distribution pattern of *F. vivipara* (Study **III**).

4.3. Endophyte mediation of grass-legume mixtures

Output of grass-endophyte interactions and distribution pattern of grasses might be significantly affected by the presence of a third organism in the environment. Grasses can be together with other families' plants including legumes. Legumes are known to be a nitrogen fixator to the soil that might facilitate grass growing beside causing plant-plant competition in grasslands. Since endophyte-produced alkaloids of the grasses might be nitrogen dependent, the invasiveness of E+ grasses may increase in a legume neighborhood compared to that of E- grasses. On the other hand, plant-plant competition of grass-legume mixtures can limit the effects of this partnership on the performance of plants. In addition, adaptive radiation of grasses has been increasingly affected by man-made arrangements, e.g., road sides, landscaping, grass production. These introduced grasses are commonly seeded with legumes and likely to distribute into nature and hybridize with wild grasses. In study **IV**, endophyte mediation was detected in grass-legume mixtures, which are widely suggested for agronomic purposes because of forage quality and economic profitability. Effects of endophytes on grass-legume mixtures depend on the grass species, proportion of the mixture and soil nutrient availability. Many studies have detected effects of environmental conditions on grass-endophyte symbiosis (from mutualism to antagonism) including nutrient availability and species dependency. My competition-based experimental design has added proportion to these well-studied variables. The proportion of grass-legume mixtures was found interactively effective with nutrient environment and study species on the endophyte-grass symbiosis outcomes.

In concordance with previous studies, endophyte infection enhanced the yield of meadow fescue in monoculture (e.g., Saikkonen et al. 2013), while in contrast to previous tall fescue studies, its monoculture yield was not increased by endophyte infection (Hill et al. 1991, Clay and Holah 1999, Saikkonen et al. 2006). Endophyte infected tall and meadow fescues were not superior over red clover at the early growth stage of grass-legume mixtures. Moreover, competitive performance of tall fescue was weaker than meadow fescue in the greenhouse conditions regardless of the presence of endophytes. In agroecosystems or natural conditions, many stress factors such as drought, flooding and herbivory may affect the competitiveness of tall fescue (Nagabyru et al. 2013). Nutrient supply positively affected red clover growth, but it appeared to suffer from inter- and intra-specific competition. In a high-nutrient environment and under increased competition with E+ meadow fescue, red clover biomass decreased suggesting that red clover may suffer from competition with E+ grasses in high nutrient soils.

4.4. Taxonomy, plant morphology, polyploidy and endophyte infection status of European red fescue

Polyploidy might be an efficient driver of plant speciation due to its genetic (e.g., increasing number of alleles, neofunctionalization of the duplicated genes) (Comai 2005) and ecological effects (e.g., habitat segregation) on plant populations. Similarly, the endophyte infection status of grasses may be expected to be related with plant speciation due to coevolutionary process of symbiosis (Thompson 2005). I expected that variation in morphological traits of grasses may be related with those characters.

I found that ploidy level was not a useful feature for taxonomic detection in red fescue complex due to variation in ploidy levels in assumed subspecies (Table 3 in study **V**). While some ploidy-specific correlations could be detected, population level analysis did not confirm association of ploidy with plant morphological traits. Local adaptation and habitat type might explain plant morphology better than ploidy level (Fig. 1 and Table 8 in study **V**).

A red fescue plant from Spanish-meadow site with two B-chromosomes had narrower leaves; shorter awn-, inflorescence-, spikelet- and glume-length; tighter tufts and fewer leaf veins than those of a Spanish-xerophytic site's plant which had no B-chromosomes in its karyotype (Study **V** Appendix 2). Similar leaf width, tuft type and B chromosomes correlation pattern were reported for *Festuca altaica* in a previous cytotaxonomic study on North American fescues (Aiken and Fedak 1992).

Higher awn-length of E+ compared to E- individuals may be related to endophyte-promoted dispersal of fescues. The importance of awns has been shown for seed dispersal of grasses due to their positive effects on burial performance of seeds to the soil (Elbaum et al. 2007). In wild plants, awn-length has been shown to be correlated with a higher proportion of buried seeds and deeper burial depth, which indicate better germination success (Garnier and Dajoz 2001). Parallel to tillering, a well-known endophyte-promoted dispersal feature of grasses depending on genetic matches of symbionts (e.g., Cheplick 2008), endophyte-induced higher awn-length of grasses may favor dispersal.

In the taxonomic study of red fescue (Study **V**), different ploidy levels were detected in different subspecies. Hence ploidy is not subspecies specific for red fescue. Morphological characters of red fescue differed between geographical locations rather than ploidy. While some ploidy-specific morphological associations and variations were found in the total data-set, these vanished within-population level comparisons.

5. CONCLUSIONS

Conditionality of grass-endophyte symbiosis has been shown in this thesis, which suggests the importance of species' evolutionary history, biotic and abiotic factors and their interactions (study **IV**). Endophyte infection frequencies are highly variable among natural populations due to different local selection forces suggesting inadequacy of global geographical and environmental generalizations (e.g., latitudinal gradients, environmental severity hypothesis for species interactions) (study **II-III**). Variation in frequencies of grass-endophytic fungi constitutes an illustrative example for a geographic mosaic theory of a coevolutionary process. Even if variation in ploidy level of grasses appeared to be positively correlated with latitude and environmental severity, these results were shown to be caused by extremes in the study area. These patterns can be better explained by local selection forces (study **II**). Furthermore, no morphological variation of plants could be associated to within-population ploidy levels of individuals. Geographical location, rather than ploidy, appeared to be significant on plant morphology (study **V**) suggesting that local selection forces favor similar morphology despite different ploidy level in evolutionary time scale. In accordance with the geographic mosaic theory, integrative studies of endophyte infections and polyploidy of grass species showed that differences among localities are likely to be based on post-glacial distribution history of plants and adaptation by local selection forces.

ACKNOWLEDGEMENTS

I’m thankful to many people during my PhD.

I’m very lucky to have three very-well cooperative supervisors who have always good connections each other: Doc. Marjo Helander (University of Turku), Doc. Irma Saloniemi (University of Turku) and Prof. Kari Saikkonen (Luke). Each has precious guidance and efforts on my doctoral studies and encouraging in every stage of my work. I had a great opportunity to learn much from them and I was influenced especially their open-minded, open-to-discuss and knowledge-sharing practices. I’ve felt that each day of my work in the ‘Fine Fescue Speciation and Their Coevolution with Systemic Fungal Endophytes Project’ has improved my skills and capacity extensively.

I would like to give very special thanks to Doc. Annamari Markkola (University of Oulu/Department of Ecology) for accepting the invitation of being my opponent, and to my thesis reviewers Prof. Helena Korpelainen (University of Helsinki/Department of Agricultural Sciences) and Doc. Katileena Lohtander-Buckbee (Finnish Environment Institute/Species Protection) for their meticulous review of my thesis. I thank to my custos Prof. Kai Norrdahl (University of Turku/Department of Biology) for his help and guidance for procedural issues of the graduation process.

During my study **I** have again great opportunity to have many collaborators from different disciplines, they really enriched and added much to my career and research adventure: I would like to emphasize Prof. Henry Väre (University of Helsinki/ Botanical Museum), Dr. Pedro E. Gundel (we worked with him in MTT Agrifood Research Finland, his current institute is University of Buenos Aires), Doc. Seppo Nokkala, Perttu Terho (we worked with him in Turku Centre for Biotechnology) and Dr Salla Riikka Vesterlund among many others. Friends in the Department of Biology, their accompany both in the university and outside events (e.g., being listed by Peggy) were really re-fueling.

I appreciate and have a great benefit from cooperative and inter-institutional academic life in Finland; I have very fruitful experience and pleasure at MTT Agrifood Research Finland facilities (now under roof of Luke); Ruissalo Botanical Garden; Turku Center for Biotechnology (Biocity) and Kevo Subarctic Research Station during my doctoral studies.

My work was financially supported by Academy of Finland and INTERACT under the European Community’s Seventh Framework Programme. Also I thank to University of Turku Foundation, Foundation Societas pro Fauna et Flora Fennica for travel grants and University of Turku Graduate School (UTUGS) BGG Doctoral Programme for the ‘Finalizing a Doctoral Dissertation´ grant beside its travel support.

Aura Professional English Consulting Ltd. (www.auraenglish.com) performed the language content editing.

My wife, Roda, is always with me and support my career steps and even catalyzing them from step to run occasionally, maybe influenced by her extensive experience on enzyme studies in her academic career.

My parents, sister and brother, although they are physically far from me (in Turkey and in Belgium) I've felt always their supportive presence in my heart and mind.

Turku, Finland, May 2016

Serdar Dirihan

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