INTRODUCTION HISTORY AND POPULATION GENETICS OF *FALCARIA VULGARIS* (APIACEAE) IN THE UNITED STATES

BY

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This thesis is approved as a credible and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABSTRACT

INTRODUCTION HISTORY AND POPULATION GENETICS OF *FALCARIA VULGARIS* (APIACEAE) IN THE UNITED STATES

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Falcaria vulgaris Bernh. (sickleweed), native to Eurasia, occurs disjunctly in the Midwest and the East Coast of the United States. In parts of Iowa, Nebraska and South Dakota, it is an aggressive weed potentially turning to invasive. The main objectives of this study were (1) to reconstruct the introduction history and spread of the plant, (2) to develop and apply molecular markers to study the genetics of sickleweed populations in the United States. I used herbarium records and a suite of molecular markers to accomplish the project objectives. I surveyed 178 US herbaria, including those in the sixteen states where USDA has reported the sickleweed occurrence. Nuclear microsatellite markers developed for *Daucus carota* were screened for their transferability to sickleweed. I also sequenced the *trnL* intron, *trnL-F* intergenic spacer, and *trnQ-rps16* intergenic spacer regions of chloroplast DNA and ITS region of nuclear ribisomal DNA to study the genetic diversity. Herbarium records indicated that sickleweed was first introduced no later than 1922, and independent introduction of this plant took place in the East Coast state and in the Midwest of the United States. The species has been documented in 37 counties of 15 states of the United States. No recent sickleweed records have been reported for the last 17 years in the U.S. except in Iowa, Nebraska and South Dakota. Nuclear microsatellite data revealed three distinct genetic races of sickleweed in the Midwest. Chloroplast sequence data revealed six chlorotypes nested into two main lineages suggesting at least two introductions of sickleweed in the

Midwest. All data sets- herbarium records, nuclear microsatellites, nuclear and Chloroplast DNA sequences support the occurrence of multiple introductions. Presence of multiple alleles in individuals suggested polyploidy in sickleweed. High genetic diversity along with evidence of multiple introductions and polyploidy suggest that sickleweed, if not controlled in a timely fashion, can emerge as a serious invader.

CHAPTER 1

LITERATURE REVIEW

Invasive species and their impacts

Invasive species are a serious threat to global biodiversity (Sala *et al.* 2000; Lee 2002; Duncan *et al.* 2004; Lachmuth *et al.* 2010; Alyokhin 2011) and are often harmful to ecosystem processes (Gordon 1998), agriculture (Cox 2004) and human health (Chauvel *et al.* 2006). Niche displacement of native plants from the oak forest by the invasive plant *Carpinus betulus* (Kwiatkowska *et al.* 1997), local extinction of native *Spartina foliosa* from Alameda Island, Coyote Hills and San Bruno marshes, and replacement of this species by introduced *S. alterniflora* and the hybrid *S. foliosa* x *S. alterniflora* (Ayres *et al.* 1999; Ayres *et al.* 2004) are two examples of invasive plants impacts on biodiversity. At the ecosystem level, invasive plants may alter the geomorphological processes, the hydrological cycle and biogeochemical cycle (Gordon 1998). In addition, these plants reduce the productivity of agricultural crops, pastures, and rangelands (Cox 2004) and some are harmful to human health (Chauvel *et al.* 2006). Billions of dollars are spent every year for the management and control of invasive species (Walker & Steffen 1997; Lee 2002; Duncan *et al.* 2004), and it is predicted that the control and management of invasive species will be one of the biggest challenges in the decades to come (Allendorf & Lundquist 2003).

Hypotheses on success of introduced species

Several hypotheses have been proposed to explain the success of introduced species in novel habitats. These hypotheses are often classified into three major groups (as summarized in Table 1.1) and are briefly described here.

Increased resource competitive ability is believed to be a driving force of invasiveness in the novel habitat as the introduced species are released from their natural enemies (Richardson *et al.* 2000a; Keane & Crawley 2002; Levine *et al.* 2004; Hierro *et al.* 2005). The enemy release hypothesis argues that fewer enemies in the introduced range give introduced species a competitive advantage over native species which already have a large number of enemies (Colautti *et al.* 2004; Torchin & Mitchell 2004). In some cases, a mutualistic relationship between an introduced species and a native species is established that helps the introduced species increase its abundance in the new range (Richardson *et al.* 2000a). Introduced plants can also succeed in the new environment by avoiding competition by extracting the unused resources (Hierro *et al.* 2005). Integration of the concept of evolution and the enemy release hypothesis forms the basis of the evolution of increased competitive ability (EICA) hypothesis (Hufbauer & Torchin 2007). EICA argues that the escape from natural enemies favors selection pressure to divert resources to the traits that promote their competitive ability (Blossey & Notzold 1995; Hufbauer & Torchin 2007). Hybridization between two species or two different source populations is another mechanism that may promote the evolution of invasiveness through the evolution of novel genotypes, fixing heterosis or by dumping genetic load (Ellstrand & Schierenbeck 2000). There are also several cases where allelopathic introduced species have succeeded in the new environments by releasing certain biochemical compounds toxic to native plants, thus supporting the novel weapon hypothesis (Callaway & Aschehoug 2000; Bais *et al.* 2003; Cappuccino & Arnason 2006).

Table 1.1 Hypotheses explaining invader success following introduction

Hypothesis	Reference
Hypotheses that explain enhanced resource competitive ability	
Enemy release	Keane and Crawley (2002)
Mutualist facilitation	Richardson *et al.* (2000a)
Biotic resistance	Levine *et al.* (2004)
Empty niche	Hierro *et al.* (2005)
Evolutionary hypotheses	
Evolution of increased competitive ability	Blossey and Notzold (1995)
Hybridization	Ellstrand and Schierenbeck (2000)
Hypotheses that explain allelopathy as the promoter of invasion	
Novel weapon hypothesis	Callaway and Aschehoug (2000)

Species introduction, naturalization and the process of invasion

According to current review by Valery *et al.* (2008), "a biological invasion consists of a species' acquiring a competitive advantage following the disappearance of natural obstacles to its proliferation, which allows it to spread rapidly and to conquer novel areas within recipient ecosystems in which it becomes a dominant population". Biological invasion consists of a series of stages where any introduced species must overcome a variety of barriers before proceeding to the next stage (Blackburn *et al.* 2011). The major steps include introduction, naturalization/establishment and spread/invasion. When a species overcomes a geographic barrier, the species is called an introduced species. Founding individuals in the introduced population usually have low genetic diversity due to a founder effect and as a result, the population may suffer from inbreeding depression (Sakai *et al.* 2001). Inbreeding depression will further minimize

population size and may lead to elimination of the species from the introduced range. Approximately 99% of the casually observed introduced species fail to establish in their introduced range (Kowarik 2005). However, genome duplication (Comai 2005) and/or asexual reproduction (Sakai *et al.* 2001; Allendorf & Lundquist 2003; Frankham 2005) can minimize the effect of inbreeding depression in some introduced species. According to Soltis and Soltis (2000) the polyploids exhibit null to negligible effects of inbreeding depression. Redundant genes in polyploids provide the potential for them to escape the effects of deleterious recessive mutations in inbreeding populations (Comai 2005). Studies have shown that polyploids can grow in diverse habitats compared to diploid individuals (Treier *et al.* 2009). Polyploid individuals can form new genotypes through genome rearrangement in a short period of time after polyploidization (Soltis & Soltis 2000). In the long run, duplicate genes may undergo adaptive divergence that may facilitate evolution of invasiveness (te Beest *et al.* 2012). Likewise, asexually reproducing species may escape inbreeding depression (Sakai *et al.* 2001) so that escaped populations start reproducing at a much higher rate (Frankham 2005).

There are also instances where the introduced populations have higher or equal genetic variation compared to their native populations (Kelager *et al.* 2013). The mechanism that facilitates the increase of genetic variation in the founding populations includes introduction of a large number of propagules (Kolar & Lodge 2001; Allendorf & Lundquist 2003), multiple introductions (Novak & Mack 1995; Sakai *et al.* 2001; McCauley *et al.* 2003; Lavergne & Molofsky 2007; Meimberg *et al.* 2010; Gaudeul *et al.* 2011), gene flow between introduced populations (Sakai *et al.* 2001) and hybridization of introduced populations with genetically divergent native populations (Ayres *et al.* 1999;

Allendorf & Lundquist 2003).Higher intra-specific genetic diversity provides better opportunities for the creation of adapted genotypes through genetic reshuffling and recombination, and also provides wide options for natural selection of genotypes suitable for the environment thereby producing a population that can better exploit resources in the introduced range (Lavergne & Molofsky 2007). Therefore, understanding the founder effect in introduced populations will allow us to predict the invasion potential of the introduced species.

When a founder population succeeds to survive and forms a self-sustaining population, the population is said to be naturalized (Sakai *et al.* 2001). If the introduced species is pre-adapted, the probability that the species will successfully naturalize increases (Bossdorf *et al.* 2008; Lachmuth *et al.* 2010). Naturalized species spread in disturbed and natural habitats after going through a lag period, the period between the introduction of a species until it becomes invasive (Richardson *et al.* 2000b; Mooney & Cleland 2001; Allendorf & Lundquist 2003). During the lag phase plants undergo genetic, ecological, and demographic changes as they become adjusted in the new environment (Ellstrand & Schierenbeck 2000; Sakai *et al.* 2001). Demographic characteristics such as high fecundity, reproduction at earlier age, short generation time, and effective dispersal mechanisms are important features that might influence the spread of a naturalized plant species (Sakai *et al.* 2001). A study by Aikio *et al.* (2010) showed that most invasive weeds have a lag period of approximately 20 to 40 years. For example, *Bromus tectorum* was first introduced to North America in1889 and remained localized around intermountain Western North America for 20 years before undergoing rapid range expansion (Mack 1981).

Understanding the introduction history and population structure of sickleweed

Study of herbarium specimens

Herbarium records are the most reliable primary source of information to reconstruct introduction and colonization history of a species when detailed historic data are not available (Strother & Smith 1970; Mack 1991; Barney 2006). Herbarium specimen labels provide valuable information that can be used to document time of introduction of non-native plants (Wester 1992; Woods *et al.* 2005; Valliant *et al.* 2007), the number of independent introductions (Barney 2006), early invasion pathways in the introduced range (Stuckey 1980; Lavoie *et al.* 2007) and distributional changes of plants over time, as in *Ambrosia artemisiifolia* (Chauvel *et al.* 2006; Lavoie *et al.* 2007), *Bouteloua curtipendula* (Laughlin 2003), *Bromus tectorum* (Novak and Mack 2001; Valliant et al. 2007), *Cortaderia selloana* and *C. jubata* (Lambrinos 2001), *Oenothera* spp. (Mihulka & Pysek 2001), *Solidago* spp. (Weber 1998), *Vincetoxicum* spp. (Sheeley & Raynal 1996) and many other species (Woods *et al.* 2005). However, specimen-based information can sometimes be misleading because of errors associated with incorrect identification and geographic and temporal biases (Delisle *et al.* 2003; Chauvel *et al.* 2006; Crawford & Hoagland 2009). In addition, results tend to be spurious if a long history of specimen collections is not considered (Pysek & Prach 1993). Therefore, herbarium data need knowledgeable and cautious interpretation. Molecular data derived from analysis of extant populations combined with herbarium data, a commonly used approach (Novak & Mack 2001; Valliant *et al.* 2007), may yield better insight into the introduction history and spread of any introduced species.

Studies based on molecular markers

The most frequently used molecular markers for the study of introduced plants include allozymes (Husband & Barrett 1991; Novak & Mack 1993; Valliant *et al.* 2007), Random Amplified Polymorphic DNA [RAPD] (Baumel *et al.* 2001), Amplified Fragment Length Polymorphism [AFLP] (Amsellem *et al.* 2000; Kang *et al.* 2007; Lachmuth *et al.* 2010) and microsatellites (Marrs *et al.* 2008; Meimberg *et al.* 2010). These markers have been used to study genetic diversity within and among populations, identify the source population, find the number of introductions [*Bromus tectorum* (Novak & Mack 1993); *Centaurea stoebemicranthos* (Marrs *et al.* 2008); *Senecioina equidens* (Lachmuth *et al.* 2010); *Ambrosia artemisiifolia* (Gaudeul *et al.* 2011)] and track routes of introduction [*Bromus tectorum* (Novak & Mack 2001; Valliant *et al.* 2007); *Rubus alceifolius* (Amsellem *et al.* 2000); *Senecio inaequidens* (Lachmuth *et al.* 2010)]. For population genetic studies, hyper-variable, co-dominant, single locus based DNA markers that are easy to develop and screen are the markers of choice (Sunnucks 2000). Microsatellite markers (also known as simple sequence repeats or SSR) possess all these characteristics (see Table 1.2); therefore, they are one of the most widely used molecular markers in population genetics. Hence, I have selected microsatellite markers to study the population genetics of sickleweed. In addition, I am also using nuclear and chloroplast sequence data in my study.

Nuclear DNA markers are bi-parental in inheritance while chloroplast DNA markers are maternally inherited; they differ in their utilities to reveal real-time processes operating in populations. Therefore, analyses of sickleweed populations from their native and introduced ranges using nuclear and chloroplast DNA markers can provide insights into sickleweed evolution (Sunnucks 2000) and invasion pathways (Estoup &

Guillemaud 2010) that may contribute in risk assessment and effective management of this species in the United States.

Table 1.2 Comparison among various molecular markers commonly used in population genetics

Marker	PCR assay	Single locus	Co-dominant	Transfer to new taxa	Polymorphism	Replicability	Recurring cost
Sequence	Yes	Yes	Yes	Yes	Low-high	High	Low
RFLP	No	Yes	Yes	Yes	Low-moderate	High	High
RAPD	Yes	No	No	Yes	High	Variable	Low
AFLP	Yes	No	No	Yes	High	High	Medium
Microsatellite	Yes	Yes	Yes	Some	High	High	Low
Allozymes	No	Yes	Yes	Yes	Low-moderate	High	Low

-Adapted from Sunnucks (2000)

Study system

Falcaria vulgaris Bernh. [family Apiaceae; 2n=22 (Goralski *et al.* 2009)], commonly known as sickleweed, is native to the central and southern parts of Western Europe, the European part of the Former Soviet Union, the Caucasus, Western Siberia, Central Asia, Asia Minor and Iran (Larina 2008). It is an introduced plant of Northern Europe (DAISIE 2008), Africa, South America (Larina 2008) and the United States (USDA 2011). In the United States, it has been reported to occur in sixteen states and exhibits disjunct distribution on the Midwest its historic range includes the states of Illinois, Iowa, Kansas, Louisiana, Missouri, Nebraska, Oklahoma, South Dakota, Wisconsin and Wyoming, and that in the East its historic range includes the states of Maryland, Massachusetts, New York, Pennsylvania, Virginia and West Virginia [Figure 1.1 (USDA 2011)].

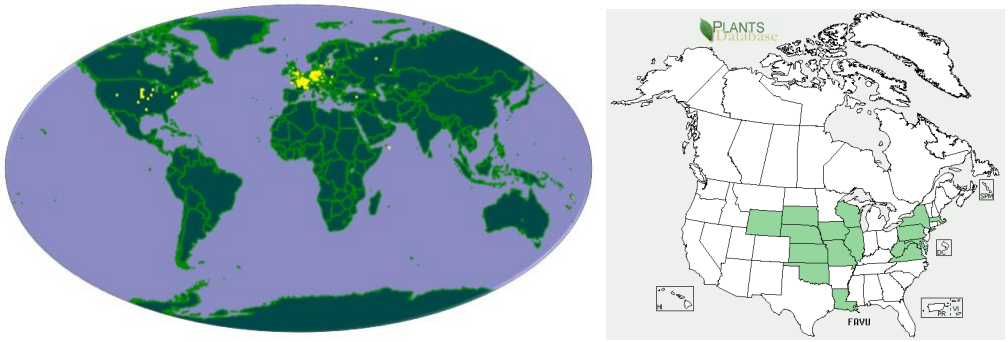


Figure 1.1 Worldwide distribution of *Falcaria vulgaris* (left, source: http://eunis.eea.europa.eu /species/152255/gbif), distribution in the United States (right, courtesy: USDA PLANTS (USDA 2011).

Information on the introduction and distribution of sickleweed in the United States is fragmented and scant. Gress (1923) first reported this species from Pennsylvania in the United States. As time progressed, the species was reported from other states as well [Kansas (Gates 1940); Missouri (Fernald 1942); Louisiana (Thomas & Raymods 1987)]. Additionally, this species has been included in the annotated checklists of some state floras- Missouri (Steyermark 1963); Iowa (Eilers & Roosa 1994); Kansas (Woods *et al.* 2005); Louisiana (MacRoberts & MacRoberts 2006); Massachusetts (Magee & Ahles 2007) and Pennsylvania (Rhoads & Block 2007). Except for these reports of the species at the regional level, there is no in-depth analysis of its introduction and distribution at the national level.

The oldest herbarium record of sickleweed in South Dakota dates back to 1961 (the record is housed at South Dakota State University). The plant was first detected at Fort Pierre National Grassland (FPNG) in 1992, and at that time this species had infested about 65 hectares of land. Now the plant has spread over more than 8000 acres in FPNG (Korman 2011). Continuous increase in area coverage of sickleweed at FPNG has attracted the attention of ecologists in the Midwest. The plant also occurs at Buffalo Gap

National Grassland (BGNG) and its control at BGNG was initiated in 2008 (Korman 2011) but the effort to eliminate sickleweed has not been successful yet. The plant has also been listed as a potentially invasive plant by Nebraska Invasive Species Council (NISC 2011).

Sickleweed is usually 30-60 cm tall with upright solid stem and fleshy tap root, leaves are primarily divided into 3-5 leaflets which are linear or linear-lanceolate with denticles along the edges giving them a sickle shape, the inflorescence is compound umbel with white flowers (Larina 2008). The flowers are andromonoecious with distinctly protandrous hermaphroditic flowers [See Knuth (1908)] which suggests that it is a cross pollinating plant. Knuth (1908) also mentions that pollination in sickleweed may be mediated by flies and beetles, and Larina (2008) refers to sickleweed as a bee-pollinated plant. Sickleweed exhibits some characteristics of invasive plant species, including the production of a large number of seeds, an effective seed dispersal mechanism whereby the seeds attached to the stem are carried away by the wind as the stems break at the nodes after senescence (Korman 2011), and the ability of the plant to reproduce asexually through root sprouting (Gress 1923; Korman 2011). These characteristics are perhaps facilitating its emergence as an aggressive weed in this part of the Midwest.

I am studying herbarium records and using molecular markers (nuclear microsatellite markers and chloroplast DNA sequences) to address the following objectives-

1. Reconstruct the introduction history and spread of sickleweed in the United States.
2. Develop molecular resources for population genetics study of sickleweed.
3. Infer the number of introductions and understand how such introduction(s) have influenced the genetic diversity and structure of sickleweed populations in the Midwest United States.

REFERENCES

Aikio S, Duncan RP, Hulme PE (2010) Lag-phases in alien plant invasions: separating the facts from the artefacts. *Oikos* **119**, 370-378.

Allendorf FW, Lundquist LL (2003) Introduction: Population biology, evolution, and control of invasive species. *Conservation Biology* **17**, 24-30.

Alyokhin A (2011) Non-natives: put biodiversity at risk. *Nature* **475**, 36-36.

Amsellem L, Noyer JL, Le Bourgeois T, Hossaert-McKey M (2000) Comparison of genetic diversity of the invasive weed *Rubus alceifolius* Poir. (Rosaceae) in its native range and in areas of introduction, using amplified fragment length polymorphism (AFLP) markers. *Molecular Ecology* **9**, 443-455.

Ayres DR, Garcia-Rossi D, Davis HG, Strong DR (1999) Extent and degree of hybridization between exotic (*Spartina alterniflora*) and native (*S. foliosa*) cordgrass (Poaceae) in California, USA determined by random amplified polymorphic DNA (RAPDs). *Molecular Ecology* **8**, 1179-1186.

Ayres DR, Zaremba K, Strong DR (2004) Extinction of a common native species by hybridization with an invasive congener. *Weed Technology* **18**, 1288-1291.

Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM (2003) Allelopathy and exotic plant invasion: from molecules and genes to species interactions. *Science* **301**, 1377-1380.

Barney JN (2006) North American history of two invasive plant species: phytogeographic distribution, dispersal vectors, and multiple introductions. *Biological Invasions* **8**, 703-717.

Baumel A, Ainouche ML, Levasseur JE (2001) Molecular investigations in populations of *Spartina anglica* C.E. Hubbard (Poaceae) invading coastal Brittany (France). *Molecular Ecology* **10**, 1689-1701.

Blackburn TM, Pysek P, Bacher S, *et al.* (2011) A proposed unified framework for biological invasions. *Trends in Ecology & Evolution* **26**, 333-339.

Blossey B, Notzold R (1995) Evolution of Increased Competitive Ability in Invasive Nonindigenous Plants - a Hypothesis. *Journal of Ecology* **83**, 887-889.

Bossdorf O, Lipowsky A, Prati D (2008) Selection of preadapted populations allowed Senecio inaequidens to invade Central Europe. *Diversity and Distributions* **14**, 676-685.

Callaway RM, Aschehoug ET (2000) Invasive plants versus their new and old neighbors: a mechanism for exotic invasion. *Science* **290**, 521-523.

Cappuccino N, Arnason JT (2006) Novel chemistry of invasive exotic plants. *Biology Letters* **2**, 189-193.

Chauvel B, Dessaint F, Cardinal-Legrand C, Bretagnolle F (2006) The historical spread of *Ambrosia artemisiifolia* L. in France from herbarium records. *Journal of Biogeography* **33**, 665-673.

Colautti RI, Ricciardi A, Grigorovich IA, MacIsaac HJ (2004) Is invasion success explained by the enemy release hypothesis? *Ecology Letters* **7**, 721-733.

Comai L (2005) The advantages and disadvantages of being polyploid. *Nature Reviews Genetics* **6**, 836-846.

Cox GW (2004) *Alien species and evolution: the evolutionary ecology of exotic plants, animals, microbes, and interacting native species.* Island Press, Washington.

Crawford PHC, Hoagland BW (2009) Can herbarium records be used to map alien species invasion and native species expansion over the past 100 years? *Journal of Biogeography* **36**, 651-661.

DAISIE (2008) *Falcaria vulgaris*. European Invasive Alien Species Gateway.

Delisle F, Lavoie C, Jean M, Lachance D (2003) Reconstructing the spread of invasive plants: taking into account biases associated with herbarium specimens. *Journal of Biogeography* **30**, 1033-1042.

Duncan CA, Jachetta JJ, Brown ML, *et al.* (2004) Assessing the economic, environmental, and societal losses from invasive plants on rangeland and wildlands. *Weed Technology* **18**, 1411-1416.

Eilers LJ, Roosa DM (1994) *The vascular plants of Iowa: an annotated checklist and natural history* University of Iowa Press, Iowa City, Iowa.

Ellstrand NC, Schierenbeck KA (2000) Hybridization as a stimulus for the evolution of invasiveness in plants? *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7043-7050.

Estoup A, Guillemaud T (2010) Reconstructing routes of invasion using genetic data: why, how and so what? *Molecular Ecology* **19**, 4113-4130.

Fernald ML (1942) Misinterpretation of Atlantic coastal plain species. *Rhodora* **44**, 238-246.

Frankham R (2005) Invasion biology - Resolving the genetic paradox in invasive species. *Heredity* **94**, 385-385.

Gates FC (1940) Recent migrational trends in the distribution of weeds in Kansas. *Transactions of the Kansas Academy of Science* **43**, 99-117.

Gaudeul M, Giraud T, Kiss L, Shykoff JA (2011) Nuclear and chloroplast microsatellites show multiple introductions in the worldwide invasion history of common ragweed, *Ambrosia artemisiifolia*. *Plos One* **6**, e17658.

Goralski G, Lubczyńska P, joachimiak AJ (2009) Chromosome Number Database.

Gordon DR (1998) Effects of invasive, non-indigenous plant species on ecosystem processes: Lessons from Florida. *Ecological Applications* **8**, 975-989.

Gress EM (1923) *Falcaria rivini*, a plant new to the United States. *Rhodora* **25**, 13-14.

Hierro JL, Maron JL, Callaway RM (2005) A biogeographical approach to plant invasions: the importance of studying exotics in their introduced and native range. *Journal of Ecology* **93**, 5-15.

Hufbauer RA, Torchin ME (2007) Integrating ecological and evolutionary theory of biological invasions. In: *Biological Invasions* (ed. Nentwig W), pp. 79-96. Springer, Berlin.

Husband BC, Barrett SCH (1991) Colonization history and population genetic structure of *Eichhornia paniculata* in Jamica. *Heredity* **66**, 287-296.

Kang M, Buckley YM, Lowe AJ (2007) Testing the role of genetic factors across multiple independent invasions of the shrub Scotch broom (*Cytisus scoparius*). *Molecular Ecology* **16**, 4662-4673.

Keane RM, Crawley MJ (2002) Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology & Evolution* **17**, 164-170.

Kelager A, Pedersen JS, Bruun HH (2013) Multiple introductions and no loss of genetic diversity: invasion history of Japanese Rose, *Rosa rugosa*, in Europe. *Biological Invasions* **15**, 1125-1141.

Knuth P (1908) *Handbook of flower pollination* Oxford, Clarendon.

Kolar CS, Lodge DM (2001) Progress in invasion biology: predicting invaders. *Trends in Ecology & Evolution* **16**, 199-204.

Korman BL (2011) *Biology and Ecology of Sickleweed (Falcaria vulgaris) in the Fort Pierre National Grassland of South Dakota,* South Dakota State University.

Kowarik I (2005) Time lags in biological invasions with regard to the success and failure of alien species. In: *Plant invasions: general aspects and special problems* eds. Phsek P, Prach K, Rejmanek M, Wade M). SPB Academic Publishers, Amsterdam, the Netherlands.

Kwiatkowska AJ, Spalik K, Michalak E, Palinska A, Panufnik D (1997) Influence of the size and density of *Carpinus betulus* on the spatial distribution and rate of deletion of forest-floor species in thermophilous oak forest. *Plant Ecology* **129**, 1-10.

Lachmuth S, Durka W, Schurr FM (2010) The making of a rapid plant invader: genetic diversity and differentiation in the native and invaded range of *Senecio inaequidens*. *Molecular Ecology* **19**, 3952-3967.

Lambrinos JG (2001) The expansion history of a sexual and asexual species of *Cortaderia* in California, USA. *Journal of Ecology* **89**, 88-98.

Larina SY (2008) *Falcaria vulgaris* Bernh. Interactive agricultural ecological atlas of Russia and neighboring countries. In: *Economic plants and their diseases, pests and weeds* eds. Afonin AN, Greene SL, Dzyubenko NI, Frolov AN).

Lavergne S, Molofsky J (2007) Increased genetic variation and evolutionary potential drive the success of an invasive grass. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 3883-3888.

Lavoie C, Jodoin Y, de Merlis AG (2007) How did common ragweed (*Ambrosia artemisiifolia* L.) spread in Quebec? A historical analysis using herbarium records. *Journal of Biogeography* **34**, 1751-1761.

Lee CE (2002) Evolutionary genetics of invasive species. *Trends in Ecology & Evolution* **17**, 386-391.

Levine JM, Adler PB, Yelenik SG (2004) A meta-analysis of biotic resistance to exotic plant invasions. *Ecology Letters* **7**, 975-989.

Mack RN (1981) Invasion of *Bromus tectorum* L. into Western North America: An ecological chronicle *Agro-Ecosystems* **7**, 145-165.

Mack RN (1991) The commercial seed trade: An early disperser of weeds in the United States. *Economic Botany* **45**, 257-273.

MacRoberts BR, MacRoberts MH (2006) An updated, annotated vascular flora of Caddo Parish, Louisiana, with notes on regional phytogeography and ecology. *SIDA Contributions to Botany* **22**, 1191-1219.

Magee DW, Ahles HE (2007) *Flora of the Northeast: a manual of the vascular flora of New Enlgand and adjacent New York*, 2nd edn. University of Massachusetts Press, Amherst, Massachusetts.

Marrs RA, Sforza R, Hufbauer RA (2008) Evidence for multiple introductions of *Centaurea stoebe micranthos* (spotted knapweed, asteraceae) to North America. *Mol Ecol* **17**, 4197-4208.

McCauley DE, Smith RA, Lisenby JD, Hsieh C (2003) The hierarchical spatial distribution of chloroplast DNA polymorphism across the introduced range of *Silene vulgaris*. *Molecular Ecology* **12**, 3227-3235.

Meimberg H, Milan NF, Karatassiou M, *et al.* (2010) Patterns of introduction and adaptation during the invasion of *Aegilops triuncialis* (Poaceae) into Californian serpentine soils. *Molecular Ecology* **19**, 5308-5319.

Mihulka S, Pysek P (2001) Invasion history of *Oenothera* congeners in Europe: a comparative study of spreading rates in the last 200 years. *Journal of Biogeography* **28**, 597-609.

Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 5446-5451.

NISC (2011) Invasive plants of Nebraska.

Novak SJ, Mack RN (1993) Genetic-Variation in *BromusTectorum* (Poaceae) - Comparison between Native and Introduced Populations. *Heredity* **71**, 167-176.

Novak SJ, Mack RN (1995) Allozyme Diversity in the Apomictic Vine *Bryonia Alba* (Cucurbitaceae) - Potential Consequences of Multiple Introductions. *American Journal of Botany* **82**, 1153-1162.

Novak SJ, Mack RN (2001) Tracing plant introduction and spread: Genetic evidence from *Bromus tectorum* (Cheatgrass). *Bioscience* **51**, 114-122.

Pysek P, Prach K (1993) Plant invasion and the role of riparian habitats: a comparison of four species alien to central Europe. *Journal of Biogeography* **20**, 413-420.

Rhoads AF, Block TA (2007) *The plants of Pennsylvania: an illustrated manual*, 2nd edn. University of Pennsylvania, Philadelphia.

Richardson DM, Allsopp N, D'Antonio CM, Milton SJ, Rejmanek M (2000a) Plant invasions - the role of mutualisms. *Biological Reviews* **75**, 65-93.

Richardson DM, Pysek P, Rejmanek M, *et al.* (2000b) Naturalization and invasion of alien plants: concepts and definitions. *Diversity and Distributions* **6**, 93-107.

Sakai AK, Allendorf FW, Holt JS, *et al.* (2001) The population biology of invasive species. *Annual Review of Ecology and Systematics* **32**, 305-332.

Sala OE, Chapin FS, Armesto JJ, *et al.* (2000) Biodiversity - Global biodiversity scenarios for the year 2100. *Science* **287**, 1770-1774.

Sheeley SE, Raynal DJ (1996) The distribution and status of species of *Vincetoxicum* in eastern North America. *Bulletin of the Torrey Botanical Club* **123**, 148-156.

Soltis PS, Soltis DE (2000) The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7051-7057.

Steyermark JA (1963) *Flora of Missouri* Iowa State University Press, Ames, Iowa.

Strother JL, Smith AR (1970) Chorology, collection dates, and taxonomic responsibility. *Taxon* **19**, 871-874.

Stuckey RL (1980) Distributional history of *Lythrum salicaria* (Purple loosestrife) in North America. *Bartonia* **47**, 18.

Sunnucks P (2000) Efficient genetic markers for population biology. *Trends in Ecology & Evolution* **15**, 199-203.

te Beest M, Le Roux JJ, Richardson DM, *et al.* (2012) The more the better? The role of polyploidy in facilitating plant invasions. *Annals of Botany* **109**, 19-45.

Thomas RD, Raymods LR (1987) *Falcaria vulgaris* Bernh. (Apiaceae): New to Louisiana. *The Southwestern Naturalist* **32**, 279.

Torchin ME, Mitchell CE (2004) Parasites, pathogens, and invasions by plants and animals. *Frontiers in Ecology and the Environment* **2**, 183-190.

Treier UA, Broennimann O, Normand S, *et al.* (2009) Shift in cytotype frequency and niche space in the invasive plant *Centaurea maculosa*. *Ecology* **90**, 1366-1377.

USDA N (2011) The Plants Database National Plant Data Center, Baton Rouge, LA 70874-4490 USA.

Valery L, Fritz H, Lefeuvre JC, Simberloff D (2008) In search of a real definition of the biological invasion phenomenon itself. *Biological Invasions* **10**, 1345-1351.

Valliant MT, Mack RN, Novak SJ (2007) Introduction history and population genetics of the invasive grass *Bromus tectorum* (Poaceae) in Canada. *American Journal of Botany* **94**, 1156-1169.

Walker B, Steffen W (1997) An overview of the implications of global change for natural and managed terrestrial ecosystems. *Conservation Ecology [online]* **1**, 2.

Weber E (1998) The dynamics of plant invasions: a case study of three exotic goldenrod species (*Solidago* L.) in Europe. *Journal of Biogeography* **25**, 147-154.

Wester L (1992) Origin and distribution of adventive alien flowering plants in Hawaii. In: *Alien plant invasions in native ecosystems of Hawaii* eds. Stone CP, Smith CW, Tunison T), pp. 99-154. University of Hawaii Cooperative National Park Resources Studies Unit, Honolulu, HI.

Woods TM, Strakosh SC, Nepal MN, *et al.* (2005) Introduced species in Kansas: floristic changes and patterns of collection based on an historical herbarium. *SIDA Contributions to Botany* **21**, 1695-1725.

CHAPTER 2

INFERRING INTRODUCTION HISTORY AND SPREAD OF *FALCARIA VULGARIS* BERNH. (APIACEAE) IN THE UNITED STATES BASED ON HERBARIUM RECORDS

ABSTRACT

Herbarium records were studied to infer the introduction history and spread of the exotic Eurasian sickleweed (*Falcaria vulgaris* Bernh.) in the United States. The spread of the plant was reconstructed using the location of early collections as possible sites of primary introduction, and the location of subsequent collections as potential pathways along which this species spread. Herbarium records indicate that sickleweed was first introduced no later than 1922, and independent introduction of this plant took place in the East and in the Midwest of the United States. The species has been found in 37 counties of 15 states of the United States. No sickleweed records have been reported for the last 17 years in the U.S., except in the state of Iowa, Nebraska and South Dakota. The plant has been characterized as an aggressive weed by experts in the latter two states, where it is locally established and has infested most notably portion of the Fort Pierre National Grassland and Buffalo Gap National Grassland in South Dakota. It is also reported from several sites in Nebraska from roadsides. It is important to verify the existence of sickle-weed in areas from where the herbarium specimens were previously collected to help identify areas at risk. Control strategies need to be implemented and policies should be developed to enlist the participation of public lands managers, transportation departments and private land-owners to control and manage this species before it becomes a more widespread invader.

Keywords: *Falcaria vulgaris*, herbarium specimen, introduced plant, sickleweed.

INTRODUCTION

A tremendous exchange of biotas has occurred since the exploration age began in the early 15th century (Mooney and Cleland 2001). Some introduced plant species were advertently introduced for their medicinal, ornamental and forage values and some were introduced for the production of fiber, timber and fuel wood (Cox 2004). In many cases, exotic plants were accidentally introduced as contaminants in crop seed or animal fodder, with domesticated animals and ship ballast, and as hitchhikers with military movements (Mack 1991; Sakai et al. 2001; Cox 2004; Chauvel et al. 2006). Theoretically, very few introduced plants become invasive; however, the number of introduced invasive plant species has reached more than 1000 in the United States alone (Mooney and Cleland 2001; USDA, ARS 2012). With the increase in the number of invasive plant species and their range expansion, the urgency to study and understand the biological process of plant introduction, establishment, spread and invasion in novel habitats is being realized (Pimentel et al. 2000).

Herbarium records are the most reliable primary source of information to reconstruct introduction and colonization history of a species when detailed historic data are not available (Strother and Smith 1970; Mack 1991; Barney 2006). Herbarium specimen labels provide valuable information that can be used to document the time of introduction of non-native plants (Wester 1992; Woods et al. 2005; Valliant et al. 2007), the number of independent introductions (Barney 2006), the early invasion pathways in the introduced range (Lavoie et al. 2007; Stuckey 1980) and distributional changes of plants over time as in *Ambrosia artemisiifolia* (Chauvel et al. 2006; Lavoie et al. 2007), *Bouteloua curtipendula* (Laughlin 2003), *Bromus tectorum* (Novak and Mack 2001;

Valliant et al. 2007), *Cortaderia selloana* and *C. jubata* (Lambrinos 2001), *Oenothera* spp. (Mihulka and Pysek 2001), *Solidago* spp. (Weber 1998), *Vincetoxicum* spp.(Sheeley and Raynal 1996) and many other species (Woods et al. 2005). However, herbarium specimen based information can sometimes be misleading because of errors associated with incorrect identification and geographic and temporal biases (Delisle et al. 2003; Chauvel et al. 2006; Crawford and Hoagland 2009). In addition, results tend to be spurious if a long history of specimen collections is not considered (Pysek and Prach 1993). Therefore, herbarium data need knowledgeable and cautious interpretation.

Falcaria vulgaris Bernh. (Syn. *F. rivini, F. sioides*; family Apiaceae; 2n = 22 [Goralski et al. 2009]), commonly known as sickleweed, is native to the European part of the former Soviet Union, the Caucasus, Western Siberia and Central Asia. It is also distributed in the central and southern parts of Western Europe, the Mediterranean, Asia Minor, and Iran. It is an introduced species in Africa, and North and South America (Larina 2008). It has been reported in sixteen states in the United States (USDA, NRCS 2011) and exhibits disjunct distribution in the midwestern and eastern USA. In the Midwest, its range includes the states of Illinois, Iowa, Kansas, Louisiana, Missouri, Nebraska, Oklahoma, South Dakota, Wisconsin and Wyoming; and in the East, it includes Maryland, Massachusetts, New York, Pennsylvania, Virginia and West Virginia. In South Dakota, it occurs in the Fort Pierre National Grassland (FPNG; ca. 3200 ha infested in 2005), Buffalo Gap National Grassland (BGNG; ca. 40 ha), and locally on the campus of South Dakota State University (Korman 2011). Several large populations of sickleweed occur in six counties in Nebraska (our field observation; R. Kaul and K. Decker, University of Nebraska) where this species has been labeled a Category II

invasive plant (invasive species whose eradication is still feasible; NISC 2011). No literature suggests invasiveness of this plant in any states other than South Dakota and Nebraska.

The sickleweed plant is usually 30-60 cm tall with upright solid stems and a fleshy tap root; leaves are pinnately divided into 3-5 leaflets that are linear or linear-lanceolate and often curved to give the leaflets a sickle shape; leaflet margins have denticles. The inflorescence is a compound umbel with white flowers (Larina 2008). The flowers are andromonoecious and protandrous (See Knuth and Muller 1908). Phenotypic plasticity of its growth habit [annual or biennial (Clapham et al. 1989) or even perennial (Bojnansky and Fargasova 2007; Korman 2011)] and reproductive system [monoecism (See Knuth and Muller 1908) and vegetative propagation through rootstocks (Gress 1923; Larina 2008; Korman 2011)] help make this plant aggressive. Korman (2011) showed that this plant is negatively impacting species diversity and forage production of native grassland at FPNG.

Information on the introduction and distribution of sickleweed in the United States is fragmented and scant. Gress (1923) first reported this species in the United States (Pennsylvania). There are also short notes on the detection of this species in other states (Gates 1940, Kansas; Fernald 1942, Missouri; Thomas and Raymond 1987 Louisiana). Additionally, this species has been included in some state floras, e.g., Steyermark 1963, Missouri; Eilers and Roosa 1994, Iowa; Woods et al. 2005, Kansas; MacRoberts and MacRoberts 2006, Louisiana; Magee and Ahles 2007, Massachusetts; Rhoads and Block 2007, Pennsylvania. Other than these reports of this species at the regional level, there is no in-depth study on its introduction and distribution at the

national level. Information for species introduction and spread in a new habitat can help predict the invasiveness of introduced species and may also be useful for control (Ricciardi et al. 2000; Kolar and Lodge 2001; Lambrinos 2001; Lavoie et al. 2003; Dybas 2004; Lerdau and Wickham 2011). In addition, this information can give clues on probable sites of invasion (Weber 1998). We are using herbarium records and relevant literature to study the introduction history and spread of sickleweed and to determine current distribution in the USA. The objectives of this study are to assess and infer 1) when and where this species was introduced, 2) current temporal spread of the species and 3) the number of independent introductions.

METHODS

Specimens from herbaria in the sixteen states, where the USDA Plants Database has reported the occurrence of sickleweed, were examined. The list of the herbaria (Appendix 1) was obtained from *Index Herbariorum* (http://sciweb.nybg.org/science2/IndexHerbariorum.asp), a directory of public herbaria of the world. Herbarium curators/collection managers of these herbaria were contacted for information on their holdings of sickleweed. The information requested for each specimen included voucher/accession number, date collected, collector(s) and collection locality. As the collections of many introduced species can be unmounted or unprocessed in herbaria, we requested information on unmounted sickelweed specimens (if any) as well. The small number of specimens and the monotypic nature of the genus led to fast communication of data from herbaria and experts. Information was also obtained from online specimen databases (BKL, HUH, ISM, KANU, KSC, LSU, Oklahoma Vascular Plant Database, RM, TROPICOS). Abbreviations for these herbaria follow those of

Holmgren et al. (1990). Most sickleweed specimens were housed in the major agricultural herbaria in the Midwest (ISC, NEB, SDC); these were visited to examine specimens. Vouchers that had been collected by the same collector from the same locality on the same date (duplicates) were regarded as one specimen following Chauvel et al. (2006). Specimens collected from countries other than the United States were not included in this study. The sites of earliest collections of herbarium specimens were considered to be the possible sites of early introduction, whereas the sites of subsequent collections were interpreted to be the possible pathways along which this species spread. Following Barney (2006), we assumed that the species is always present once it was collected from a county. Based on this assumption, a temporal distribution map of the species (at county level) was constructed using ArcGIS 9.3 (ESRI 2008).

RESULTS

Falcaria vulgaris specimens in herbaria

Among the 178 herbaria contacted, we received responses from 76 herbaria. Among these, 42 herbaria (BALT, BDWR, BTJW, BUPL, CAMU, CORT, DEK, DWC, DWU, ECH, EMNH, FWVA, ILL, ISU, KEN, KNOX, KSTC, LAF, LSUS, LYN, MCN, MOAR, MOR, MVSC, MWI, NWOSU, NYS, ORU, PHIL, PLAT, RMS, RUHV, SDU, SEMO, SMS, TAWES, URV, VAS, WARM, WILLI, WVW and YELLO) had no sickleweed specimens. At the remaining 34 herbaria (BH, BHSC, BKL, CM, CSCN, DUR, F, FARM, GH, ISC, ISM, KANU, KSC, LSU, MASS, MO, NEB, NEBC, NLU, NO, ODU, OKL, OKLA, OMA, PA, PH, RM, SDC, UMO, UWM, UWSP, VPI, WIS and WVA), we found 195 sickleweed specimens collected from the United States. After excluding the duplicate specimens, we examined a total of 143 sickleweed specimens.

These specimens were collected from 1922 to 2011 from 32 counties of 15 states. We noted 5 more counties where sickleweed has been reported (J. T. Kartesz, Biota of North America) but for which we found no specimen evidence. Among the 16 states from which USDA Plants Database reported the occurrence of sickleweed, we were unable to locate sickleweed specimens from Maryland in any of the herbaria we contacted. To our knowledge, sickleweed has been reported from 37 counties in 15 states in the United States. The majority of specimens examined were from Iowa, Nebraska, and South Dakota, and there were no collections from any other state made during the last 17 years (Table 2.1). GH houses the highest number of specimens collected from different states, including the oldest collections from Iowa, Oklahoma, Massachusetts, Nebraska, Pennsylvania, and Wisconsin. SDC houses the highest number of recently collected specimens.

Information from Herbarium Records

Collection history of *Falcaria vulgaris*—Sickleweed was first reported by Gress (1923) as a new species to the United States. He was the collector of the oldest specimen of sickleweed, which was collected from the agricultural field at Mercersberg, Franklin County, Pennsylvania, on September 2, 1922 (Figure 2.1). This specimen is housed at Gray Herbarium (Gress, s.n., GH) with a duplicate at Carnegie Museum of Natural History (Gress, s.n., CM). Sickleweed was subsequently reported in New York (August 1923; Holtzoff, 289002, BKL), West Virginia (August 1954; Hicks and Bartley, 32, WVA), Virginia (June 1974; Harril and Wise, 31616, VPI), and Massachusetts (August 1989; Sorrie and Weatherbee, 4884, GH; NEBC; MASS). The specimen collected from

Massachusetts in August 1989 (Sorrie and Weatherbee, 4884, GH; NEBC; MASS) represents the most recent collection from the eastern USA.

Table 2.1 Earliest and the most recent specimen records of *Falcaria vulgaris* in the United States

State	Oldest herbarium record	Voucher information	Most recent herbarium record	Voucher information
Illinois	28 June 1955	Rexroat, 49125, ISM	11 July 1957	Martens, s.n., SOTO
Iowa	1 Oct 1930	Harmon, s.n., GH; ISC	20 Aug 2011	Nepal, Neupane and Piya, 101, SDC
Kansas	29 May 1932	Anthony, s.n., KANU	28 June 1951	Blocker, 26719, KSC
Louisiana	28 Apr 1984	Thomas and Raymond, 88299,NLU	15 May 1984	Thomas and Taylor, 88740,NLU
Massachusetts	25 Aug 1989	Sorrie and Weatherbee, 4884, GH; NEBC; MASS	25 Aug 1989	Sorrie and Weatherbee, 4884, GH; NEBC; MASS
Missouri	19 July 1941	Miller, 35958, UMO	10 Sept 1991	Ellis, s.n., MO
Nebraska	16 Sept 1946	Kinch, s.n., GH	20 Aug 2011	Nepal, Neupane and Piya, 101, SDC
New York	1 Aug 1923	Holtzoff, 289002, BKL	30 May 1928	Holtzoff, 289003, BKL
Oklahoma	30 July 1957	Engleman, 105664, OKL	1 July 1974	Hamman,s.n.,DUR
Pennsylvania	2 Sept 1922	Gress, s.n., GH	29 July 1962	PH
South Dakota	9 June 1961	Unknown, s.n., SDC	20 May 2009	Korman, 470, SDC
Virginia	27 June 1974	Harril and Wise, 31616, VPI	16 Aug 1980	Wieboldt, 71984, VPI
West Virginia	5 Aug 1954	Hicks and Bartley, 32, WVA	5 Aug 1954	Hicks and Bartley, 32, WVA
Wisconsin	29 July 1981	Thompson, 0013345, WIS	11 Aug 1991	Thompson, 0013345, WIS
Wyoming	9 Sept 1995	Dorn, 600764, RM	9 Sept 1995	Dorn, 600764, RM

The oldest specimen from the Midwest is from Sioux County, Iowa, collected on October 1, 1930 (Harmon, s.n., GH, ISC). The next report was from Atchison County, Kansas, in 1932. The species was then reported from Missouri (July 1941; Miller, 35958, UMO), Nebraska (September 1946; Kinch, s.n., GH), Illinois (June 1955; Rexroat, 49125, ISM), Oklahoma (July 1957; Engleman, 105664, OKL), South Dakota (June 1961; Unknown, s.n., SDC), Wisconsin (July 1981; Thompson, 0013345, WIS), Louisiana (April 1984; Thomas and Raymond, 88299, NLU) and Wyoming (September 1995; Dorn, 600764, RM). After 1995, specimens were collected only from the states of Iowa, Nebraska and South Dakota. The oldest and latest herbarium specimens collected from different states in the United States are presented in Table 2.1.

Collection site—The first herbarium specimens from the East (Pennsylvania) and the Midwest (Iowa) were both collected from agricultural fields. In Pennsylvania, the first sickleweed specimen was collected from a field where clover (*Trifolium* spp.) and timothy grass (*Phleum pratense*) were being cultivated. Similarly, the oldest herbarium specimen from South Dakota was collected from an agricultural field, but most of the recent specimens are from grasslands (FPNG and BGNG, South Dakota). In Nebraska, most specimens were collected from the roadsides with the specific locality not provided. Table 2.2 shows the number of sickleweed specimens collected from different habitats at different time periods.

Introduction, spread and naturalization— On most of the sickleweed herbarium labels, the species is noted to be introduced and native to Europe and Asia, but without a mention of the actual country of origin of the accession. The status of the plant was given as “naturalized”, “adventive” or “common” in most of the counties at the time of

collection, but in few cases the plant was described as “rare”. There is no information on how this species was introduced to the United States. There exists, however, some literature about collector’s observation of the species in the field that provides some valuable clues. Gress (1923) reported this species as new to the United States, whereas Cratty (1930), Gates (1940), Thomas and Raymond (1987) mentioned this species as new to Iowa, Kansas and Louisiana, respectively. Collectors have also mentioned that the species was new to the state on some of the herbarium labels. In most cases, the species was detected long after introduction and by the time it had become established as a relatively large population (Fernald 1942; Thomas and Raymond 1987). A century long collection of sickleweed specimens in the USA shows that the spread of the species is concentrated mainly in the Midwest (Figure 2.2a-d).

Table 2.2 Number of sickleweed specimens collected from different habitats during different time periods

Year	Agricultural land	Railroad/Roadway	Grassland	Unknown	Total
1920-1940	5	2		2	9
1941-1960		2	3	6	11
1961-1980	1	1	1	2	5
1981-2000		10	3	2	15
2001-2011		5	150		155

Figure 2.1 Earliest record of *F. vulgaris* from Pennsylvania- collector Gress. (Image Source: Emily Wood, GH).

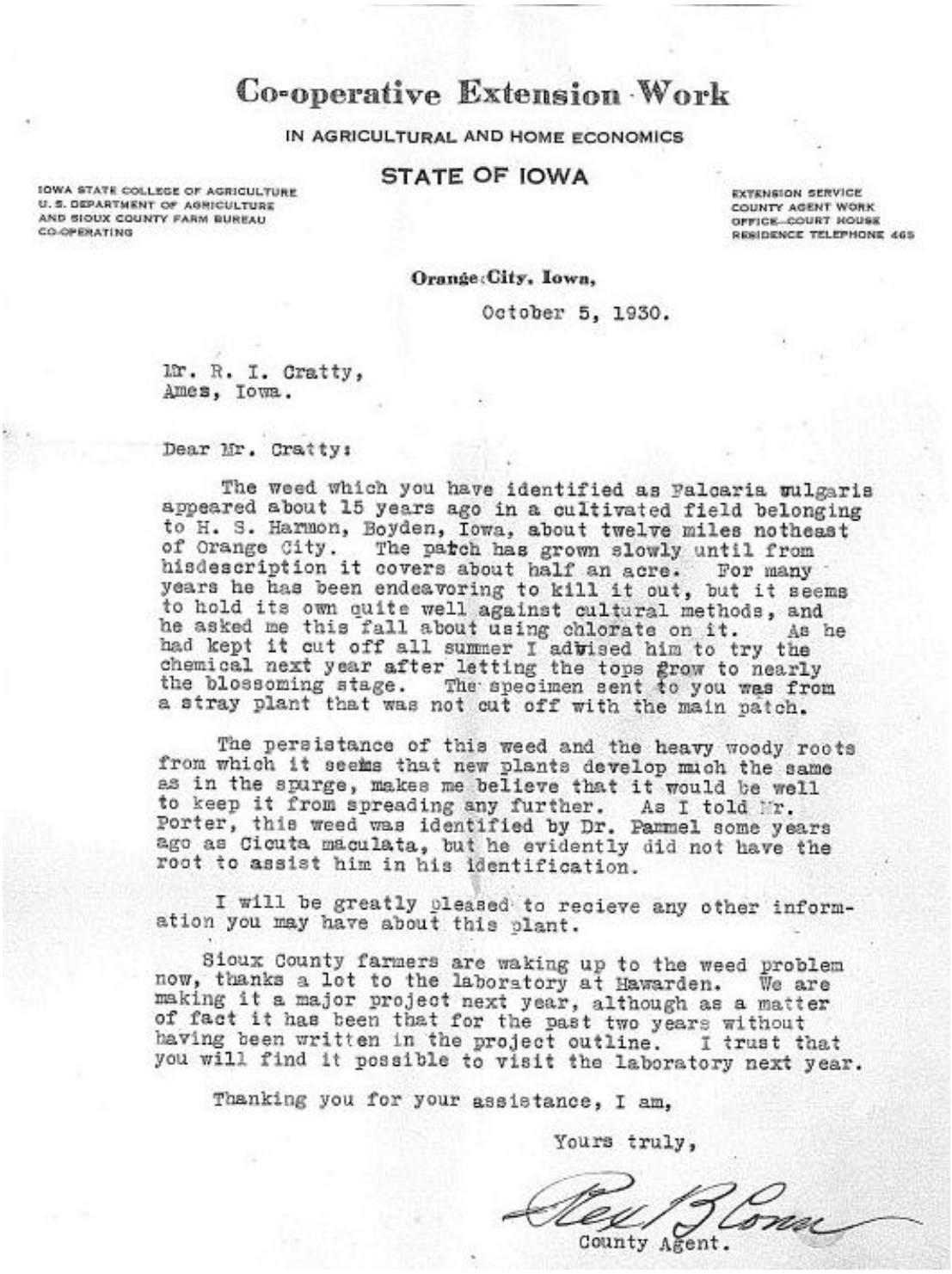
Figure 2.2 Spatial distributions of sickleweed records in the United States counties up to (a) 1950 (b) 1975 (c) 2000 and (d) 2012.The arrows in Fig. 2(a) indicate the counties where sickleweed was first detected. The distribution maps were created using our data, information from herbarium records and geo reference data from BONAP (Biota of North America Program).

DISCUSSION

***Sickleweed herbarium records in the introduced range*—**In the United States, sickleweed has been collected since 1922, but the number of sickleweed collections is relatively small (Table 2.2). There is no record of collection from the East and some states in the Midwest (except Iowa, Nebraska and South Dakota) for the last 17 years. Usually, adventive species are repeatedly collected if they persist and are abundant (Wester 1992). In some cases, the lower genetic diversity of introduced plants results in inbreeding depression thereby causing the species to disappear (Ellstrand and Elam 1993). In the Czech Republic, Pergl et al. (2012) reported the disappearance of *Heracleum mantegazzianum* from 76% of the sites where the species had once colonized. In United States, sickleweed may likewise have disappeared from several sites where it had once colonized. Mitchell and Tucker (2000) and Weldy and Werier (2012) reported that sickleweed has disappeared from New York. However, it is not possible to ensure the extinction of a species from a specific locality merely on the basis of herbarium data. Sometimes collectors show no interest in collecting a species once it is represented in the herbarium from a particular locality (Stuckey 1980; Chauvel et al. 2006). This is true for many exotic plant species. Although no sickleweed specimen has been collected recently in Massachusetts and Pennsylvania, the plant has been listed in a recent publication as an adventive species for Massachusetts and Pennsylvania (Magee and Ahles 2007; Rhoads and Block 2007; The Pennsylvania Flora Project) recognizing that the plant may still occur in these states. To reconstruct the spread of the plant, we assumed that the species was potentially present in each of the counties where it was once collected.

Introduction and spread of sickleweed—Sickleweed was introduced in the first quarter of the twentieth century in the United States. According to Gress (1923), the farmer whose field in Pennsylvania was the source of the earliest USA collection had detected the plant about five years before the collection date. In the Midwest, sickleweed was first collected in 1930 from a farm field in Iowa. A letter sent by the county agent of Sioux County to the curator of ISC, R. I. Cratty (Figure 2.3), mentions that the species was previously misidentified as *Cicuta maculata*. Also, Cratty (1930) mentions that the farmer of the field where the specimen was collected had detected this weed about 15 years before the collection date of the specimen. It is not unusual that introduced plants are noticed only after they are well established and cover a large area (Wester 1992). It appears that sickleweed was detected approximately at the same time in the early 20th century in the East and in the Midwest of the United States. Cox (2004) reported that several ruderal plants have been introduced accidentally to the USA from Europe as contaminants in crop seed and animal fodder (Cox 2004). Since sickleweed was first reported as a weed from agricultural fields in both East and Midwest states, we assume that this species was introduced accidentally, and perhaps as a seed contaminant.

If introduced species have commercial value, then they could have been purposely introduced; otherwise they could be assumed to be accidentally introduced (Wester 1992). In some parts of its native range, sickleweed has been traditionally used as a medicinal herb to treat skin ulcers, stomach disorders, liver diseases, and kidney and bladder stones, and has also been eaten as a vegetable (See Khazaei and Salehi 2006). Fernald (1942) reported the occurrence of a sickleweed population in Missouri near a



Co-operative Extension Work

IN AGRICULTURAL AND HOME ECONOMICS

STATE OF IOWA

IOWA STATE COLLEGE OF AGRICULTURE
U. S. DEPARTMENT OF AGRICULTURE
AND SIOUX COUNTY FARM BUREAU
CO-OPERATING

EXTENSION SERVICE
COUNTY AGENT WORK
OFFICE—COURT HOUSE
RESIDENCE TELEPHONE 465

Orange City, Iowa,

October 5, 1930.

Mr. R. I. Cratty,
Ames, Iowa.

Dear Mr. Cratty:

The weed which you have identified as Falcaria vulgaris appeared about 15 years ago in a cultivated field belonging to H. S. Harmon, Boyden, Iowa, about twelve miles notheast of Orange City. The patch has grown slowly until from hisdescription it covers about half an acre. For many years he has been endeavoring to kill it out, but it seems to hold its own quite well against cultural methods, and he asked me this fall about using chlorate on it. As he had kept it cut off all summer I advised him to try the chemical next year after letting the tops grow to nearly the blossoming stage. The specimen sent to you was from a stray plant that was not cut off with the main patch.

The persistance of this weed and the heavy woody roots from which it seems that new plants develop much the same as in the spurge, makes me believe that it would be well to keep it from spreading any further. As I told Mr. Porter, this weed was identified by Dr. Pammel some years ago as Cicuta maculata, but he evidently did not have the root to assist him in his identification.

I will be greatly pleased to recieve any other information you may have about this plant.

Sioux County farmers are waking up to the weed problem now, thanks a lot to the laboratory at Hawarden. We are making it a major project next year, although as a matter of fact it has been that for the past two years without having been written in the project outline. I trust that you will find it possible to visit the laboratory next year.

Thanking you for your assistance, I am,

Yours truly,

Rex B. Conn

County Agent.

Figure 2.3 Letter sent by county agent Rex B. Conn to R. I. Cratty (ISC curator) informing about the occurrence of sickleweed in Sioux County, Iowa.

community with a large number of German immigrants, suggesting the population could have been intentionally introduced from central Europe.

Based on the distribution map constructed using herbarium specimens (Figure 2.2), we see two disjunct distributions with earliest detections in both the East and in the Midwest being nearly simultaneous. We therefore propose two primary introductions of sickleweed in the United States. The sickleweed population in Franklin County, Pennsylvania, is possibly the source population for the eastern USA and the population in Sioux County, Iowa for the Midwest. Propagules then may have been dispersed from these primary sites to the other sites (Figure 2.2a) through various mechanisms. Transportation of plant propagules may occur through the attachment of seeds to muddy vehicles or tires used for human and freight transportation (Kowarik and von der Lippe 2007). Additionally, the cutting and transporting of hay that included sickleweed with mature seed could account for the spread. Furthermore, when the sickleweed plant senesces, it breaks at the nodes, and plant segments tumble in the wind to disperse the seed (See Limpert et al. 2004; Korman 2011). Also, the seeds might have been transported from the primary sites by mammals and birds. For example, there is some evidence of sickleweed seeds being transported from the plant's native range to other countries within Europe by ducks and other water birds (Brochet et al. 2009). If molecular data derived from analysis of herbarium specimens and extant populations were combined with the herbarium data, is a commonly used approach (see Novak and Mack 2001; Valliant et al. 2007), better insight into the entry and spread of sickleweed in the USA could be had.

Current control efforts and future prospects—Attempts to control sickleweed at FPNG in South Dakota have shown how difficult this weed can be once it becomes established. It was first detected at FPNG in 1992, and at that time this species had infested only 65 hectares of land. But attempts to manage the outbreak began only after a decade had passed and the plant was spreading aggressively and overtaking grassland vegetation. An attempt to control the species spread using prescribed fire proved ineffective. Herbicide treatment with Dupont Telar XP® has been practiced since 2004 to control spread of the weed and has proven effective with repeat applications, although not all of the area infested has been treated and new patches are being found outside of treated areas (Korman 2011) with current infestation now an estimated 8,000 acres. This example illustrates the need to eradicate exotic plant infestations as soon as they are detected. Eradication of invasive species can be easy when a few plants are found early by appropriate survey, or the population size is small and confined to a small area (Wester 1992), but when the area of infestation increases, the cost of control and management increases exponentially (Rejmanek and Pitcairn 2002).

In Nebraska, we observed the occurrence of several large populations of sickleweed along roadsides. According to Pysek and Prach (1993), if invasive species occur along roadsides or railroad tracks, these sites not only harbor the plant, but also serve as corridors for their spread. Plants growing along roadsides are more likely to be transported by vehicles and may also spread to nearby pastures and hay fields by means of wind or other agents. In Nebraska, sickleweed is listed as a Category II invasive plant by the Nebraska Invasive Species Council (2011). Thus far, no major program has been launched for its control and management (K. Decker, University of Nebraska - Lincoln),

but recently the large population in Lancaster County, Nebraska, has been treated with herbicide and all plants appear to be dead (R Kaul, University of Nebraska- Lincoln). Infestations at FPNG and BGNG in South Dakota and along roadsides in Nebraska that represent diverse habitats make it necessary that control strategies involve cooperation among public land managers, transportation departments, and private landowners to hope for effective long term control and management. This study on the distribution and spread of sickleweed is pursued for purposes of realizing better control strategies and management practices in the region. In this paper, we present information compiled to interpret the status of recent sickleweed populations relative to those of the past. The absence of recent sickleweed records from states other than Iowa, Nebraska, and South Dakota suggests that this species is growing undetected or not currently present in those states. However, should it become established over a period of time, it may become invasive elsewhere when a sufficient number of propagules is transported to congenial environments as discussed by Kolar and Lodge (2001). In this study we used herbarium data to reconstruct the introduction history of sickleweed and its subsequent dispersal in the United States. Additionally, knowing the environmental limits of this plant along with the dispersal pathways will help us predict areas vulnerable to future invasion.

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LITERATURE CITED

Barney, J.N. 2006. North American history of two invasive plant species: phytogeographic distribution, dispersal vectors, and multiple introductions. Biological Invasions 8:703-717.

Bojnansky, V., and A. Fargasova. 2007. Atlas of seeds and fruits of Central and East European flora. Springer, The Netherlands.

Brochet, A., M. Guillemain, H. Fritz, M. Gauthier-Clerc, and A.J. Green. 2009. The role of migratory ducks in the long-distance dispersal of native plants and the spread of exotic plants in Europe. Ecography 32:919-928.

Chauvel, B., F. Dessaint, C. Cardinal-Legrand, and F. Bretagnolle. 2006. The historical spread of *Ambrosia artemisiifolia* L. in France from herbarium records. Journal of Biogeography 33:665-673.

Clapham, R., T.G. Tutin, and D.M. Moore.1989. Flora of the British Isles. Cambridge University Press, NY.

Cox, G.W. 2004. Alien species and evolution. Island press, Washington DC.

Cratty, R.I. New weed is given name of sicklewort. Des Moines Register, 23 November, 1930.

Crawford, H.C., and B.W. Hoagland. 2009. Can herbarium records be used to map alien species invasion and native species expansion over the past 100 years? Journal of Biogeography 36:651-661.

Delisle, F., C. Lavoie, M. Jean, and D. Lachance. 2003. Reconstructing the spread of invasive plants: taking into account biases associated with herbarium specimens. Journal of Biogeography 30:1033-1042.

Dybas, C.L. 2004. Invasive species: The search for solutions. Bioscience 54:615-621.

Eilers, L.J., and D.M. Roosa. 1994. The vascular plants of Iowa: an annotated checklist and natural history. University of Iowa Press, Iowa City, IA.

Ellstrand, N.C., and D.R. Elam. 1993. Population genetic consequences of small population size: implications for plant conservation. Annual Review of Ecology and Systematics 24:217–42.

ESRI. 2008. ArcGIS, version 9.3. Environmental Systems Research Institute, Redlands, CA.

Fernald, M.L. 1942. Misinterpretation of Atlantic coastal plain species. Rhodora 44:238-246.

Gates, F.C. 1940. Recent migrational trends in the distribution of weeds in Kansas. Transactions of the Kansas Academy of Science 43:99-117.

Goralski, G., P. Lubczyńska, and A.J. Joachimiak. 2009. Chromosome Number Database. Available at http://www.binoz.uj.edu.pl:8080/chromosomes/ [Cited April 06, 2011].

Gress, E.M. 1923. *Falcaria rivini* a plant new to the United States. Rhodora 25:13-14.

Holmgren, P.K., N.H. Holmgren, and L.C. Barnett. 1990. Index Herbariorum. Part I, The herbaria of the world, 8th ed. IAPT and the New York Botanical Garden, NY.

Khazaei, M., and H. Salehi. 2006. Protective effect of *Falcaria vulgaris* extract on ethanol induced gastric ulcer in rat. Iranian Journal of Pharmacology and Therapeutics 5:43-46.

Knuth, P., and H. Muller. 1908. Handbook of flower pollination. Clarendon Press, Oxford.

Kolar, C.S., and D.M. Lodge. 2001. Progress in invasion biology: predicting invaders. Trends in Ecology and Evolution 16:199-204.

Korman, B.L. 2011. Biology and ecology of Sickleweed (*Falcaria vulgaris*) in the Fort Pierre National Grassland of South Dakota. Thesis. South Dakota State University, Brookings, SD.

Kowarik, I., and M. von der Lippe. 2007. Pathways in plant invasions. Pages 29-47 in W. Nentwig, editor. Ecological Studies. Springer, Berlin.

Lambrinos, J.G. 2001. The expansion history of a sexual and asexual species of *Cortaderia* in California, USA. Journal of Ecology 89:88-98.

Larina, S.Y. 2008. *Falcaria vulgaris* Bernh. Interactive agricultural ecological atlas of Russia and neighboring countries. In A.N. Afonin, S.L. Greene, N.I. Dzyubenko and A.N. Frolov, editors. Economic plants and their diseases, pests and weeds. Available at http://www.agroatlas.ru/en/content/weeds/Falcaria_vulgaris/ [Cited June 04, 2011.]

Laughlin, D.C. 2003. Geographic distribution and dispersal mechanisms of *Bouteloua curtipendula* in the Appalachian Mountains. American Midland Naturalist 149:268-281.

Lavoie, C., M. Jean, F. Delisle, and G. Letourneau. 2003. Exotic plant species of the St Lawrence River wetlands: a spatial and historical analysis. Journal of Biogeography 30:537-549.

Lavoie, C., Y. Jodoin, and A.G. de Merlis. 2007. How did common ragweed (*Ambrosia artemisiifolia* L.) spread in Quebec? A historical analysis using herbarium records. Journal of Biogeography 34:1751-1761.

Lerdau, M., and J.D. Wickham. 2011. Non-natives: four risk factors. Nature 475:36-37.

Limpert, E., K. Ammann, P. Bartos, W.K. Graber, G. Kost, and J.G. Fuchs. 2004. Airborne migration of obligate nomads demonstrates gene flow across Eurasia. Pages 339-352 in D. Werner, editor. Biological Resources and Migration. Springer, NY.

Mack, R.N. 1991. The commercial seed trade: An early disperser of weeds in the United States. Economic Botany 45:257-273.

MacRoberts, B.R., and M.H. MacRoberts. 2006. An updated, annotated vascular flora of Caddo Parish, Louisiana, with notes on regional phytogeography and ecology. SIDA Contributions to Botany 22:1191-1219.

Magee, D.W., and H.E. Ahles. 2007. Flora of the Northeast: a manual of the vascular flora of New Enlgand and adjacent New York, 2nd ed. University of Massachusetts Press, Amherst, MA.

Mihulka, S., and P. Pysek. 2001. Invasion history of *Oenothera* congeners in Europe: a comparative study of spreading rates in the last 200 years. Journal of Biogeography 28:597-609.

Mitchell, R.S., and G.C. Tucker. 2000. Revised checklist of New York state plants. New York State Museum, Albany.

Mooney, H.A., and E.E. Cleland. 2001. The evolutionary impact of invasive species. Proceedings of the National Academy of Sciences 98:5446-5451.

NISC (2011) Invasive plants of Nebraska. Available at http://snr5.unl.edu/invasives/pdfs/Invasive%20Plant%20Lists/NE%20Invasive%20Plants%20List%20Only%204-14-11.pdf [Cited 5 November, 2011].

Novak, S.J., and R.N. Mack. 2001. Tracing plant introduction and spread: Genetic evidence from *Bromus tectorum* (Cheatgrass). Bioscience 51:114-122.

Pergl, J., P. Pysek, I. Perglova, and V. Jarosik. 2012. Low persistence of a monocarpic invasive plant in historical sites biases our perception of its actual distribution. Journal of Biogeography 39: 1293-1302.

Pimentel, D., L. Lach, R. Zuniga, and D. Morrison. 2000. Environmental and economic costs of non-indigenous species in the United States. Bioscience 50:53-65.

Pysek, P., and K. Prach. 1993. Plant invasion and the role of riparian habitats: a comparison of four species alien to central Europe. Journal of Biogeography 20:413-420.

Rejmanek, M., and M.J. Pitcairn. 2002. When is eradication of exotic pest plants a realistic goal? Pages 249-253 in C.R. Veitch and M.N. Clout, editors. Turning the

tide: the eradication of invasive species. IUCN SSC Invasive Specialist Group, Gland, Switzerland and Cambridge, UK.

Rhoads, A.F., and T.A. Block. 2007. The plants of Pennsylvania: an illustrated manual, 2nd ed. University of Pennsylvania Press, Philadelphia.

Ricciardi, A., W.W.M. Steiner, R.N. Mack, and D. Simberloff. 2000. Toward a global information system for invasive species. Bioscience 50:239-244.

Sakai, A.K., F.W. Allendorf, J.S. Holt, D.M. Lodge, J. Molofsky, K.A. With, S. Baughman, R.J. Cabin, J.E. Cohen, N.C. Ellstrand, D.E. Mncauley, P. O'Neil, I.M. Parker, J.N. Thomson, and S.G. Weller. 2001. The population biology of invasive species. Annual Review of Ecology and Systematics 32:305-332.

Sheeley, S.E., and D.J. Raynal. 1996. The distribution and status of species of *Vincetoxicum* in eastern North America. Bulletin of the Torrey Botanical Club 123:148-156.

Steyermark, J.A. 1963. Flora of Missouri. Iowa State University Press, Ames, Iowa.

Strother, J.L., and A.R. Smith. 1970. Chorology, collection dates, and taxonomic responsibility. Taxon 19:871-874.

Stuckey, R.L. 1980. Distributional history of *Lythrum salicaria* (Purple loosestrife) in North America. Bartonia. Proceedings of the Philadelphia Botanical Club 47:18.

Thomas, R.D., and L.R. Raymond. 1987. *Falcaria vulgaris* Bernh. (Apiaceae): New to Louisiana. The Southwestern Naturalist 32:279.

USDA, ARS. 2012. National Genetic Resources Program. Germplasm resources information network (Online Database). National germplasm resources laboratory,

Beltsville, Maryland. Available at http://www.ars-grin.gov/cgi-bin/npgs/html/noxweed.pl [Cited 14 March 2012].

USDA, NRCS. 2011. The Plants Database. National Plant Data Center, Baton Rouge, LA 70874-4490 USA. Available at http://plants.usda.gov [Cited 16 April 2011].

Valliant, M.T., R.N. Mack, and S.J. Novak. 2007. Introduction history and population genetics of the invasive grass *Bromus tectorum* (Poaceae) in Canada. American Journal of Botany 94:1156-1169.

Weber, E. 1998. The dynamics of plant invasions: a case study of three exotic goldenrod species (*Solidago* L.) in Europe. Journal of Biogeography 25:147-154.

Weldy, T., and D. Werier. 2012. New York flora atlas. Available at http://newy-ork.plantatlas.usf.edu/ [Cited 2 February, 2012].

Wester, L. 1992. Origin and distribution of adventive alien flowering plants in Hawaii. Pages 99-154 in C.P. Stone, C.W. Smith and J.T. Tunison, editors. Alien plant invasions in native ecosystems of Hawaii: Management and research. University of Hawaii, Manoa.

Woods, T.M., S.C. Strakosh, M.P. Nepal, S. Chakrabati, N.B. Simpson, M.H. Mayfield, and C.J. Ferguson. 2005. Introduced species in Kansas: floristic changes and patterns of collection based on an historical herbarium. SIDA Contributions to Botany 21:1695-1725.

CHAPTER 3

CHARACTERIZATION OF NUCLEAR AND CHLOROPLAST MICROSATELLITE MARKERS FOR *FALCARIA VULGARIS* (APIACEAE)

ABSTRACT

Falcaria vulgaris (sickleweed) is native to Eurasia and a potential invasive plant of the United States. No molecular markers have been developed so far for sickleweed. Characterization of molecular markers for this plant would allow investigation into its population structure and biogeography thereby yielding insights into risk analysis and effective management practices of the plant. In order to characterize the molecular markers, DNA samples were collected from eight populations in Iowa, Nebraska and South Dakota. Nuclear microsatellite markers developed for other Apiaceae taxa were screened and tested for inter-generic transferability to sickleweed. The chloroplast *trnL* intron and *trnL-F* intergenic spacer regions were sequenced and the sequences were used to design primers to amplify the microsatellites present within each region. We characterized eight polymorphic microsatellite markers for sickleweed that included six nuclear and two chloroplast markers. Our result showed inter-generic transferability of six nuclear microsatellite markers from *Daucus carota* to *F. vulgaris.* The markers we characterized are useful for population genetics study of *F. vulgaris*.

Keywords: *Falcaria vulgaris*; invasive species; microsatellite; *trnL* intron; *trnL-trnF* intergenic spacer; sickleweed

INTRODUCTION

Sickleweed (*Falcaria vulgaris* Bernh.; Apiaceae) is native to Europe and Asia [1] and was introduced to the United States in the early 1920s [2]. It has been reported from 35 counties across 16 states in the United States and exhibits disjunct distribution in midwestern and eastern USA [3]. Sickleweed exhibits some characteristics of invasive plant species, including the production of a large number of seeds, an effective seed dispersal mechanism whereby the seeds attached to the stem are carried away by the wind as the stems break at the nodes after the plant senescence, and the ability of the plant to reproduce asexually through root sprouting. These characteristics are perhaps facilitating its emergence as an aggressive weed in the Midwest [4]. Continuous increase in areal coverage of sickleweed in Fort Pierre National Grassland (FPNG) and Buffalo Gap national Grassland (BGNG) of South Dakota has attracted attention of ecologists in the Midwest. The plant has also been listed as potentially invasive plant by the Nebraska Invasive Species Council [5].

Both nuclear and chloroplast DNA markers are commonly used for the genetic analysis of invasive plant populations particularly to predict the invasiveness of the introduced species, identify the source populations and to help design effective control programs for invasive species [6]. Microsatellite markers are one of the most preferred molecular markers because they are co-dominant, hyper-variable and are highly reproducible [7, 8]. However, development of novel microsatellite markers is an expensive and laborious task [9]. Cross-species transfering of microsatellite markers and identification of chloroplast microsatellites using universal chloroplast markers are methods that can avoid high cost and long time needed for marker development [10, 11].

Markers are useful for investigating population structure and phylogeography of introduced species, and are also useful for comparative studies of different species [10], and analyzing the process of population divergence and speciation [12]. No molecular markers have previously been developed for sickleweed that would allow us to study the genetic structure of this plant. Here we report on inter-generic transferability of six nuclear microsatellite markers from *Daucus carota* to *F. vulgaris,* and two polymorphic chloroplast microsatellite markers**.**

METHODS

Screening of microsatellite markers

We reconstructed a phylogeny of the family Apiaceae based on nuclear ribosomal Internal Transcribed Spacer (ITS) DNA sequences available in GenBank and we searched for microsatellite markers developed for taxa closely related to *Falcaria vulgaris*. Based on our phylogenetic analysis, we decided to screen microsatellite markers developed for *Daucus carota* and *Heracleum mantegazzianum*. We selected 85 microsatellite markers with di-, tri- and tetra- nucleotide repeats developed for *Daucus* [13,14] and six markers developed for *Heracleum* [15] for the genetic analysis of sickleweed. Fresh leaf tissues were collected in silica gel from eight populations (**Table 3.1)** from Iowa (one population), Nebraska (three populations) and South Dakota (four populations). Voucher specimens except for the populations from Boyd County, Nebraska were deposited at South Dakota State University Herbarium (SDC). The silica gel dried leaf samples were ground to a fine powder and total DNA was extracted using DNeasy Plant Minikit (Qiagen corp., Valencia, CA).

For screening the microsatellite markers, PCR was carried out in a reaction mixture of 15µl containing 50ng genomic DNA, 3µl of 5X buffer (Promega), 1.2µl of 10mM dNTPs (Promega), 2µl of 25mM $MgCl_2$ (Promega), 1µl each of 10pM forward and reverse primers and 2 units of Jump Start Taq polymerase (Sigma-Aldrich). The PCR conditions were an initial denaturation of 4 minutes at 94^0C followed by 40 cycles of 1 minute denaturation at 94^0C, 20 seconds of annealing temperature and 1 minute extension at 72^0 C, and final extension of 5 minutes at 72^0C. Electrophoresis was carried out in 1.2% agarose gel to evaluate the quality of PCR products and the presence of repeat motif in the amplicons was verified by re-sequencing the PCR products.

Table 3.1 Collection sites of the sickleweed population

SN	Collection site	Population name	Latitude	Longitude	Sample Size
1	Onawa, Iowa	IA	$42^0$57'9.62"	$96^0$7'34.68"	12
2	Wayne, Nebraska	WA	42°14'7.18"	97°8'32.24"	12
3	Boyd, Nebraska	NE29	$42^0$52'2.99"	$98^0$45'7.22"	12
4	Boyd, Nebraska	NE30	$42^0$57'9.63"	$98^0$40'9.93"	12
5	Alkali West FPNG, South Dakota	AW	$44^0$10'57.85'	$100^0$17'42.87"	12
6	Grass Creek FPNG, South Dakota	GC	$44^0$11'45.35"	$100^0$18'26.49"	12
7	BGNG, South Dakota	BG	$43^0$55'56.12"	$102^0$24'8.54"	12
8	Brookings, South Dakota	BRK	$44^0$18'53"	$96^0$47'38"	12

For chloroplast microsatellite, we sequenced the *trnL* intron and *trnL-F* intergenic spacer (using the primer pair 5'-CGAAATCGGTAGACGCTACG-3' and 5'-ATTTGAACTGGTGACACGAG-3') of chloroplast DNA. We searched for nucleotide repeats within the region and found two mononucleotide repeats with more than ten mononucleotide repeats and designed the primers using primer3 software (http://primer3.wi.mit.edu/) to amplify these mono-nucleotide repeats.

Genotyping, test for potential artifacts and data analyses

For genotyping, PCR was carried out in a reaction mixture of 15µl containing 50ng genomic DNA, 3µl of 5X buffer, 1.2µl of dNTPs, 2µl of 25mM $MgCl_2$, 0.5µl of 10pM forward primer, 0.5µl of 10pM forward primer tagged with M13 tail (ACGACGTTGTAAAACGAC), 1µl of 10pM reverse primers each and 2 units of *Taq* polymerase. The PCR conditions were similar to that of primer screening PCR conditions. The PCR products were genotyped using 3730x1 DNA analyzer (Applied Biosystems) at the Iowa State University DNA Facility.

Genemarker V2.4.0 (Softgenetics) was used to visualize the genotyping data and create allele reports. The possible genotyping artifacts such as stuttering, large allele drop-out and presence of null alleles were tested using Micro-Checker [16]. The analysis of microsatellite polymorphisms including number of alleles, observed and expected heterozygosity were performed using Arlequin V3.1 [17], the polymorphism information content (PIC) value was computed using the Excel Microsatellite Toolkit [18] and haploid diversity for chloroplast microsatellite was computed using Genalex V. 6.41 [19].

RESULTS AND DISCUSSIONS

The probability of microsatellite marker transferability reduces with distance of phylogenetic relationship [20]. There are several examples of microsatellite marker transferability within a genus but few at higher taxonomic levels. For example, microsatellite transferability success rate within genera of eudicots is approximately 60% compared to 10% across genera [10]. Therefore, it is always beneficial to identify phylogenetically close relatives before screening microsatellite markers for cross species transferability when microsatellites markers are not available for sister species within the genus. In our phylogenetic analysis, we found *Apium graveolens*, *D. carota*, *Eryngium alpinum* and *H. mantegazzianum* were the only taxa with microsatellite markers developed within the Apiaceae clade and they were all distantly related to *F. vulgaris*. Previous studies have shown transferability of *Daucus* and *Heracleum* microsatellite markers to species in other genera [13, 15]; therefore, we chose microsatellite markers developed for these two genera to test their transferability to sickleweed.

None of the six *H. mantegazzianum* microsatellites marker amplified the *F. vulgaris* DNA; however, all six markers did amplify the DNA of *H. maximum* which was used as a positive control in this test. The transferability of these microsatellite markers (see [15] for primer sequences) from *H. mantegazzianum* to *H. maximum* has not been previously reported. With *Daucus* microsatellite primers, 26% of the primers amplified *Falcaria* DNA. *Daucus* primers tested for the amplification of *Falcaria* were classified based on the quality of electrophoretic bands: six primers produced clean band with nearly expected amplicon size (7%), 16 primers produced multiple bands (19%) and 63 primers produced no band (74%). The six markers that produced clean bands are

presented in **Table 3.2**. The six identified nuclear microsatellites and two chloroplast microsatellite primers were used for genotyping sickleweed samples collected from eight populations.

The signals of the genotyping results were clean and did not show stuttering bands. Micro-Checker showed no evidence of scoring error due to stuttering and large allele drop-out. The program identified no genotyping error due to null alleles either. Our test of genotyping artifacts suggests that any deviation from Hardy-Weinberg equilibrium in our analysis is the result of change in allele frequency and not due to genotyping artifacts.

All six nuclear microsatellite loci were polymorphic with mean number of allele per locus of 8.8 (range 3 to 19) and most of these loci, except ESSR80, had high polymorphism content value (**Figure 3.1**). Three out of six nuclear microsatellite loci were monomorphic for the population from Iowa. This could be because of small sample size of the population. Two, three and five loci showed significant deviation from Hardy-Weinberg equilibrium for the populations from Iowa, Nebraska and South Dakota, respectively (**Table 3.3**).

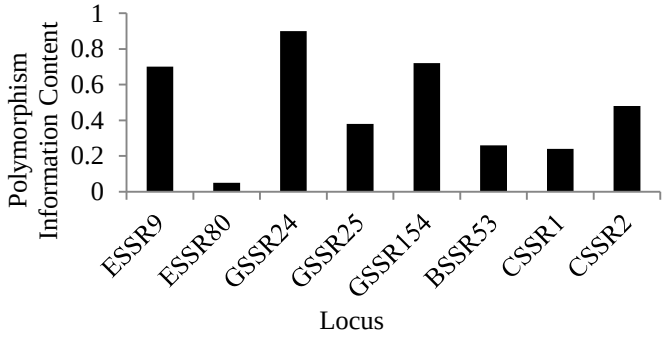

Figure 3.1 Polymorphism information content values displayed by the microsatellite loci

Table 3.2 Characteristics of microsatellite markers for *F. vulgaris*

Locus[a]	Primer sequences (5'-3')	Repeat motif	Tm (^{0}C)	Size range (bp)
Nuclear microsatellites				
ESSR9 (FAM)	F: ATCTGGGGAACTTGCTGTTG R: AGCATCAGCAGCAGCTACAA	(TGC)6	58	290-311
ESSR80 (FAM)	F: ACAGCCAGATGAGCAGGACT R: GAGATTTGGCAATGTGGGAT	(CA)9	53	235-239
GSSR24 (FAM)	F:GCCAACCATCAAAATCACTTCT R:GAATAACTGCCTGCAATACCG	(TC)12	51	281-321
GSSR25 (FAM)	F:CCAGAAACTGATTTTTAATTTAGGC R:CGTTTTTCAATAAAACCTCAATC	(CATA)21	53	166-222
GSSR154 (FAM)	F: CTTATATGTGATGGCGTCGAAA R: GACTGCACCGCTCCTAACTC	(TC)11	53	302-316
BSSR53 (FAM)	F:GCTTTAGAACTTCTTCTAGTCGTCCA R:CTCATGAGCTCACTTCATCTAACTCC	**(**AT)8	53	193-211
Chloroplast microsatellites				
CSSR1 (FAM)	F: GTTCAAATGGGGAGTCCTTG R: TAT CCC CAA AAA GCC CAT T	(A)11	53	376-377
CSSR2 (FAM)	F: CGG AAG TTT CAA TGG AAG GA R: TAA TTC CGG GGT TTC TCT GA	(T)11	53	177-179

[a]The fluorescent dye used to label forward primer is given in parentheses.

Table 3.3 Number of samples (n), Number of alleles (A), observed heterozygosity (Ho) and expected heterozygosity (He) of different nuclear microsatellite markers for the samples from Iowa, Nebraska and South Dakota

	Iowa				Nebraska				South Dakota			
Locus	n	A	H_o	H_e	n	A	H_o	H_e	n	A	H_o	H_e
ESSR9	12	3	na	na	36	7	0.52*	0.80	48	5	0.73*	0.71
ESSR80	12	1	na	na	36	2	0.02	0.03	48	3	0.02*	0.06
GSSR24	12	3	1.00*	0.52	36	12	0.80*	0.84	48	11	0.91*	0.92
GSSR25	12	4	0.33	0.42	36	3	0.27	0.33	48	9	0.48	0.43
GSSR154	12	3	1.00*	0.65	36	7	0.69*	0.80	48	5	0.43*	0.68
BSSR53	12	1	na	na	36	3	0.06	0.05	48	6	0.16*	0.39

* Departure from Hardy-Weinberg equilibrium at $p<0.05$.

na – Locus is monomorphic and test for H-W equilibrium was not done

Among six nuclear microsatellite loci, three loci (ESSR9, GSSR24 and GSSR25) produced three to four alleles for some samples (**Figure 3.2**). These multiple alleles per sample indicates that sickleweed in the novel range might have undergone gene duplications perhaps through polyploidization [21].

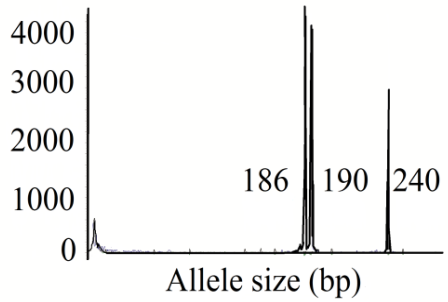

Figure 3.2 Electropherograms of a sample at GSSR 25 locus. Similar multiple peaks were present at GSSR24 and ESSR9 loci. Multiple peaks displayed by several loci suggest that sickleweed in the United States might have undergone polyploidization

Chloroplast DNA markers are often used for the studies at higher taxonomic levels as they have very slow evolutionary rates and do not reveal much variation within species [22]. However, chloroplast microsatellites have been effectively used for the study of intra-specific variation in plants [11]. The two chloroplast microsatellite markers used in our study were also polymorphic and detected two (CSSR1) and three alleles (CSSR2). The average mean haploid diversity for these two loci was 0.23 and these two loci detected three chloroplast haplotypes. The number of alleles and the haploid diversity of these two loci in different populations are presented in **Table 3.4**.

Table 3.4 Number of alleles (n) and haploid diversity (h) of two chloroplast microsatellite markers

	Iowa		Nebraska		South Dakota	
Locus	n	h	n	h	n	h
CSSR1	2	0.32	1	0	1	0
CSSR2	2	0.32	2	0.27	2	0.47

These polymorphic microsatellite markers can be used for the study of population structure and gene flow in the introduced as well as in the native range and will ultimately be useful in the identification of source population(s). Use of non-recombinant chloroplast microsatellite markers and nuclear microsatellite markers will exactly determine if the gene flow is the result of seed or pollen flow [11].

The nuclear and chloroplast microsatellite markers together can provide important insights about the genetic structure of populations. Because nuclear DNA markers are bi-parental while the chloroplast DNA markers are maternally inherited, they differ in their utilities to reveal real-timeprocesses operating in the populations. Therefore, analyses of sickleweed populations from native and introduced ranges using these markers will provide insights into sickleweed evolution [7] and invasion pathways [23] and may contribute in the risk assessment and effective management of this species in the United States.

Screening of more microsatellite markers, particularly markers based on Expressed Sequence Tags (ESTs) from other species of Apiaceae family, may reveal other microsatellites markers that may be useful for sickleweed because studies have shown that ESTs based microsatellite markers show greater transferability than anonymous microsatellites as the genes are highly conserved across different genera [24, 25]. Similarly, there are several other universal chloroplast primers (see [11]) that can be sequenced to identify chloroplast microsatellite markers. These microsatellite markers are useful not only for the population genetics study of sickleweed but may be useful for the comparative study of species across Apiaceae.

CONCLUSIONS

Our results demonstrated successful inter-generic transferability of microsatellite markers from *Daucus carota* to *Falcaria vulgaris*. Since these two species belong to two distantly related genera, transferability of microsatellite markers between these species indicates that these microsatellite markers may work for other genera within Apiaceae. We are also reporting two chloroplast microsatellite markers for sickleweed. Sequencing other chloroplast regions using universal chloroplast markers may reveal more chloroplast microsatellite markers. These nuclear and chloroplast microsatellite markers are polymorphic and there are useful for population genetics and phylogeographic studies of sickleweed.

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REFERENCES

[1] S. Y. Larina, "*Falcaria vulgaris* Bernh. Interactive Agricultural Ecological Atlas of Russia and Neighboring Countries," 2009. http://www.agroatlas.ru/en/content/weeds/Falcaria_vulgaris/

[2] E. M. GRESS, "*Falcaria rivini*, A Plant New to the United States," *Rhodora*, vol. 25, 1923, pp. 13-14.

[3] S. Piya, M. P. Nepal, A. Neupane, G. E. Larson and J. L. Butler, "Inferring Introduction History and Spread of *Falcaria vulgaris* Bernh. (Apiaceae) in the United States Based on Herbarium Records," *Proceedings of the South Dakota Academy of Sciences*, Vol. 91, 2013, pp. 113-129.

[4] B. L. Korman, "Biology and Ecology of Sickleweed (*Falcaria vulgaris*) in the Fort Pierre National Grassland of South Dakota" M.Sc. Thesis, South Dakota State University, Brookings, 2011.

[5] NISC, "Invasive Plants of Nebraska" 2011. http://snr5.unl.edu/invasives/pdfs/Invasive%20Plant%20Lists/NE%20Invasive%20Plants%20List%20Only%204-14-11.pdf

[6] A. K. Sakai, F. W. Allendorf, J. S. Holt, D. M. Lodge, J. Molofsky, K. A. With, *et al*., "The Population Biology of Invasive Species," *Annual Review of Ecology and Systematics*, Vol. 32, 2001, pp. 305-332. doi:10.1146/annurev.ecolsys.32.081501.114037

[7] Sunnucks, "Efficient Genetic Markers for Population Biology," *Trends in Ecology and Evolution*, Vol. 15, No. 5, 2000, pp. 199-203. doi:10.1016/S0169-5347(00)01825-5

[8] K. A. Selkoe and R. J. Toonen, "Microsatellites for Ecologists: A Practical Guide to Using and Evaluating Microsatellite Markers," *Ecology Letters*, Vol. 9, No. 5, 2006, pp. 615-629. doi:10.1111/j.1461-0248.2006.00889.x

[9] J. Squirrell, P. M. Hollingsworth, M. Woodhead, J. Rus-sell, A. J. Lowe and M. Gibby, "How Much Effort Is Required to Isolate Nuclear Microsatellites from Plants?" *Molecular Ecology*, Vol. 12, 2003, pp. 1339-1348. doi:10.1046/j.1365-294X.2003.01825.x

[10] T. Barbara, C. Palma-Silva, G. M. Paggi, F. Bered, M. F. Fay and C. Lexer, "Cross-Species Transfer of Nuclear Microsatellite Markers: Potential and Limitations," *Molecular Ecology*, Vol. 16, No. 18, 2007, pp. 3759-3767. doi:10.1111/j.1365-294X.2007.03439.x

[11] J. Provan, W. Powell and P. M. Hollingsworth, "Chloroplast Microsatellites: New Tools for Studies in Plant Ecology and Evolution," *Trends in Ecology and Evolution*, Vol. 16, No. 3, 2001, pp. 142-147. doi:10.1016/S0169-5347(00)02097-8

[12] M. A. F. Noor and J. L. Feder, "Speciation Genetics: Evolving Approaches" *Nature Reviews Genetics, Vol.* 7, No. 11, 2006, pp. 851-861. doi:10.1038/nrg1968

[13] P. F. Cavagnaro, S. M. Chung, S. Manin, M. Yildiz, A. Ali, M. S. Alessandro, *et al.*, "Microsatellite Isolation and Marker Development in Carrot - Genomic Distribution, Linkage Mapping, Genetic Diversity Analysis and Marker Transferability Across Apiaceae," *BMC Genomics*, Vol. 12, No. 386, 2011. doi:10.1186/1471-2164-12-386

[14] M. Iorizzo, D. A. Senalik, D. Gizebelus, M. Bowman, P. F. Cavagnaro, M. Mativienko, *et al.*, "De- Novo Assembly and Characterization of the Carrot Transcriptome Reveals Novel Genes, New Markers, and Genetic Diversity," *BMC Genomics, Vol.* 12, No. 389, 2011. doi:10.1186/1471-2164-12-389

[15] P. Henry, J. Provan, J. Goudet, A. Guisan, S. Jahodova and G. Besnard, "A Set of Primers for Plastid Indels and Nuclear Microsatellites in the Invasive Plant *Heracleum mantegazzianum* (Apiaceae) and Their Transferability to *Heracleum sphondylium*," *Molecular Ecology Resources, Vol.* 8, No. 1, 2008, pp. 161-163. doi:10.1111/j.1471-8286.2007.01911.x

[16] C. Van Oosterhout, W. F. Hutchinson, D. P. M. Wills and P. Shipley, "Micro-Checker: Software for Identifying and Correcting Genotyping Errors in Microsatellite Data," *Molecular Ecology Notes*, Vol. 4, No. 3, 2004, pp. 535-538. doi:10.1111/j.1471-8286.2004.00684.x

[17] L. Excoffier, L. G. Laval and S. Schneider, "Arlequin ver. 3.0: An Integrated Software Package for Population Genetics Data Analysis," *Evolutionary Bioinformatics Vol.* 1, 2005, pp. 47-50.

[18] S. D. E. Park, "Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection," Ph.D Thesis, University of Dublin, Dublin, 2001.

[19] R. Peakall and P. E. Smouse, "GenAlEx 6: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research," *Molecular Ecology Notes*, Vol. 6, No. 1, 2006, pp. 288-295. doi:10.1111/j.1471-8286.2005.01155.x

[20] R, K. Varshney, A. Garner and M. E. Sorrells, "Genic Microsatellite Markers in Plants; Features and Applications," Vol. 23, No. 1, *Trends in Biotechnology*, Vol. 23, No. 1, 2005, pp. 48-55. doi:10.1016/j.tibtech.2004.11.005

[21] J. A. Coyer, G. Hoarau, G. A. Pearson, E. A. Serrao, W. T. Stam and J. L. Olsen, "Convergent Adaptation to a Marginal Habitat by Homoploid Hybrids and Polyploid Ecads in the Seaweed Genus *Fucus*," *Biology Letters*, Vol. 2, No. 3, 2006, pp. 405-408. doi:10.1098/rsbl.2006.0489

[22] P. Taberlet, L. Gielly, G. Pautou and J. Bouvet, " Universal Primers for Amplification of Three Non-Coding Regions of Chloroplast DNA," *Plant Molecular Biology*, Vol. 17, No. 5, 1991, pp. 1105-1110. doi:10.1007/BF00037152

[23] A. Estoup and T. Guillemaud, "Reconstructing Routes of Invasion Using Genetic Data: Why, How and So What?," *Molecular Ecology*, Vol. 19, No. 19, 2010, pp. 4113–4130. doi:10.1111/j.1365-294X.2010.04773.x

[24] P. C. H. Pashley, J. R. Ellis, D. E. McCauley and J. M. Burke, "EST Databases as a Source for Molecular Markers: Lessons from *Helianthus*," *Journal of Heredity*, Vol. 97, No. 4, 2006, pp. 381-388. doi:10.1093/jhered/esl013

[25] S. B. Savadi, B. Fakrudin, H. L. Nadaf and M. V. C. Gowda, "Transferability of Sorghum Genic Microsatellite Markers to Peanut," *American Journal of Plant Sciences*, Vol. 3, No. 9, 2012, pp. 1169-1180. doi:10.4236/ajps.2012.39142

CHAPTER 4

POPULATION GENETICS OF *FALCARIA VULGARIS* BERNH (APIACEAE) IN THE UNITED STATES

ABSTRACT

Sickleweed *(*Apiaceae; *Falcaria vulgaris* Bernh.), native to Europe and Asia, is an introduced species in the United States and grows as an aggressive weed in certain areas of Iowa, Nebraska and South Dakota. In this Chapter, I am reporting on the application of the molecular markers characterized in Chapter 3 for studying the population genetic structure of sickleweed in the United States. I used six microsatellite markers to genotype 96 individuals from eight populations in the Midwest. I also developed nuclear Internal Transcribed Spacer (ITS) and chloroplast *trnL-trnF* and *trnQ-rps16* DNA sequences to study the sequence variation in sickleweed populations. Microsatellite data identified three genetic races to which sickleweed individuals in the Midwest belong to. Overall population genetic diversity was very high (H' =0.76): two populations from Fort Pierre National Grassland exhibited the highest genetic diversity (0.99), while two populations, one from Brookings County, South Dakota (0.56) and one from Onawa County, Iowa (0.41), exhibited the lowest genetic diversity. Total genetic differentiation among populations was also high (0.20) compared to values reported for most of plant species. There was no correlation between geographical distance and genetic distance suggesting human mediated dispersal. Chloroplast DNA sequence data revealed six chloroplast haplotypes nested into two main lineages suggesting at least two independent introductions of sickleweed in the Midwest. High genetic differentiation and high genetic diversity also support the occurrence of multiple introductions. Presence of multiple

alleles across multiple microsatellite loci suggested polyploidy, which is probably a post introduction evolutionary strategy of sickleweed to alleviate the effect of inbreeding. Higher genetic variation in the introduced range attributable to multiple introductions, polyploidy and clonal reproduction may be inducing the evolution of invasiveness in sickleweed. The results from this study provide valuable insights into the rapid evolution of sickleweed in the Midwest with the potential implications to management practices that may prevent this species from becoming more serious invader.

Keywords: *Falcaria vulgaris*, microsatellite, multiple introductions, polyploidy, *trnL-trnF, trnQ-rps16*

INTRODUCTION

Introduced species can have severe repercussions on global biodiversity (Sala *et al.* 2000; Lee 2002; Provan *et al.* 2005; Lachmuth *et al.* 2010; Alyokhin 2011), therefore it is necessary to be vigilant about every plant introduced to a plant community (Simberloff *et al.* 2011). The negative effects of introduced plant species after they become invasive include loss of native biodiversity (Allendorf & Lundquist 2003), alteration of geo-morphological processes, modification of hydrological and biogeochemical cycles (Gordon 1998); and reduction in productivity of agricultural crops, pastures, and rangelands (Cox 2004). Increasing international commerce has facilitated tremendous exchange of biotas (Mooney & Cleland 2001) that is expected to increase with time; therefore, the number of invasive plants is also expected to rapidly increase (Frankham 2005). It is impossible to control the introduction of plant species but timely identification of an introduced plant that can emerge as invasive can ward off huge ecological and economic losses. Therefore, it has become essential to identify the plants

that can become invasive as early as possible (Kolar & Lodge 2001) so that proper management can be developed before the species starts spreading. Knowledge of the genetic structure of the extant populations and gene flow among the populations is important to predict the long term persistence of the invading species (Nater *et al.* 2013).

Conservation biology theory suggests that a population with low genetic diversity would tend to undergo extinction due to genetic drift and inbreeding depression (Ellstrand & Elam 1993; Young *et al.* 1996); however, there exists a genetic paradox for invasive species (Allendorf & Lundquist 2003). Introduced species that represent a subset of their source population, usually have reduced genetic diversity compared to the source population, but they may be capable of successfully expanding their range in the novel habitat by outcompeting and replacing the locally adapted native species (Allendorf & Lundquist 2003). A major obstacle for the establishment of introduced species in the new range is to overcome the negative effects associated with small population size and low genetic diversity (Leung *et al.* 2004; Taylor & Hastings 2005). Multiple introductions (Lavergne & Molofsky 2007), a large number of founder individuals (Allendorf & Lundquist 2003), gene flow among populations (Slatkin 1987), polyploidy (Comai 2005) and asexual reproduction (Sakai *et al.* 2001; Frankham 2005) are some of the mechanisms how invasive species can offset the negative consequences of small population size and reduced genetic diversity.

Multiple introductions and high propagule pressure increase the genetic diversity of the introduced population (Allendorf & Lundquist 2003). High genetic diversity and gene flow among populations are two important factors that may promote successful spread of an established population (Sakai *et al.* 2001). Higher intra-specific genetic

diversity would provide better opportunities for the creation of adapted genotypes through genetic reshuffling and recombination, and also provide options for natural selection of genotypes better suited for the environment thereby producing a population that can better exploit the novel conditions of the introduced range (Lavergne & Molofsky 2007). Therefore, studies on the population genetic structure of introduced plants are useful to predict their invasiveness (Sakai *et al.* 2001; Valliant *et al.* 2007). At the same time, such studies may also be useful to identify source populations (Novak & Mack 2001; Genton *et al.* 2005; Provan *et al.* 2005; Valliant *et al.* 2007) and to help design effective control measures for invasive species (Sakai *et al.* 2001).

Polyploidy and the ability to reproduce asexually are two important phenomena that are common among invasive plants (Sakai *et al.* 2001; Pandit *et al.* 2011). The duplicate genes in polyploids allow them to escape the effects of deleterious recessive alleles common to bottlenecked population (Comai 2005). In the long run, the duplicate genes may undergo adaptive divergence that may facilitate evolution of invasiveness (te Beest *et al.* 2012). Likewise, asexually reproducing species, in addition to escaping from inbreeding depression (Sakai *et al.* 2001), usually develop more compatibility with the habitat in which the plant grows (Rejmanek 2000), and propagate faster than normal rate (Frankham 2005).

Most of the current studies on invasion biology are focused on invasive plants with severe impacts (Pysek *et al.* 2008) and our knowledge of potentially invasive naturalized plants that are at lag phase of establishment is very limited (Kowarik 2005). Therefore, recently naturalized plants should also be studied to determine their potential for invasion and improve management strategies on time (Wilson *et al.* 2011). In this

chapter, I am reporting on the population structure of the naturalized species- *Falcaria vulgaris* Bernh. (Family Apiaceae). Sickleweed, as the plant is known by common name, is native to southern and central Europe and extends eastward through central Asia and the Middle East. It is an introduced species in Africa, northern Europe, and North and South America (DAISIE 2008; Larina 2008). It has been reported in sixteen states in the United States (USDA, NRCS 2011) and exhibits disjunct distribution in the midwestern, southern and eastern regions. However, field observations and herbarium records suggest that this plant no longer occurs in the eastern and southern regions and has been reported recently only from three states in the Midwest that include Iowa, Nebraska and South Dakota (Piya *et al.* 2013). In South Dakota, Fort Pierre National Grassland (FPNG) and Buffalo Gap National Grassland (BGNG) are the two grasslands where this plant has expanded its distribution and abundance (Korman 2011). Several large populations of sickleweed have also been reported along roadsides in Nebraska where the Nebraska Invasive Species Council has listed this plant as a potentially invasive plant (NISC 2013). The plant also occurs in much smaller areas in Onawa, IA and Brookings, SD.

Sickleweed is described as annual, biennial or perennial (Clapham *et al.* 1987; Korman 2011). It produces protandrous andromonoecious flowers for sexual reproduction (Knuth & Muller 1908) and produces large numbers of seeds. The plant exhibits a characteristic seed dispersal mechanism: when the plant senescences, the stems break at the nodes and tumble in wind to disperse the seed (Korman 2011). In addition, the plant can reproduce asexually through its root stock (Gress 1923; Korman 2011). The plasticity in life history traits (habit, seed dispersal mechanism, and clonal reproduction) is perhaps attributable to its emergence as an aggressive weed in the Midwest.

In this study, I am using DNA markers with different modes of inheritance (biparental: nuclear ribosomal ITS DNA sequences and microsatellites; maternal: chloroplast DNA sequences) to address the following objectives:

I. Study the genetic structure of Midwest populations of sickleweed in the United States.

II. Evaluate the potential relationship between genetic diversity of sickleweed and its observed aggressiveness. I hypothesized that those populations that exhibited the most aggressive expansion (FPNG and BGNG) would exhibit high genetic diversity as estimated by expected heterozygosity and effective population size.

III. Determine whether sickleweed populations in the United States were established from a single introduction.

METHODS

Sample collection

Information about the sample collection sites is presented in Table 3.1 (Chapter 3). Fresh leaf tissues were collected in silica gel from 12 plants per population. Samples were collected from individuals growing >10 meters apart to avoid sampling clones of the same individual. The silica gel dried leaf sample was ground to a fine powder using mortar and pestle and DNA was isolated from the ground leaf tissue using DNeasy Plant Minikit (Qiagen corp., Valencia, CA).

Microsatellites genotyping

As described in Chapter 3, six microsatellite markers developed for *Daucus carota* were successfully transferred to sickleweed (Piya & Nepal 2013), therefore, I used

these markers to genotype sampled individuals to accomplish the objectives as outlined above. For genotyping, PCR was carried out in a reaction mixture of 15µl containing 50ng genomic DNA, 3µl of 5X buffer, 1.2µl of dNTPs, 2µl of 25mM $MgCl_2$, 0.5µl of 10pM forward primer, 0.5µl of 10pM forward primer tagged with M13 tail, 1µl of 10pM reverse primers each and 2 units of *Taq* polymerase. The PCR conditions were an initial denaturation of 4 minutes at 94^0C followed by 40 cycles of 1 minute denaturation at 94^0C, 20 seconds (varies with primer pairs; see Table 3.2) of annealing temperature and 1 minute extension at 72^0 C, and final extension of 5 minutes at 72^0C. The PCR products were genotyped using 3730x1 DNA Analyzer (Applied Biosystems) at Iowa State University DNA Facility.

GeneMarker V2.4.0 (Softgenetics) was used to visualize the genotyping data and create allele reports. Possible genotyping artifacts such as stuttering, large allele drop-out and presence of null allele were tested using MICRO-CHECKER (Van Oosterhout *et al.* 2004).

Data analyses

Population genetic parameters such as average number of alleles (n_a), effective number of alleles (n_e) and Shannon diversity index (H') for each locus were computed using PopGene (Yeh & Boyle 1997). Total gene diversity (H_T) was partitioned into within population (H_S) and among population gene diversity (D_{ST}). G_{ST}, the proportion of genetic diversity that resides among populations, was computed as the ratio of D_{ST} to H_T. Observed and expected heterozygosity, test for deviation from Hardy-Weinberg equilibrium, and linkage disequilibrium for each locus were calculated using the program Arlequin V3.0 (Excoffier *et al.* 2005). Population fixation indices (F_{IS}) for each

population were calculated using FSTAT V1.2 (Goudet 1995). Expected heterozygosity (H_E) was used to estimate effective population size (N_E) under two different mutation models: infinite allele model (IAM) and stepwise mutation model (SMM). Mathematically, the population genetic parameters were calculated as follows.

i. Average number of alleles per locus (N_a)

$N_a = (1/K)\sum_{i=1}^{K} n_i$

Where K is the total number of loci and n_i is the total number of alleles detected per locus.

ii. Effective number of alleles (A_e)

$A_e = 1/(1 - h) = 1/\Sigma p_i$

Where, $h = 1 - \Sigma p_i^2$ = heterozygosity in a locus and p_i is frequency of the i^{th} allele in a locus.

iii. Shannon diversity Index (H')

$$H' = -\sum_{i=1}^{R} p_i \ln p_i$$

Where H is the Shannon diversity index and p_i is the proportion of individuals belonging to i^{th} genotype and R is the total number of genotypes.

iv. Effective population size (N_e)

Infinite Allele Model (IAM)

$N_e = (H_e/1\text{-}H_e)/4\mu$ (Crow & Kimura 1970)

Stepwise mutation model (SMM)

$N_e = [1/(1\text{-}H_e)^2 - 1]/8\mu$ (Ohta & Kimura 1973)

Where, μ is mutation rate and H_e is the expected heterozygosity.

Private alleles for each population (alleles that are present only in one population), if present, were determined using Genalex 6.5 (Peakall & Smouse 2006). Arlequin (Excoffier *et al.* 2005) was used to calculate pair-wise genetic differentiation (F_{ST}) and perform analysis of molecular variance (AMOVA). Bayesian analysis of spatial genetic structure of the sampled populations was carried out using the program STRUCTURE Version 2.2 (Pritchard *et al.* 2000). I performed 20 independent runs for each value of K ranging from 1 through 11. Each run included 10,000 burn-in iterations and 50,000 replicates. The best K value was selected by computing ΔK following Evanno *et al.* (2005). The individuals with the proportion of inferred ancestry (Q) ≥0.7 were assigned to a cluster and those with Q less than 0.7 were not assigned to any cluster. Mantel's test for isolation by distance (Mantel 1967) was performed using IBDWS 3.23 (http://ibdws.sdsu.edu/~ibdws/) to test the relationship between natural log of geographical distance and genetic distance ($F_{ST}/1-F_{ST}$).

Chloroplast and Nuclear DNA sequence data

Six samples from each of the populations were randomly chosen to amplify the chloroplast *trnL* intron, *trnL-F* intergenic spacer and *trnQ-rps16* intergenic spacer. One sample per population was used to amplify the nuclear ribosomal Internal Transcribed Spacer (ITS) DNA sequence. Aforementioned nuclear and chloroplast DNA regions were amplified by PCR in a reaction mixture of 25µl containing 50ng genomic DNA, 5µl of 5X buffer, 1µl of dNTPs, 2.5µl of 25mM $MgCl_2$, 2.5µl of 10pM primers and 2 units of *Taq*. The PCR conditions were an initial denaturation of 5 minutes at 94^0C followed by 27 cycles of 1 minute denaturation at 94^0C, 1 min of annealing at 55^0C and 2 minutes extension at 72^0C, and final extension of 10 minutes at 72^0C. Primer pairs used were

TCCTCCGCTTATTGATATGC and GGAAGTAAAAGTCGTAACAAGG for ITS, CGAAATCGGTAGACGCTACG and ATTTGAACTGGTGACACGAG for *trnL-trnF* and GCGTGGCCAAGCGGTAAGGC and GTTGCTTTCTACCACATCGTTT for *trnQ-rps16* intergenic spacer, respectively. The PCR products were purified using QIAquick PCR purification kit (Qiagen Corp., Valencia, CA). The purified PCR products were sent to the DNA facility at Iowa State University for sequencing using a 3730x1 DNA Analyzer (Applied Biosystems).

DNA sequences were edited using the program Sequencher 5.0 (GeneCodes). Sequences were aligned using ClustalX (Larkin *et al.* 2007). Nucleotide diversity, number of haplotypes and haplotypes diversity of these sequences were computed using DnaSP5 (Librado & Rozas 2009). Network tree of the chloroplast haplotypes were constructed using Network (http://www.fluxus-engineering.com/sharenet.htm). Maximum parsimony analysis of chloroplast DNA sequences was performed in the program PAUP 4.0 (Swafford 2003).

RESULTS

Microsatellite markers

The characteristics of the microsatellite markers are described in Chapter 3. All sampled populations deviated from Hardy-Weinberg equilibrium at the majority of the loci and no significant linkage disequilibrium was observed. The average number of alleles detected per microsatellite locus was 8.83 with the range of 3 to 19. GSSR24 was the most polymorphic locus among the six loci (see Table 3.3, Chapter 3). Total gene diversity (H_T) of these six loci averaged 0.524 with 0.428 within population gene diversity and 0.096 among populations gene diversity (Table 4.1). Total allelic diversity partitioned among population (G_{ST}) was 0.184.

Table 4.1 Nei (1973, 1977) gene diversity indices for sickleweed samples collected from eight populations

Locus	H_T	H_S	D_{ST}	G_{ST}
ESSR9	0.756	0.543	0.213	0.281
ESSR80	0.041	0.041	0.000	0.004
GSSR24	0.919	0.768	0.151	0.164
GSSR25	0.429	0.414	0.014	0.033
GSSR154	0.759	0.595	0.164	0.216
BSSR53	0.238	0.203	0.035	0.147
Overall	0.524	0.428	0.096	0.184

Note: H_T- Total gene diversity; H_S- within population component; D_{ST}- Among population component; G_{ST}- Total allelic diversity partitioned among populations

Within and among populations genetic diversity

Three populations in South Dakota (AW, GC and BG) and one population in Nebraska (NE30) exhibited higher genetic diversity than the other four populations (Figure 4.1a -c). Two populations in Nebraska (NE29 and WA) exhibited moderate genetic diversity values and one population in Iowa (IA) and one population in South Dakota (BRK) had the least genetic diversity. The number of alleles detected per population varied from 12 (Iowa) to 25 (NE30). Effective number of alleles ranged from 1.67 (IA) to 2.93 (BG) and Shannon diversity indices ranged from 0.41 (IA) to 0.99 (AW and GC). The expected mean heterozygosity across all populations was 0.42 (range 0.30-0.55). Mean effective population size was 362 (range 214 to 611) and 616 (range 325-1230) for IAM and SMM mutation models, respectively. Only one population (AW) had significant within-population fixation indices (F_{IS}). The level of inbreeding coefficient

ranged from -0.450 to 0.159. Private alleles were detected in five populations: one in AW, five in GC and three in each of BG, NE29 and NE30.

Table 4.2 Within-population genetic diversity of eight sickleweed populations

Population	n	H_o	H_e	Ne (IAM)	Ne (SMM)	F_{IS}
NE29	12	0.37	0.39	319	527	0.042
AW	12	0.45	0.53	563	1102	0.159*
NE30	12	0.49	0.48	461	843	-0.037
BG	12	0.51	0.45	409	720	-0.127
BRK	12	0.28	0.31	224	343	0.104
GC	12	0.52	0.55	611	1230	0.057
IA	12	0.42	0.30	214	325	-0.450
WA	12	0.33	0.35	269	427	0.062
Mean		0.42	0.42	362	616	-0.023

Note: n- sample size; N- Total number of alleles detected; H_o- Observed heterozygosity; H_e- Expected heterozygosity; N_e- Effective population size; F_{IS} - Correlation of genes within individuals within populations

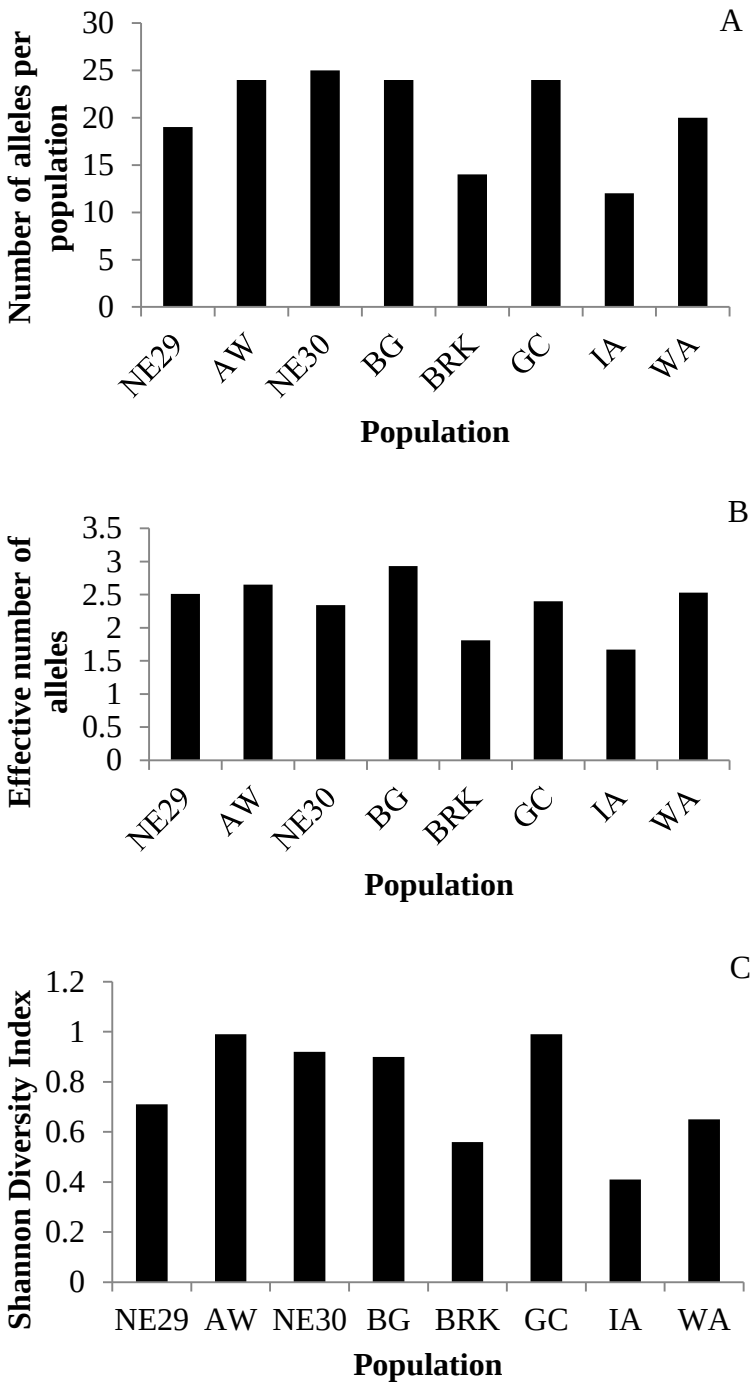

Figure 4.1 Genetic diversity parameters among different populations- A) Number of alleles per population in different population; B) Effective number of alleles displayed by different populations; and C) Shannon Diversity Index of different populations.

Analysis of molecular variance revealed that the overall mean F_{ST} value was 0.20. Of the total variation, 20.80% variation was present among populations and the remaining 79.20% variation was present within populations (Table 4.3).

Population structure

Mantel's test of isolation by distance (IBD) showed no correlation (r = 0.15, p= 0.23) between genetic distance ($F_{ST}/1-F_{ST}$) and geographical distance. In addition no geographical pattern in the distribution of chloroplast haplotypes was observed. In fact, population from different geographical regions clustered together.

The highest value of ΔK in the STRUCTURE analysis was obtained at K=3 suggesting three clusters (color coded: red[1], green[2] and blue[3], hereafter called races) of sickleweed as shown in Figure 4.2. Ninety-four percent of the individuals were assigned to one of the three races based on the coefficient of inferred ancestry (Q- value). The BRK population contained individuals from all three races, AW, NE30 and BG had two races and NE29, GC, IA and WA did not exhibit any genetic admixture (Table 4.4).

Table 4.3 Analysis of molecular variance (AMOVA) within and among populations

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among populations	7	64.260	0.330	20.80
Within populations	184	231.292	1.257	79.20
Total	191	295.552	1.587	

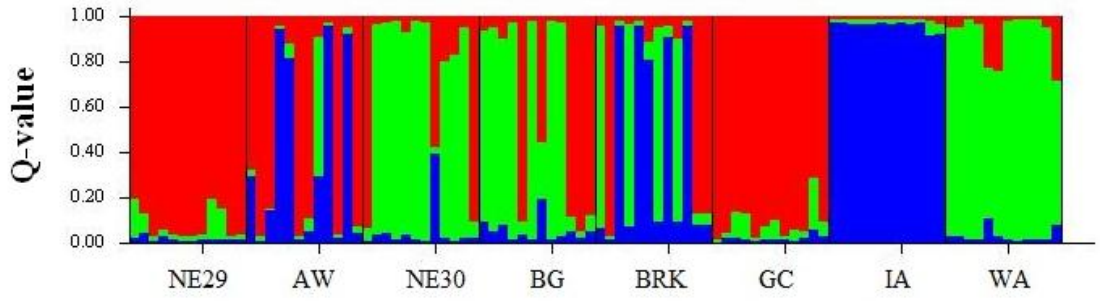

Figure 4.2 Bayesian clustering of *Falcaria vulgaris* based on microsatellite data. Each population is separated by vertical line. Population name is along the horizontal line and the number in the vertical line indicates coefficient of inferred ancestry (Q). Each cluster is color-coded.

Table 4.4 Assignment of individuals from eight populations to three genetic clusters based on microsatellite data

	Cluster			unassigned	% assigned
Population	1 (red)	2 (green)	3 (blue)		
NE29	12				100
AW	6		4	2	84
NE30	2	9		1	91
BG	4	7		1	91
BRK	3	4	5		100
GC	12				100
IA			12		100
WA		10		2	84
Total	39	30	21		94

Detection of polyploidy

Multiple alleles were observed across five loci in four samples from NE30 populations (Table 4.5). However, none of the samples exhibited multiple alleles at BSSR53 locus. As shown in Table 4.5, each of loci ESSR9, ESSR80, GSSR24 and GSSR154 had three alleles each while GSSR25 had four alleles. Presence of multiple alleles in these samples indicates at least partial genome duplication and firmly implies polyploidy. The amplified fragment size of these putative polyploid samples equaled to the amplicon size of diploid samples ruling out the possible amplification of random fragments from other region within the genome.

Table 4.5 Allele fragment size (bp) and number of alleles of putative polyploid samples at five different loci in samples collected from the NE30 population

Sample/Locus	ESSR9	ESSR80	GSSR24	GSSR25	GSSR154
B9	305, 308, 317	187, 191, 237	291, 313, 315	182, 186, 190, 206	302, 308, 330
B1	293, 305, 317	187, 191, 237	283, 313, 315	182, 186, 190, 206	302, 308, 330
B4	293, 305, 317	187, 191, 237	283, 291	182, 186, 190, 206	310, 316, 330
B7	290, 293, 317	187, 191, 237	291, 315, 317	182, 186, 190, 206	302, 306, 330

DNA sequence variation

Chloroplast DNA sequences: The total length of *trnL* intron and *trnL-F* intergenic spacer was 909 bp and that of *trnQ-rps16* intergenic spacer was 884 bp (Table 4.6). The combined data matrix thus comprised of 48X1793 bp. This matrix contained 16 variable sites among which, 13 were parsimony informative. Two types of sequence variation were observed in the aligned matrix: insertion/deletion (indels) and single nucleotide polymorphisms (SNPs). Among the variable sites, five were indels and the remaining eight were SNPs. All these SNPs exhibited two-base polymorphism. Based on these variable sites, six chloroplast haplotypes were identified among 48 sickleweed accessions. Distribution of these chlorotypes and network tree is shown in Figure 4.3. Unrooted cladogram (Figure 4.4) divided these haplotypes into two distinct maternal lineages.

The *trnL-trnF* and *trnQ-rpl16* regions had the nucleotide diversity of 0.00278 and 0.00208, respectively. The combined sequences had the nucleotide diversity values of 0.00194. Haplotype diversity for *trnL-trnF* and *trnQ-rps16* were 0.588 and 0.421 (Table 4.6).

Table 4.6 Polymorphism information of sickleweed based on chloroplast DNA sequences

Region	n	L (bp)	π	h	hd
trnL-trnF	48	909	0.00278	4	0.588
trnQ-rps16	48	884	0.00208	3	0.421
Combined	48	1793	0.00194	6	0.608

Note: n- number of samples; L-length of the sequence; π- nucleotide diversity; h- number of haplotypes; hd- haplotype diversity.

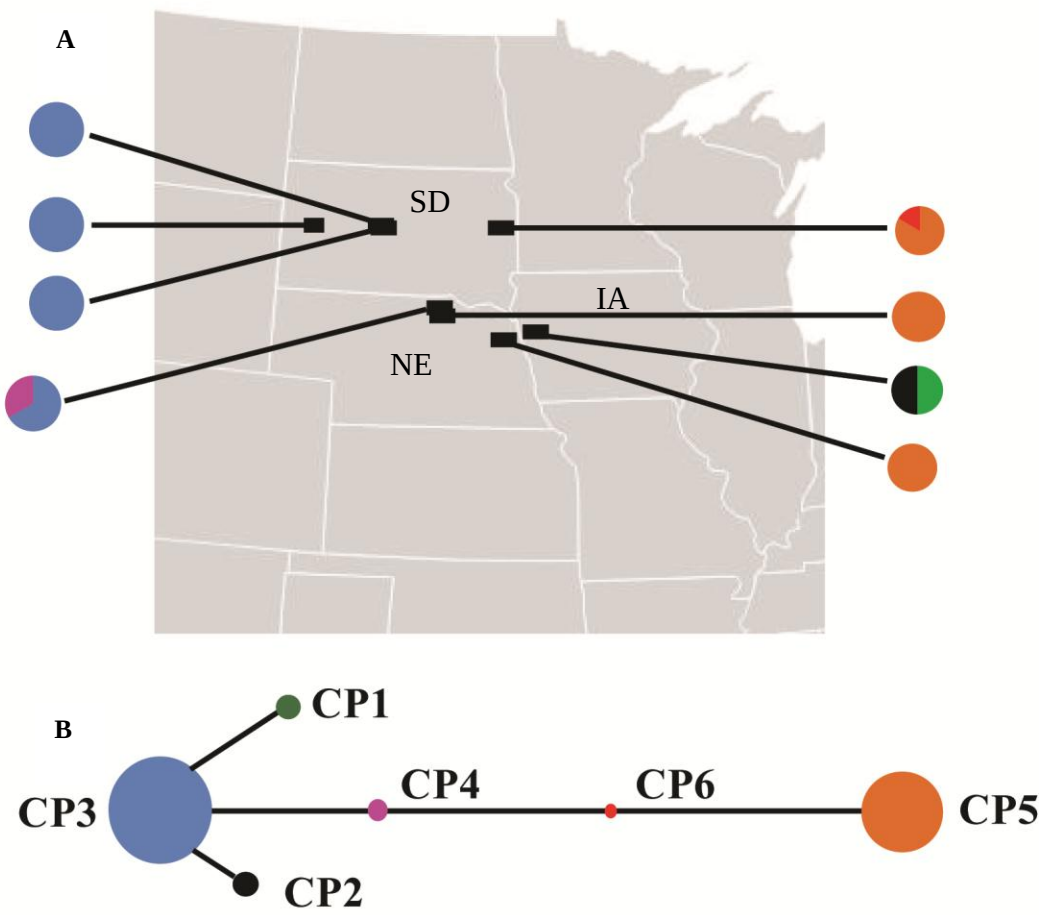

Figure 4.3 Chlorotype variations in *Falcaria vulgaris*. A) Distribution map of chlorotypes in Iowa, Nebraska and South Dakota and B) Network of the chlorotypes. Each chlorotype is represented by a circle whose size is proportional to the relative frequency of the chlorotype

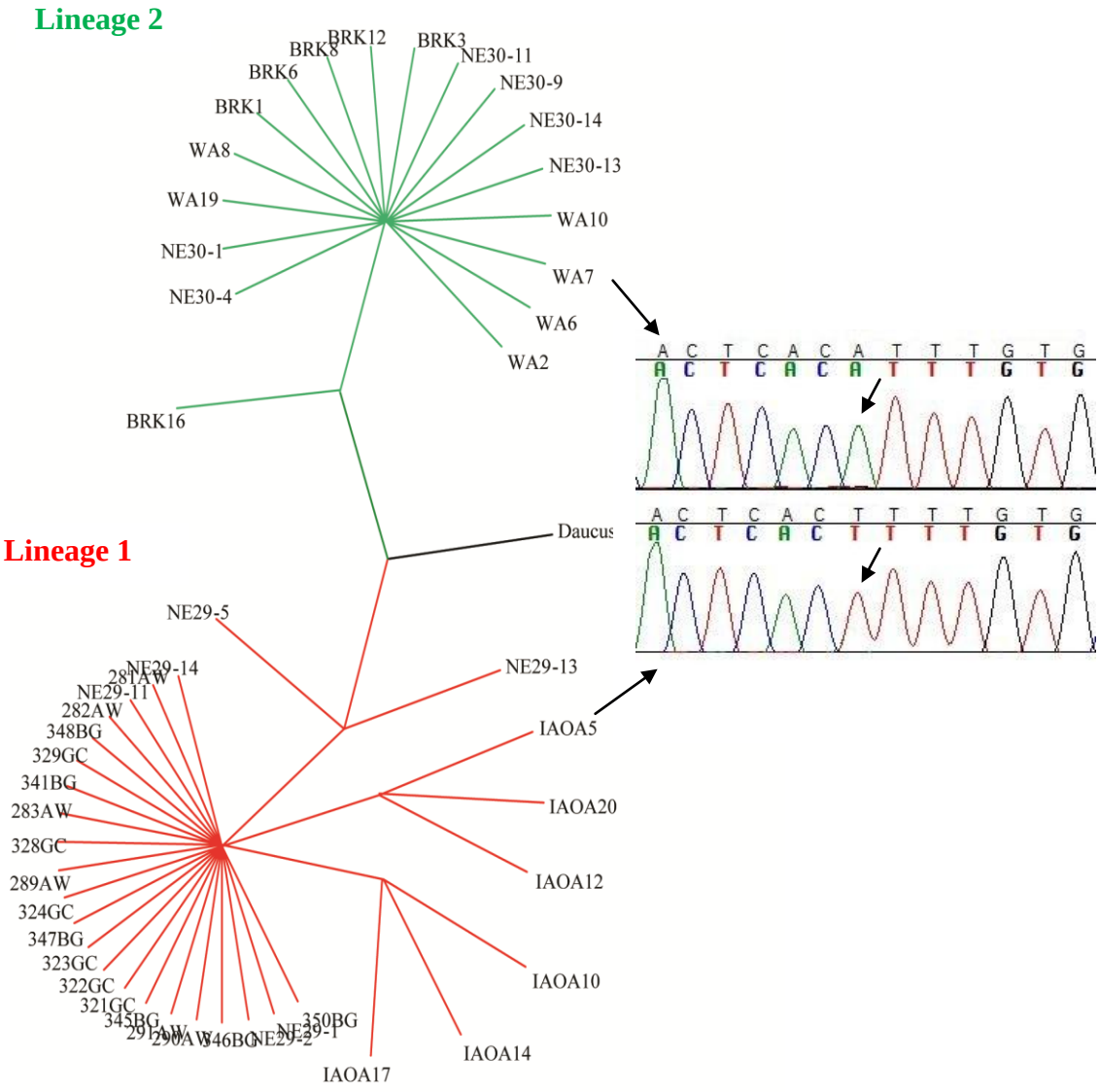

Figure 4.4 Unrooted cladogram based on the combined data matrix of chloroplast DNA sequences (*trnL-trnF* and *trnQ-rpl16* regions). On the right is the chromatogram that display variation in the nuclear DNA sequences (ITS region) between two lineages

The total length of ITS sequences in *Falcaria vulgaris* was 686 bp and the matrix was comprised of 8X686 bp. This matrix contained one variable site as shown by the chromatogram in Figure 4.4. The sequences nested into lineage -1 had a T and those into lineage-2 had an A at the 513th position of the aligned data matrix.

DISCUSSION

This study is the first attempt to explore the population genetics of sickleweed. Molecular markers characterized in this study will be useful for future studies on sickleweed and comparison of the results from this study with the data from old world populations may help identify the source population(s) for these introduced populations. In addition, the data generated from cross-species transferred microsatellite markers from *Daucus carota* (transferable to other Apiaceae members such as celery, parsley and cilantro (Cavagnaro *et al.* 2011) may be useful for comparative studies among various Apiaceae species.

Genetic diversity and mating system in sickleweed populations

While sickleweed grows as an aggressive weed in the Midwest, there is no report of recent occurrence of sickleweed in the eastern USA (Piya *et al.* 2013). Several introduced populations fail at different stages of invasion and may disappear (Richardson *et al.* 2000). Sickleweed populations might have faced the similar fate in the eastern USA, where there is no recent report of sickleweed occurrence. However, in Iowa, Nebraska and South Dakota, some populations have naturalized and are rapidly expanding their range. The current sickleweed distribution pattern perhaps signifies the importance of species introductions at multiple sites with different conditions whereby some of the introduced populations succeed to pass all barriers of the introduction-naturalization-invasion continuum (Lockwood *et al.* 2005; Simberloff 2009; Blackburn *et al.* 2011).

The extant sickleweed populations in the Midwest are of different population size. Among the eight sampled populations, the population at FPNG is the most aggressively

spreading followed by the BGNG population. In Nebraska, aggressively growing sickleweed populations have covered significant area along roadsides and nearby agricultural fields. These populations also exhibited higher genetic diversity. Comparatively, Brookings, SD and Onawa, IA populations of sickleweed are much smaller in size and have the least genetic diversity. Therefore, microsatellite markers showed that allelic richness, effective population size and other indices of genetic diversity of sickleweed populations were proportional to the plant aggressiveness in the Midwest. These findings were consistent to Gaudeul *et al.* (2011) who reported higher genetic diversity in more aggressive populations in *Ambrosia artemisiifoia* from Europe.

The nucleotide diversity of chloroplast DNA, however, did not correspond with the population size of the plant. The chloroplast DNA sequences used in the present study are evolutionarily more conserved than the nuclear microsatellites and ITS sequences. Three populations from FPNG and BGNG, which represent the largest populations among the sampled populations, were monomorphic and shared the same chloroplast haplotypes. Occurrence of monomorphic chloroplast DNA sequences in these sites suggests a single introduction. The sickleweed populations at FPNG and BGNG have covered more acreage in the last decades than other areas in the Midwest (Korman 2011). If there were a single introduction, perhaps these populations had a larger number of founding members or repeated introduction events increasing the propagule pressures, which is considered a critical factor for the establishment of an introduced plant (Kolar & Lodge 2001). An alternative explanation might be selection of pre-adapted sickleweed genotypes (CP3) at an early stage of introduction which resulted in elimination of other chloroplast haplotypes from these populations.

In Nebraska, sampled sickleweed populations occur along the roadsides. Roadsides not only harbor the plant, but also serve as corridors for their spread (Pysek & Prach 1993) which can easily be transported by vehicles. Therefore, these populations are more likely to spread to other sites. Among these populations, NE30 had the highest genetic diversity followed by NE29 (~25 miles away from NE30) and WA (~125 miles away from NE30). Three chlorotypes were detected from Nebraska (CP3 and CP4 from NE29, and CP5 from NE30 and WA). Populations NE29 and NE30 are geographically closer but contain individuals genetically distinct from one another. Microsatellite data revealed that the NE29 population contained individuals of one genetic race (first cluster, Figure 4.2) while NE30 contained most individuals of another genetic race (second cluster). Exchange of propagules between these two genetic races with distinct chlorotypes, could produce a genetic admixture resulting in aggressive genotypes with better adaptability.

Populations from Onawa, IA and Brookings, SD are small and isolated with low genetic diversity which strongly suggests that these populations are affected by the founder effect (Lachmuth *et al.* 2010). Such small isolated populations are assumed to be established by rare long distance dispersal (Austerlitz *et al.* 2000). Both of these populations have two chlorotypes each, however. Genetic recombination between the genotypes with two different chlorotypes might facilitate in the evolution of more aggressive genotypes.

According to Handbook of flower pollination (Knuth 1908), sickleweed is markedly protrandrous. Three populations IA, NE30 and BG showed negative inbreeding coefficient (F_{IS}) as expected for cross pollinating species (Hartl & Clark 2007). While

BRK, GC, NE29 and WA populations showed positive F_{IS} suggesting some level of inbreeding in these populations. Only the Alkali West (AW) population showed significant heterozygous deficiency (Table 4.2). This could be due to the Wahlund Effect (heterozygous deficiency as a result of pooling sub-structured populations with different allele frequencies and that do not randomly mate with each other as a single unit). Because the plant has spread over a large area and samples were collected from individuals growing more distantly than those in other populations, samples might have come from different sub-structured populations resulting in significant heterozygote deficiency. These findings are consistent to the population structure as described for invasive ragweed in Europe (Gaudeul *et al.* 2011).

Genetic structure of sickleweed in the United States

Tang *et al.* (2009) predicted that human mediated long distance dispersal might have contributed in the spread of *Parthenium hysterophorus* in China as they did not detect any geographical structure in genetic variation. Bayesian clustering of the populations from different regions showed no pattern. Mantel's test of isolation by distance (IBD) in sickleweed populations showed no correlation ($r = 0.15$, $p = 0.23$) between genetic distance ($F_{ST}/1\text{-}F_{ST}$) and geographical distance suggesting that there is no genetic isolation by distance. In addition, no geographical pattern was observed in the distribution of chloroplast haplotypes. In fact, populations from different geographical regions clustered together. Absence of geographical structure in the genetic variation of sickleweed suggests human mediated long distance dispersal in the spread of this plant. However, two populations of FPNG (AW and GC), which are less than two miles apart, had very low genetic differentiation ($F_{ST} = 0.073$). These two populations also share a

common chlorotype and most likely they have established by natural expansion through seed dispersal. Korman (2011) reported that sickleweed can effectively disperse its seeds with the help of wind: when the plants senescence, the stems break at the nodes and tumble in the wind to disperse the seed.

Multiple introductions of sickleweed in the United States

Six chlorotypes with two distinct maternal lineages were observed (Figure 4.4) suggesting at least two introductions of sickleweed in the Midwest. Interestingly, these two lineages differ in their ITS DNA sequences. Since the chloroplast gene regions used in this region are often conserved even at higher taxonomic levels, it is very unlikely that these different chloroplast haplotypes are a result of post introduction evolution of sickleweed. If we assume two lineages as two separate introductions, the propagule size of each of the two introductions should have been large as described by Gaskin et al. (2005) that included several chloroplast haplotypes. However, four chlorotypes of lineage 1 occur in three different populations [CP1 and CP2 (IA), CP3 (AW, GC, BG, NE29) and CP4 (NE29)] that strongly suggest more than two introductions in the Midwest U.S.

The nuclear microsatellite data in the present study also correspond with multiple introductions of sickleweed in the United States. The Bayesian analyses showed three distinct genetic races (Figure 4.2) suggesting multiple introductions of sickleweed. In addition, this study showed a high genetic differentiation of 0.20 among the populations. Based on herbarium records, all the populations that I included in this study are approximately 30 years old or younger; therefore, it is unlikely that a single introduction of sickleweed would create so much differentiation in such a short period of time. Therefore, the current distribution of sickleweed in the Midwest United States is more

likely a result of multiple introductions. Multiple introduction events are also supported by high genetic diversity indices e.g. high expected hetetozygosity, and a large number of alleles in the introduced sickleweed populations. Expected heterozygosity is one of the indicators of genetic diversity. A study by Henry *et al.* (2009) showed multiple introductions of *Heracleum mantegazzianum* in the western Swiss Alps, where the expected heterozygosity in the invasive range was 0.35 based on nuclear microsatellite markers. Likewise,Gaudeul *et al.* (2011) showed expected heterozygosity of 0.73 in invasive populations of *Ambrosia artemisiifolia* in Europe resulted from multiple introductions. The expected heterozygosity in sickleweed is 0.42, which lies in the range of the studies described above. To sum up, chloroplast DNA sequence and nuclear DNA data strongly suggest multiple introductions of sickleweed in the United States.

Evidence of polyploidy

Consistent presence of multiple alleles across microsatellite loci in individuals strongly suggests the occurrence of polyploidy (Coyer *et al.* 2006) in sickleweed. There is no previous report of polyploidy in this species. This evidence of polyploidization in sickleweed could be a post introduction evolutionary strategy to adapt in the new environment and minimize the effect of inbreeding depression due to founder events. When the population size is small, redundant genes in the polyploids allow them to escape from the deleterious effects of inbreeding depression (Comai 2005). Polyploidy may also result in the transformation of morphology, phenology, physiology and ecology of plants within few generations (Levin 2002) making them more competent to exploit the particular habitat, giving them competitive advantages over native plants (Leitch & Leitch 2008) and ultimately facilitating easy progression along introduction-

naturalization-invasion pathway (Richardson *et al.* 2000). Further exploration using flow cytometry backed by karyotyping is necessary to verify the occurrence of polyploidy in sickleweed populations.

CONCLUSIONS

In absence of samples from native range, it was not possible to reconstruct the complete invasion route of sickleweed in the United States. In order to identify source populations and reconstruct the routes of sickleweed introduction, a more comprehensive study that includes samples both from native and introduced range and uses a large number of molecular markers is warranted.

Results of this study indicate multiple introductions and genetic admixture in sickleweed which has resulted in higher genetic diversity in the introduced populations. Genetic diversity of a population has been considered a key factor for successful invasion. Therefore, results of this study indicate that sickleweed in the Midwest has potential to emerge as a successful invader. Microsatellite genotyping result suggested the occurrence of polyploidy in sickleweed which was previously unknown. Therefore, it is important to explore if polyploidy in sickleweed is present in the native range or if it is just a post introduction evolutionary strategy of the plant to overcome inbreeding due to the founder effect. It is also necessary to verify polyploidy in sickleweed in the United States using karyotyping and/or flow cytometry.

The genetic data from this study provide opportunities to understand ecological and evolutionary changes that undergo during the invasion process and these data can be indirectly implemented for the management of invasive plants. Therefore, results of this

study are useful for planning the management strategies of sickleweed. Some possible applications of these genetic data include predicting the invasive potential of sickleweed population to control its further expansion, identifying source populations to determine effective bio-control agents and predicting the potential of introduced plants to develop resistance against biological control or herbicide treatment.

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REFERENCES

Allendorf FW, Lundquist LL (2003) Introduction: Population biology, evolution, and control of invasive species. *Conservation Biology* **17**, 24-30.

Alyokhin A (2011) Non-natives: put biodiversity at risk. *Nature* **475**, 36-36.

Austerlitz F, Mariette S, Machon N, Gouyon PH, Godelle B (2000) Effects of colonization processes on genetic diversity: Differences between annual plants and tree species. *Genetics* **154**, 1309-1321.

Blackburn TM, Pysek P, Bacher S, *et al.* (2011) A proposed unified framework for biological invasions. *Trends in Ecology & Evolution* **26**, 333-339.

Cavagnaro PF, Chung SM, Manin S, *et al.* (2011) Microsatellite isolation and marker development in carrot - genomic distribution, linkage mapping, genetic diversity analysis and marker transferability across Apiaceae. *Bmc Genomics* **12**.

Clapham AR, Tutin TG, Moore DM (1987) *Flora of the British Isles*, 3rd edn. Cambridge University Press, Cambridge Cambridgeshire ; New York, NY, USA.

Comai L (2005) The advantages and disadvantages of being polyploid. *Nature Reviews Genetics* **6**, 836-846.

Cox GW (2004) *Alien species and evolution: the evolutionary ecology of exotic plants, animals, microbes, and interacting native species.* Island Press, Washington.

Coyer JA, Hoarau G, Pearson GA, *et al.* (2006) Convergent adaptation to a marginal habitat by homoploid hybrids and polyploid ecads in the seaweed genus *Fucus*. *Biology Letters* **2**, 405-408.

Crow JF, Kimura M (1970) *An Introduction to Population Genetics Theory* Harper and Row Publishers, New York.

DAISIE (2008) *Falcaria vulgaris*. European Invasive Alien Species Gateway.

Ellstrand NC, Elam DR (1993) Population Genetic Consequences of Small Population Size: Implications for Plant Conservation. *Annual Review of Ecology and Systematics* **24**, 217-242.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611-2620.

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics* **1**, 47-50.

Frankham R (2005) Invasion biology - Resolving the genetic paradox in invasive species. *Heredity* **94**, 385-385.

Gaudeul M, Giraud T, Kiss L, Shykoff JA (2011) Nuclear and chloroplast microsatellites show multiple introductions in the worldwide invasion history of common ragweed, *Ambrosia artemisiifolia*. *Plos One* **6**, e17658.

Genton BJ, Shykoff JA, Giraud T (2005) High genetic diversity in French invasive populations of common ragweed, *Ambrosia artemisiifolia*, as a result of multiple sources of introduction. *Molecular Ecology* **14**, 4275-4285.

Gordon DR (1998) Effects of invasive, non-indigenous plant species on ecosystem processes: Lessons from Florida. *Ecological Applications* **8**, 975-989.

Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate F-statistics. *Journal of Heredity* **86**, 485-486.

Gress EM (1923) *Falcaria rivini*, a plant new to the United States. *Rhodora* **25**, 13-14.

Hartl DL, Clark AG (2007) *Principles of population genetics*, 4th edn. Sinauer Associates, Sunderland, Mass.

Henry P, Le Lay G, Goudet J, *et al.* (2009) Reduced genetic diversity, increased isolation and multiple introductions of invasive giant hogweed in the western Swiss Alps. *Molecular Ecology* **18**, 2819-2831.

Knuth P (1908) *Handbook of flower pollination* Oxford, Clarendon.

Knuth P, Muller H (1908) *Handbook of flower pollination* Clarendon Press, Oxford.

Kolar CS, Lodge DM (2001) Progress in invasion biology: predicting invaders. *Trends in Ecology & Evolution* **16**, 199-204.

Korman BL (2011) *Biology and Ecology of Sickleweed (Falcaria vulgaris) in the Fort Pierre National Grassland of South Dakota*, South Dakota State University.

Kowarik I (2005) Time lags in biological invasions with regard to the success and failure of alien species. In: *Plant invasions: general aspects and special problems* eds. Phsek P, Prach K, Rejmanek M, Wade M). SPB Academic Publishers, Amsterdam, the Netherlands.

Lachmuth S, Durka W, Schurr FM (2010) The making of a rapid plant invader: genetic diversity and differentiation in the native and invaded range of *Senecio inaequidens*. *Molecular Ecology* **19**, 3952-3967.

Larina SY (2008) *Falcaria vulgaris* Bernh. Interactive agricultural ecological atlas of Russia and neighboring countries. In: *Economic plants and their diseases, pests and weeds* eds. Afonin AN, Greene SL, Dzyubenko NI, Frolov AN).

Larkin MA, Blackshields G, Brown NP, *et al.* (2007) Clustal W and clustal X version 2.0. *Bioinformatics* **23**, 2947-2948.

Lavergne S, Molofsky J (2007) Increased genetic variation and evolutionary potential drive the success of an invasive grass. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 3883-3888.

Lee CE (2002) Evolutionary genetics of invasive species. *Trends in Ecology & Evolution* **17**, 386-391.

Leitch AR, Leitch IJ (2008) Perspective - Genomic plasticity and the diversity of polyploid plants. *Science* **320**, 481-483.

Leung B, Drake JM, Lodge DM (2004) Predicting invasions: Propagule pressure and the gravity of allee effects. *Ecology* **85**, 1651-1660.

Levin DA (2002) *The role of chromosomal change in plant evolution* Oxford University Press, Oxford.

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451-1452.

Lockwood JL, Cassey P, Blackburn T (2005) The role of propagule pressure in explaining species invasions. *Trends in Ecology & Evolution* **20**, 223-228.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Res* **27**, 209-220.

Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 5446-5451.

Nater A, Arora N, Greminger MP, *et al.* (2013) Marked Population Structure and Recent Migration in the Critically Endangered *Sumatran Orangutan* (Pongo abelii). *Journal of Heredity* **104**, 2-13.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proc Natl Acad Sci U S A* **70**, 3321-3323.

Nei M (1977) F-statistics and analysis of gene diversity in subdivided populations. *Ann Hum Genet* **41**, 225-233.

NISC (2013) Invasive plants of Nebraska. http://www.neweed.org/neweed/Documents/Watchlist.pdf.

Novak SJ, Mack RN (2001) Tracing plant introduction and spread: Genetic evidence from *Bromus tectorum* (Cheatgrass). *Bioscience* **51**, 114-122.

Ohta T, Kimura M (1973) A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. *Genetical Research* **22**, 201-204.

Pandit MK, Pocock MJO, Kunin WE (2011) Ploidy influences rarity and invasiveness in plants. *Journal of Ecology* **99**, 1108-1115.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288-295.

Piya S, Nepal MN (2013) Characterization of Nuclear and Chloroplast Microsatellite Markers for *Falcaria vulgaris* (Apiaceae). *American Journal of Plant Sciences* **4**.

Piya S, Neupane A, Larson GE, Butler J, Nepal MN (2013) Inferring introduction history and spread of *Falcaria vulgaris* bernh. (Apiaceae) in the United States based on herbarium records. *Proceedings of the South Dakota Academy of Science, In press*.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.

Provan J, Murphy S, Maggs CA (2005) Tracking the invasive history of the green alga *Codium fragile* ssp tomentosoides. *Molecular Ecology* **14**, 189-194.

Pysek P, Prach K (1993) Plant invasion and the role of riparian habitats: a comparison of four species alien to central Europe. *Journal of Biogeography* **20**, 413-420.

Pysek P, Richardson DM, Pergl J, *et al.* (2008) Geographical and taxonomic biases in invasion ecology. *Trends in Ecology & Evolution* **23**, 237-244.

Rejmanek M (2000) Invasive plants: approaches and predictions. *Austral Ecology* **25**, 497-506.

Richardson DM, Pysek P, Rejmanek M, *et al.* (2000) Naturalization and invasion of alien plants: concepts and definitions. *Diversity and Distributions* **6**, 93-107.

Sakai AK, Allendorf FW, Holt JS, *et al.* (2001) The population biology of invasive species. *Annual Review of Ecology and Systematics* **32**, 305-332.

Sala OE, Chapin FS, Armesto JJ, *et al.* (2000) Biodiversity - Global biodiversity scenarios for the year 2100. *Science* **287**, 1770-1774.

Simberloff D (2009) The Role of Propagule Pressure in Biological Invasions. *Annual Review of Ecology Evolution and Systematics* **40**, 81-102.

Simberloff D, Alexander J, Allendorf F, *et al.* (2011) Non-natives: 141 scientists object. *Nature* **475**, 36-36.

Slatkin M (1987) Gene flow and the geographic structure of natural populations. *Science* **236**, 787-792.

Swafford DL (2003) *PAUP4. Phylogenetic Analysis Using Parsimony* Sinauer Associates, Sunderland, Massachusetts.

Tang SQ, Wei F, Zeng LY, *et al.* (2009) Multiple introductions are responsible for the disjunct distributions of invasive *Parthenium hysterophorus* in China: evidence from nuclear and chloroplast DNA. *Weed Research* **49**, 373-380.

Taylor CM, Hastings A (2005) Allee effects in biological invasions. *Ecology Letters* **8**, 895-908.

te Beest M, Le Roux JJ, Richardson DM, *et al.* (2012) The more the better? The role of polyploidy in facilitating plant invasions. *Annals of Botany* **109**, 19-45.

Valliant MT, Mack RN, Novak SJ (2007) Introduction history and population genetics of the invasive grass *Bromus tectorum* (Poaceae) in Canada. *American Journal of Botany* **94**, 1156-1169.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**, 535-538.

Wilson JRU, Gairifo C, Gibson MR, *et al.* (2011) Risk assessment, eradication, and biological control: global efforts to limit Australian acacia invasions. *Diversity and Distributions* **17**, 1030-1046.

Yeh FC, Boyle TJB (1997) Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129**, 157.

Young A, Boyle T, Brown T (1996) The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology & Evolution* **11**, 413-418.

APPENDICES

Appendix- 1

List of herbaria contacted

State	Acronyms of the herbaria
Illinois	CACS, EIU, CHIC, F, ILLS,ISM, ISU, KNOX, MOR, NRRL, DEK, SIU,CEL, WARK
Iowa	MOVC, GRI, ILH, ISC, GRI, LCDI, BDI, SICH, ISTC, WET
Kansas	KSTC, FHKSC, KSC, SAL, KANU, WASH
Louisiana	LSU, LSUS, LTU, MCN, THIB, NATC, SELU, NO, USLH, NLU, NOLS
Maryland	BALT, MARY, SUHC, TAWES, US
Massachusetts	AC, CUW, WMGC, GH, NASC, NMMA, NEBC, HNUB, PM, SCHN, SPR, HDSM, MASS, WELC
Missouri	MCJ, MODNR, MO, MWSJ, NEMO, NMSU, SEMO, SMS, SOTO, UMO, WARM, WJC
Nebraska	CSCN, HNWU, NEBK, OMA, NEB,
New York	BKL, GRCH, BH, ECH, HHH, DH, SOUT, HPH, NY, NYS, ROCH, SBU, CORT, SYRF, BING, PLAT, SUCO, SIM, SYR, VAS
Oklahoma	ECSC, NOSU, NWOSU, OKLA, ORU, DUR, WHO, CSU, OKL, OCLA, TULS
Pennsylvania	ANSP, BUPL, CM, IUP, KEN, MVSC, MOAR, MCA, FMC, PAM, PACMA, RPM, LAT, SLRO, SWC, ABFM, PHIL, DWC
South Dakota	DWU, AUG, BHSC, SDC, SDU
Virginia	CVCW, HAVI, EHCV, GMUF, JMUH, FARM, LFCC, LYN, MWCF, ODU, RUHV, SARC, WILLI, URV, VA, VCU, VDAC, VIL, VPI, VSUH, ROAN, WYCO
West Virginia	DEWV, FWVA, MUHW, MVC, WVA, WVW
Wisconsin	CART, MIL, SNC, UWW, FDLW, UWEC, UWJ, UWL, UWM, WIS, OSH, UWGB, USWP, SUWS
Wyoming	BTJW, CWC, RMS, RM, YELLO

Appendix- 2

Pair wise genetic differentiation (FST) of sickleweed populations

	NE29	AW	NE30	BG	BRK	GC	IA	WA
NE29	-	3.177	1.897	4.07	2.139	3.143	0.973	1.421
AW	0.135	-	3.180	3.359	3.727	6.257	1.653	1.740
NE30	0.208	0.135	-	3.428	1.629	2.227	1.366	1.889
BG	0.109	0.129	0.127	-	2.187	2.350	1.917	2.268
BRK	0.189	0.118	0.234	0.186	-	2.054	1.034	1.415
GC	0.137	0.073	0.183	0.175	0.195	-	1.143	1.699
IA	0.339	0.232	0.267	0.206	0.325	0.304	-	1.211
WA	0.206	0.223	0.209	0.180	0.260	0.227	0.292	-

Note: Above diagonal line is the number of migrants per generation between the populations and below the diagonal line is the pairwise F_{ST} value between populations.

Appendix- 3

Chloroplast tRNA-Leu (*trnL*) gene, partial sequence; *trnL-trnF* intergenic spacer, complete sequence; and tRNA-Phe (*trnF*) gene, partial sequence; of different chlorotypes

1. Chlorotype 1 (CP1)

   Total length= 908

   trnL gene= 1-524

   trnL tRNA = 470-524

   trnL-F intergenic spacer = 525-880

   trnF gene = 881-909

   tRNA = 881-908

   ACTAAGTGAGAACTTTCAAATTCAGAGAACCCCGGAATTAATAAAAAT
   GGGCAATCCTGAGCCAAATCCTATTTTCAAAAAAAAAAAACAAATGC
   CCAAAAGGTGAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTC
   TAACAAATGGAATTGACTGTGTTGCATTGGTAGAGGAATCCTTCCATT
   GAAACTTCCGAAAAGATGAAGGATATACGTATATACATACGTACTGA
   AATACTCTATCAAATGATTAATGACGACCTTAATCTGTATTTTTCTATG
   AAAAGGGGAAAAATTGTTGTGAATCGATTCCAGATTTAAGAAATAATC
   GAATATTCATTGATCAAAGCATTCACCCACACAGTCTGATAGTTCTTTT
   GCAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCGTTCTAC
   ATGTCAATACCGGCAACAATGAAATTTATAGTAAGAGGAAAATCCGTT
   GACTTTAAAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAAAAAGCCC
   ATTTGACTCCTTAACTATTCACTTTTTATCTTATCTTTTTTTTTTTCGTTA
   GTAGTTCAAAATTTGTTATCTTTCTTATTCACCCTACTCTTTTACAAAG
   GGATCCGAGATAAAAATTTGTCTCTTATGACAAGTCTTGTGATATATG
   ATACATGTACAAATGACCGTCTTTAACCAAGGACTCCCCATTTGAACG
   AGTCACGGTCAATATCATTATTCATATTATTCATACTGAAACTGACAA
   AGTCTTCCTTTTTTGAAGATCCAAGAAATTCTAGCACCTAGATAAGAA
   TTTGTAATACCCTTTGCATTGACATAGACCTAAGTTATCTAGTAAAATG
   AGGATGCGGTATCGGGAATGAGAATGGTCGGGATAGCTCAG

2. Chlorotype 2 (CP2)

   Total length= 909

   trnL gene= 1-524

   trnL tRNA = 470-524

   trnL-F intergenic spacer = 525-881

   trnF gene = 882-909

   tRNA = 882-909

   ACTAAGTGAGAACTTTCAAATTCAGAGAAACCCCGGAATTAATAAAA
   ATGGGCAATCCTGAGCCAAATCCTATTTTCAAAAAAAAAAAACAAAT
   GCCCAAAAGGTGAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGT
   TCTAACAAATGGAATTGACTGTGTTGCATTGGTAGAGGAATCCTTCCA
   TTGAAACTTCCGAAAAGATGAAGGATATACGTATATACATACGTACTG
   AAATACTCTATCAAATGATTAATGACGACCTTAATCTGTATTTTTCTAT
   GAAAAGGGGAAAAATTGTTGTGAATCGATTCCAGATTTAAGAAATAA
   TCGAATATTCATTGATCAAAGCATTCACCCACACAGTCTGATAGTTCTT
   TTGCAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCGTTCTA
   CATGTCAATACCGGCAACAATGAAATTTATAGTAAGAGGAAAATCCGT
   TGACTTTAAAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAAAAAGCC
   CATTTGACTCCTTAACTATTCACTTTTTATCTTATCTTTTTTTTTTTCGTT
   AGTAGTTCAAAATTTGTTATCTTTCTTATTCACCCTACTCTTTTACAAA
   GGGATCCGAGATAAAAATTTGTCTCTTATGACAAGTCTTGTGATATAT
   GATACATGTACAAATGACCGTCTTTAACCAAGGACTCCCCATTTGAAC
   GAGTCACGGTCAATATCATTATTCATATTATTCATACTGAAACTGACA
   AAGTCTTCCTTTTTTGAAGATCCAAGAAATTCTAGCACCTAGATAAGA
   ATTTGTAATACCCTTTGCATTGACATAGACCTAAGTTATCTAGTAAAAT
   GAGGATGCGGTATCGGGAATGAGAATGGTCGGGATAGCTCAG

3. Chlorotype 3 (CP3)

   Total length= 907

   trnL gene= 1-523

   trnL tRNA = 469-523

   trnL-F intergenic spacer = 524-879

   trnF gene = 880-907

   tRNA = 880-907

ACTAAGTGAGAACTTTCAAATTCAGAGAAACCCCGGAATTAATAAAAATG
GGCAATCCTGAGCCAAATCCTATTTTCAAAAAAAAAAACAAATGCCCAAA
AGGTGAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAA
TGGAATTGACTGTGTTGCATTGGTAGAGGAATCCTTCCATTGAAACTTCCG
AAAAGATGAAGGATATACGTATATACATACGTACTGAAATACTCTATCAA
ATGATTAATGACGACCTTAATCTGTATTTTTCTATGAAAAGGGGAAAAAT
TGTTGTGAATCGATTCCAGATTTAAGAAATAATCGAATATTCATTGATCAA
AGCATTCACCCCCACAGTCTGATAGTTCTTTTGCAGAACTAATTAATCGGA
CGAGAATAAAGATAGAGTCCCGTTCTACATGTCAATACCGGCAACAATGA
AATTTATAGTAAGAGGAAAATCCGTTGACTTTAAAAATCGTGAGGGTTCA
AGTCCCTCTATCCCCAAAAAGCCCATTTGACTCCTTAACTATTCACTTTTT
ATCTTATCTTTTTTTTTTCGTTAGTAGTTCAAAATTTGTTATCTTTCTTATTC
ACCCTACTCTTTTACAAAGGGATCCGAGATAAAAATTTGTCTCTTATGACA
AGTCTTGTGATATATGATACATGTACAAATGACCGTCTTTAACCAAGGAC
TCCCCATTTGAACGAGTCACGGTCAATATCATTATTCATATTATTCATACT
GAAACTGACAAAGTCTTCCTTTTTTGAAGATCCAAGAAATTCTAGCACCT
AGATAAGAATTTGTAATACCCTTTGCATTGACATAGACCTAAGTTATCTAG
TAAAATGAGGATGCGGTATCGGGAATGAGAATGGTCGGGATAGCTCAG

4. Chlorotype 4 (CP4)

   Total length= 909

   trnL gene= 1-523

   trnL tRNA = 469-523

   trnL-F intergenic spacer = 524-881

   trnF gene = 882-909

   tRNA = 882-909

ACTAAGTGAGAACTTTCAAATTCAGAGAAACCCCGGAATTAATAAAAATG
GGCAATCCTGAGCCAAATCCTATTTTCAAAAAAAAAAAAACAAATGCCCAA
AAGGTGAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAA
ATGGAATTGACTGTGTTGCATTGGTAGAGGAATCCTTCCATTGAAACTTCC
GAAAAGATGAAGGATATACGTATATACATACGTACTGAAATACTCTATCA
AATGATTAATGACGACCTTAATCTGTATTTTTCTATGAAAAGGGGAAAAA
TTGTTGTGAATCGATTCCAGATTTAAGAAATAATCGAATATTCATTGATCA
AAGCATTCACCCACACAGTCTGATAGTTCTTTTGCAGAACTAATTAATCGG
ACGAGAATAAAGATAGAGTCCCGTTCTACATGTCAATACCGGCAACAATG
AAATTTATAGTAAGAGGAAAATCCGTTGACTTTAAAAATCGTGAGGGTTC
AAGTCCCTCTATCCCCAAAAAGCCCATTTGACTCCTTAACGATTCACTTTT
TATCTTATCTTTTTTTTTTTCGTTAGTAGTTCAAAATTCGTTATCTTTCTTAT
TCACCCTACTCTTTTACAAAGGGATCCGAGATAAAAATTTGTCTCTTATGA
CAAGTCTTGTGATATATGATACATGTACAAATGACCGTCTTTAACCAAGG
ACTCCCCATTTGAACGAGTCACGGTCAATATCATTATTCATATTATTCATA
CTGAAACTGACAAAGTCTTCCTTTTTTGAAGATCCAAGAAATTCTAGCACC
TAGATAAGAATTTGTAATACCCTTTGCATTGACATCGACCTAAGTTATCTA
GTAAAATGAGGATGCGGTATCGGGAATGAGAATGGTCGGGATAGCTCAG

5. Chlorotype 5 (CP5)

   Total length= 907

   trnL gene= 1-522

   trnL tRNA =467 -522

   trnL-F intergenic spacer = 523-879

   trnF gene = 880-907

   tRNA = 880-907

   ACTAAGTGAGAACTTTCAAATTCAGAGAAACCCCGGAATTAATAAAA
   ATGGGCAATCCTGAGCCAAATCCTATTTTCAAAAAAAAAACAAATGCC
   CAGAAGGTGAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCT
   AACAAATGGAATTGACTGTGTTGCATTGGTAGAGGAATCCTTCCATTG
   AAACTTCCGAAAAGATAAAGGATATACGTATATACATACGTACTGAA
   ATACTCTATCAAATGATTAATGACGACCTTAATCTGTATTTTTCTATGA
   AAAGGGGAAAAATTGTTGTGAATCGATTCCAGATTTAAGAAATAATCG
   AATATTCATTGATCAAAGCATTCACCCACACAGTCTGATAGTTCTTTTG
   CAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCGTTCTACAT
   GTCAATACCGGCAACAATGAAATTTATAGTAAGAGGAAAATCCGTTG
   ACTTTAAAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAAAAAGCCCA
   TTTGACTCCTTAACGATTCACTTTTTATCTTATCTTTTTTTTTTTCGTTAG
   TAGTTCAAAATTCGTTATCTTTCTTATTCACCCTACTCTTTTACAAAGG
   GATCCGAGATAAAAATTTGTCTCTTATGACAAGTCTTGTGATATATGA
   TACATGTACAAATGACCGTCTTTAACCAAGGACTCCCCATTTGAACGA
   GTCACGGTCAATATCATTATTCATATTATTCATACTGAAACTGACAAA
   GTCTTCCTTTTTTGAAGATCCAAGAAATTCTAGCACCTAGATAAGAATT
   TGTAATACCCTTTGCATTGACATCGACCTAAGTTATCTAGTAAAATGA
   GGATGCGGTATCGGGAATGAGAATGGTCGGGATAGCTCAG

6. Chlorotype 6 (CP6)

   Total length= 907

   trnL gene= 1-522

   trnL tRNA = 467-522

   trnL-F intergenic spacer = 523-879

   trnF gene = 880-907

   tRNA = 880-907

   ACTAAGTGAGAACTTTCAAATTCAGAGAAACCCCGGAATTAATAAAA
   ATGGGCAATCCTGAGCCAAATCCTATTTTCAAAAAAAAAACAAATGCC
   CAGAAGGTGAAAAAGGATAGGTGCAGAGACTCAATGGAAGCTGTTCT
   AACAAATGGAATTGACTGTGTTGCATTGGTAGAGGAATCCTTCCATTG
   AAACTTCCGAAAAGATAAAGGATATACGTATATACATACGTACTGAA
   ATACTCTATCAAATGATTAATGACGACCTTAATCTGTATTTTTCTATGA
   AAAGGGGAAAAATTGTTGTGAATCGATTCCAGATTTAAGAAATAATCG
   AATATTCATTGATCAAAGCATTCACCCACACAGTCTGATAGTTCTTTTG
   CAGAACTAATTAATCGGACGAGAATAAAGATAGAGTCCCGTTCTACAT
   GTCAATACCGGCAACAATGAAATTTATAGTAAGAGGAAAATCCGTTG
   ACTTTAAAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAAAAAGCCCA
   TTTGACTCCTTAACGATTCACTTTTTATCTTATCTTTTTTTTTTTCGTTAG
   TAGTTCAAAATTCGTTATCTTTCTTATTCACCCTACTCTTTTACAAAGG
   GATCCGAGATAAAAATTTGTCTCTTATGACAAGTCTTGTGATATATGA
   TACATGTACAAATGACCGTCTTTAACCAAGGACTCCCCATTTGAACGA
   GTCACGGTCAATATCATTATTCATATTATTCATACTGAAACTGACAAA
   GTCTTCCTTTTTTGAAGATCCAAGAAATTCTAGCACCTAGATAAGAATT
   TGTAATACCCTTTGCATTGACATCGACCTAAGTTATCTAGTAAAATGA
   GGATGCGGTATCGGGAATGAGAATGGTCGGGATAGCTCAG

Appendix- 4

Chloroplast *trnQ-rps16* intergenic spacer, partial DNA sequence of different chlorotypes

1. Chlorotype 1 (CP1)

Total length= 883

trnQ-rps16 intergenic spacer, partial sequence = 1-883

GAGACATTATATCAGAGTCAAAGTTTATTTTGAAAGTAACGTTATGAAAA

AATTCCTAATTATTTGAGAGAATGTCATTCTAGTTTCTTTTAGAATTTTG

AATGATTCTTTTATGAATCATATCTTTGTTTTTCACAACATACAGATATT

ACTAAATAGATTTGTTTGTGATCAAGATATGATGGTAACATAGGTCACAC

CCTAGTTGAATTCAACTAATTAAAAAAGAAAGTATGGCGGATTTTTCATC

ATACGTTCATTCCCTTCATAGTGAAGGGGTTTAGAGCTACTTAATTTGAA

GAAAAAACCTTACGTCTCATATCTTTTTGAATTTTCAACGATACGTTTTA

TTTTTAATTACACTAAGAAAGAGCACTTCTTTCCTATATCTTATTCTTTA

TCCATTCAAATTAAACGTCAAAAAATGGGTAGCCAAATTATTAGGATCT

CTTTATTCTAAACAAGAGGGGGGTTATTACATTCAATTAATGGGGATTAA

GTCTCTTAAGACAAGACTGGGTTTGGGTAAACTAAAAAATTAGGAGCAAG

CGCCTAATTTGGACTTGTTTGTCTTTGTCCCTAGAGAGTATCCTTTCCCC

AAAAGGGATCCTTTTTTGTTTTTGTTCTAATTAAGCATTCCCAGAGAGTC

GTGGGATAGATAGTTCTTAATTAGGAACAGTAATAGGAGGTCTTCATTTA

AATATGTATATCTGTGTATGTCCGAATCTCATTAATAACGAATCAAGTTA

CATATGTAACGGATCCATAATAAAGACTGTAGTTCAGTCTATCTGATAGG

TACGAAAAACCCCTAATTCGTTCATAATATCTTATTAAGAAAATTCGAAT

CGAAAGAACAAGTACTTAAATATTCTCTTTGATC

2. Chlorotype 2 (CP2)

Total length= 883

trnQ-rps16 intergenic spacer, partial sequence = 1-883

GAGACATTATATCAGAGTCAAAGTTTATTTTGAAAGTAACGTTATGAAAA
AATTCCTAATTATTTGAGAGAATGTCATTCTAGTTTCTTTTAGAATTTTG
AATGATTCTTTTATGAATCATATCTTTGTTTTTCACAACATACAGATATT
ACTAAATAGATTTGTTTGTGATCAAGATATGATGGTAACATAGGTCACAC
CCTAGTTGAATTCAACTAATTAAAAAAGAAAGTATGGCGGATTTTTCATC
ATACGTTCATTCCCTTCATAGTGAAGGGGTTTAGAGCTACTTAATTTGAA
GAAAAAACCTTACGTCTCATATCTTTTTGAATTTTCAACGATACGTTTTA
TTTTTAATTACACTAAGAAAGAGCACTTCTTTCCTATATCTTATTCTTTA
TCCATTCAAATTAAACGTCAAAAAATGGGTAGCCAAATTATTAGGATCT
CTTTATTCTAAACAAGAGGGGGGTTATTACATTCAATTAATGGGGATTAA
GTCTCTTAAGACAAGACTGGGTTTGGGTAAACTAAAAAATTAGGAGCAAG
CGCCTAATTTGGACTTGTTTGTCTTTGTCCCTAGAGAGTATCCTTTCCCC
AAAAGGGATCCTTTTTTGTTTTTGTTCTAATTAAGCATTCCCAGAGAGTC
GTGGGATAGATAGTTCTTAATTAGGAACAGTAATAGGAGGTCTTCATTTA
AATATGTATATCTGTGTATGTCCGAATCTCATTAATAACGAATCAAGTTA
CATATGTAACGGATCCATAATAAAGACTGTAGTTCAGTCTATCTGATAGG
TACGAAAAACCCCTAATTCGTTCATAATATCTTATTAAGAAAATTCGAAT
CGAAAGAACAAGTACTTAAATATTCTCTTTGATC

3. Chlorotype 3 (CP3)

Total length= 883

trnQ-rps16 intergenic spacer, partial sequence = 1-883

GAGACATTATATCAGAGTCAAAGTTTATTTTGAAAGTAACGTTATGAAAA
AATTCCTAATTATTTGAGAGAATGTCATTCTAGTTTCTTTTAGAATTTTG
AATGATTCTTTTATGAATCATATCTTTGTTTTTCACAACATACAGATATT
ACTAAATAGATTTGTTTGTGATCAAGATATGATGGTAACATAGGTCACAC
CCTAGTTGAATTCAACTAATTAAAAAAGAAAGTATGGCGGATTTTTCATC
ATACGTTCATTCCCTTCATAGTGAAGGGGTTTAGAGCTACTTAATTTGAA
GAAAAAACCTTACGTCTCATATCTTTTTGAATTTTCAACGATACGTTTTA
TTTTTAATTACACTAAGAAAGAGCACTTCTTTCCTATATCTTATTCTTTA
TCCATTCAAATTAAACGTCAAAAAATGGGTAGCCAAATTATTAGGATCT
CTTTATTCTAAACAAGAGGGGGGTTATTACATTCAATTAATGGGGATTAA
GTCTCTTAAGACAAGACTGGGTTTGGGTAAACTAAAAAATTAGGAGCAAG
CGCCTAATTTGGACTTGTTTGTCTTTGTCCCTAGAGAGTATCCTTTCCCC
AAAAGGGATCCTTTTTTGTTTTTGTTCTAATTAAGCATTCCCAGAGAGTC
GTGGGATAGATAGTTCTTAATTAGGAACAGTAATAGGAGGTCTTCATTTA
AATATGTATATCTGTGTATGTCCGAATCTCATTAATAACGAATCAAGTTA
CATATGTAACGGATCCATAATAAAGACTGTAGTTCAGTCTATCTGATAGG
TACGAAAAACCCCTAATTCGTTCATAATATCTTATTAAGAAAATTCGAAT
CGAAAGAACAAGTACTTAAATATTCTCTTTGATC

4. Chlorotype 4 (CP4)

Total length= 883

trnQ-rps16 intergenic spacer, partial sequence = 1-883

GAGACATTATATCAGAGTCAAAGTTTATTTTGAAAGTAACGTTATGAAAA
AATTCCTAATTATTTGAGAGAATGTCATTCTAGTTTCTTTTAGAATTTTG
AATGATTCTTTTATGAATCATATCTTTGTTTTTCACAACATACAGATATT
ACTAAATAGATTTGTTTGTGATCAAGATATGATGGTAACATAGGTCACAC
CCTAGTTGAATTCAACTAATTAAAAAAGAAAGTATGGCGGATTTTTCATC
ATACGTTCATTCACTTCATAGTGAAGGGGTTTAGAGCTACTTAATTTGAA
GAAAAAACCTTACGTCTCATATCTTTTTGAATTTTCAACGATACGTTTTA
TTTTTAATTACACTAAGAAAGAGCACTTCTTTCCTATATCTTATTCTTTA
TCCATTCAAATTAAACGTCAAAAAATGGGGTAGCCAAATTATTAGGATCT
CTTTATTCTAAACAAGAGGGGGGTTATTACATTCAATTAATGGGATTAA
GTCTCTTAAGACAAGACTGGGTTTGGGTAAACTAAAAAATTAGGAGCAAG
CGCCTAATTTGGACTTGTTTGTCTTTGTCCCTAGAGAGTATCCTTTCCCC
AAAAGGGATCCTTTTTTGTTTTTGTTCTAATTAAGCATTCCCAGAGAGTC
GTGGGATAGATAGTTCTTAATTAGGAACAGTAATAGGAGGTCTTCATTTA
AATATGTATATATGTGTATGTCCGAATCTCATTAATAACGAATCAAGTTA
CATATGTAACGGATCCATAATAAAGACTGTAGTTCAGTCTATCTGATAGG
TACGAAAAACCCCTAATTCGTTCATAATATCTTATTAAGAAAATTCGAAT
CGAAAGAACAAGTACTTAAATATTCTCTTTGATC

5. Chlorotype 5 (CP5)

Total length= 883

trnQ-rps16 intergenic spacer, partial sequence = 1-883

GAGACATTATATCAGAGTCAAAGTTTATTTTGAAAGTAACGTTATGAAAA
AATTCCTAATTATTTGAGAGAATGTCATTCTAGTTTCTTTTAGAATTTTG
AATGATTCTTTTATGAATCATATCTTTGTTTTTCACAACATACAGATATT
ACTAAATAGATTTGTTTGTGATCAAGATATGATGGTAACATAGGTCACAC
CCTAGTTGAATTCAACTAATTAAAAAAGAAAGTATGGCGGATTTTTCATC
ATACGTTCATTCACTTCATAGTGAAGGGGTTTAGAGCTACTTAATTTGAA
GAAAAAACCTTACGTCTCATATCTTTTTGAATTTTCAACGATACGTTTTA
TTTTTAATTACACTAAGAAAGAGCACTTCTTTCCTATATCTTATTCTTTA
TCCATTCAAATTAAACGTCAAAAAATGGGGTAGCCAAATTATTAGGATCT
CTTTATTCTAAACAAGAGGGGGGTTATTACATTCAATTAATGGGATTAA
GTCTCTTAAGACAAGACTGGGTTTGGGTAAACTAAAAAATTAGGAGCAAG
CGCCTAATTTGGACTTGTTTGTCTTTGTCCCTAGAGAGTATCCTTTCCCC
AAAAGGGATCCTTTTTTGTTTTTGTTCTAATTAAGCATTCCCAGAGAGTC
GTGGGATAGATAGTTCTTAATTAGGAACAGTAATAGGAGGTCTTCATTTA
AATATGTATATATGTGTATGTCCGAATCTCATTAATAACGAATCAAGTTA
CATATGTAACGGATCCATAATAAAGACTGTAGTTCAGTCTATCTGATAGG
TACGAAAAACCCCTAATTCGTTCATAATATCTTATTAAGAAAATTCGAAT
CGAAAGAACAAGTACTTAAATATTCTCTTTGATC

6. Chlorotype 6 (CP6)

Total length= 883

trnQ-rps16 intergenic spacer, partial sequence = 1-883

GAGACATTATATCAGAGTCAAAGTTTATTTTGAAAGTAACGTTATGAAAA
AATTCCTAATTATTTGAGAGAATGTCATTCTAGTTTCTTTTAGAATTTTG
AATGATTCTTTTATGAATCATATCTTTGTTTTTCACAACATACAGATATT
ACTAAATAGATTTGTTTGTGATCAAGATATGATGGTAACATAGGTCACAC
CCTAGTTGAATTCAACTAATTAAAAAAGAAAGTATGGCGGATTTTTCATC
ATACGTTCATTCCCTTCATAGTGAAGGGGTTTAGAGCTACTTAATTTGAA
GAAAAAACCTTACGTCTCATATCTTTTTGAATTTTCAACGATACGTTTTA
TTTTTAATTACACTAAGAAAGAGCACTTCTTTCCTATATCTTATTCTTTA
TCCATTCAAATTAAACGTCAAAAAATGGGTAGCCAAATTATTAGGATCT
CTTTATTCTAAACAAGAGGGGGGTTATTACATTCAATTAATGGGGATTAA
GTCTCTTAAGACAAGACTGGGTTTGGGTAAACTAAAAAATTAGGAGCAAG
CGCCTAATTTGGACTTGTTTGTCTTTGTCCCTAGAGAGTATCCTTTCCCC
AAAAGGGATCCTTTTTTGTTTTTGTTCTAATTAAGCATTCCCAGAGAGTC
GTGGGATAGATAGTTCTTAATTAGGAACAGTAATAGGAGGTCTTCATTTA
AATATGTATATCTGTGTATGTCCGAATCTCATTAATAACGAATCAAGTTA
CATATGTAACGGATCCATAATAAAGACTGTAGTTCAGTCTATCTGATAGG
TACGAAAAACCCCTAATTCGTTCATAATATCTTATTAAGAAAATTCGAAT
CGAAAGAACAAGTACTTAAATATTCTCTTTGATC

Appendix- 5
Internal Transcribed Spacer (ITS) DNA sequence variation between two chloroplast lineages

1. Lineage 1

Total length=686

18s rRNA gene, partial sequence= 1-39

ITS1= 40-255

5.8s rRNA gene= 256-418

ITS2= 419-641

28s rRNA gene, partial sequence= 642-686

AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAATCCTGC
GATAGCAGAACGACCCGCTAACTCGTAAAAACATCGGGCAAGCTCACGGG
GATTCCGTCCCCTGTTGGCGAACCCTCGGTAGGTGGTCGCCCTTCGGTGG
CCACCGGCCCACGAAATCATCCGGGCGCGGCATGCGCCAAGGAAATTAAA
ACTGAATTGATGACCGCTTCCCGTTAGCCGGGTAGTGGCGTCATTCTAAA
ACACAAACGACTCTCGACAACGGATATCTCGGCTCTCGCATCGATGAAGA
ACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCAT
CGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGT
CTGCCTGGGTGTCACGCATCGTGTTGCCCCCAAACACTCACTCCTTCTGG
AAATGTTTCGTTTTTGGGGGCGGAAATTGGCCTCCCGTGCCTTTTGGTGC
GGTTGGCACAAAAGTGAGTCTCCGGCGACGGACGTCGCGACATCGGTGGT
TGTAAAAAGACCTTCTTGTCTTGTCGCGCGAATCCCTGTCACCTTAGCGA
GCTCCAGGATCCTTAGGCGCCACATGTTGTGTGCGCTCCGAATGTGACCC
CAGGTCAGGCGGGACTACCCGCTGAGTTTAAGCATA

2. Lineage 2

Total length=686

18s rRNA gene, partial sequence= 1-39

ITS1= 40-255

5.8s rRNA gene= 256-418

ITS2= 419-641

28s rRNA gene, partial sequence= 642-686

AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAATCCTGC
GATAGCAGAACGACCCGCTAACTCGTAAAAACATCGGGCAAGCTCACGGG
GATTCCGTCCCCTGTTGGCGAACCCTCGGTAGGTGGTCGCCCTTCGGTGG
CCACCGGCCCACGAAATCATCCGGGCGCGGCATGCGCCAAGGAAATTAAA
ACTGAATTGATGACCGCTTCCCGTTAGCCGGGTAGTGGCGTCATTCTAAA
ACACAAACGACTCTCGACAACGGATATCTCGGCTCTCGCATCGATGAAGA
ACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCAT
CGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCATTAGGCCGAGGGCACGT
CTGCCTGGGTGTCACGCATCGTGTTGCCCCCAAACACTCACTCCTTCTGG
AAATGTTTCGTTTTTGGGGGCGGAAATTGGCCTCCCGTGCCTTTTGGTGC
GGTTGGCACAAATGTGAGTCTCCGGCGACGGACGTCGCGACATCGGTGGT
TGTAAAAAGACCTTCTTGTCTTGTCGCGCGAATCCCTGTCACCTTAGCGA
GCTCCAGGATCCTTAGGCGCCACATGTTGTGTGCGCTCCGAATGTGACCC
CAGGTCAGGCGGGACTACCCGCTGAGTTTAAGCATA