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Author: Doorduin, Lena Johanna
Title: Rapid evolution or preadaptation in invasive Jacobaea vulgaris
Issue Date: 2012-06-26

Rapid evolution or preadaptation in invasive *Jacobaea vulgaris*

Leonie Doorduin

Doorduin, Lena Johanna

Rapid evolution or preadaptation in invasive *Jacobaea vulgaris*

PhD thesis Leiden University, The Netherlands

An electronic version of this thesis can be downloaded from:
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Cover design and thesis lay-out by Rene Glas (www.reneglas.com).
Printed by Offsetdrukkerij Nautilus, Leiden

ISBN: 978-90-8570-833-9

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Rapid evolution or preadaptation in invasive *Jacobaea vulgaris*

PROEFSCHRIFT

Ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. P. F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op dinsdag 26 juni 2012

klokke 15:00 uur

door

Lena Johanna Doorduin

Geboren te Rozenburg

in 1983

P r o m o t i e c o m m i s s i e

Promotor • Prof. dr. P.G.L. Klinkhamer

Copromotor • Dr. K. Vrieling

Overige leden • Prof. dr. E. van der Meijden

Prof. dr. C.J. ten Cate

Dr. A. Biere (NIOO)

Prof. dr. J. Joshi (Universität Potsdam)

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General Introduction

In 1901, the water hyacinth (*Eichhornia crassipes*) was introduced from South America into China as a good fodder plant. However, one hundred years later this plant has led to serious environmental- and economical problems in China. Because of large coverage of this weed on water, sunlight penetration as well as oxygen content in the water is reduced, which has a severe negative impact on the water ecosystem. For example 60% of the local species living in Dianchi Lake (Yunnan Province) was wiped out by the invasion of the water hyacinth. Furthermore these waters are difficult to cross by ship and many canals have irrigation and drainage problems. In Shanghai more than 10 million USD is spent on this pest species each year (Villamagna and Murphy 2010).

The introduction of species from one area into another is a natural process that has always been a part of evolutionary history. However, the deliberate and undeliberate transport of species by humans, starting circa 10.000 years ago during the Neolithic Revolution added considerably to the frequency of new introductions. As an early example, around 4.000 B.C. domesticated pigs were introduced in Europe from Asia and crossed with wild pigs (Larson et al. 2007). The increase of human migrations and trade in the 19th century was accompanied by the spread of domesticated species like cereals, rice and cattle (Di Castri 1989) and the accidental spread of natural species as contaminants, like the brown rat (Atkinson 1977) and the zebra mussel (Mooney and Cleland 2001). With these activities, species were even able to spread from one continent to the other, crossing almost insurmountable biogeographical barriers (Mooney and Cleland 2001) and maintained themselves in these new environments in many cases.

Invasive plant species are defined as species that manage to cope with the new environment, disperse to other local communities and become extraordinarily prominent in their range (Moutou and Pastoret 2010). Species that adjust to other climate conditions and therefore have a possibility to shift ranges can also be considered as potential invaders (Engelkes et al. 2008). Species can receive a pest status if they have a negative impact on human health, are a pest in agricultural crops, lead to a loss of native biodiversity due to competition or predation, or cause habitat degradation and disruption. Several examples document these negative impacts. The invasive black mustard (*Brassica nigra*) increased the herbivore pressure on a native bunchgrass in the United States (Orrock et al. 2008). Kudzu (*Pueraria lobata*) introduced in the United States for erosion control is now a pest species threatening native ecosystems due to its rapid growth rate (Forseth and Innis 2004). Common ragweed (*Ambrosia artemisiifolia*) originated from North America was introduced undeliberately into Europe and is nowadays presenting a major health problem because of its highly allergenic pollen. Furthermore ragweed is estimated to reduce corn crops yield by 55% (Makra et al. 2005).

Besides the impact on the environment, invasions can have an economic impact in two ways. Firstly invasive species may negatively affect crop and forestry production and grazing capacity. Secondly there are the direct costs of combating invasions like control and quarantine measures (Mack et al. 2000). For the United States the annual cost of all invasive species (plants, animals and microorganisms) is estimated to exceed 138 billion dollar per year (Pimentel et al. 2005).

In this thesis I will focus on the mechanisms that contribute to the invasiveness of a plant species and on finding the source populations of invasive plant species. This is important in light of predicting a potential pest species.

Theories on invasive plant species

To become invasive, a plant has to go through four stages; transport, colonization, establishment and becoming abundant and widespread (Vermeij 1996). Although many species are introduced, only a small number of them can establish and even a smaller proportion can maintain and spread in the new area. Several theories aim to explain the success of invasive plant species (Lodge 1993, Mack et al. 2000, Eppinga et al. 2006, Ren and Zhang 2009, Verhoeven et al. 2010).

In this thesis I will focus on theories that emphasize the plant's release from herbivores of the native area. The *Enemy Release Hypothesis* (ERH) (Keane and Crawley 2002) states that when plants are introduced into the new area they leave behind their specialist herbivores and therefore experience a reduced herbivore pressure in the invasive area. Building on the ERH, the *Evolution of Increased Competitive Ability* (EICA) hypothesis (Blossey and Notzold 1995) predicts that, under reduced enemy pressure, selection may shift the resource allocation of invasive plant species from defence to growth. This allocation to growth also results in a higher reproduction, giving the invasive plants a competitive advantage over local plants. So in contrast with the ERH, which is based on an ecological change, the EICA hypothesis is also based on an evolutionary change. Although natural selection may decrease defence compounds of plants in the new area, plants are still in need for defences against generalist herbivores in the invasive area. Plants employ different defences against generalist and specialist herbivores. Feeny (1976) and Rhoades & Cates (1976) simultaneously developed the *Apparency theory*, which makes a distinction between "quantitative" and "qualitative" defences (Feeny 1976, Rhoades and Cates 1976). Quantitative defences are digestibility reducers (e.g. tough leaves, thorns) and occur in high concentrations which make them expensive to produce. Quantitative defences act against specialist as well as generalist herbivores. Qualitative defences are toxins (e.g. phenolics, alkaloids) and occur in relatively low quantities, which make them a cheaper defence compared to quantitative defences. They act against generalist herbivores but specialist herbivores are often adapted to these defences and can even use these chemicals as a cue to locate their host plant as a feeding or oviposition stimulant or sequestrate them for their own defence (Bernays et al. 2003, Macel and Vrieling 2003). Digestibility reducers provide protection against both generalist and specialist herbivores but have a high allocation cost (Glawe et al. 2003). On the other hand toxins have a lower allocation cost and defend the plant against generalists but simultaneously makes the plant more vulnerable to adapted specialist herbivores. This dilemma is referred to as the *specialist- generalist dilemma* (van der Meijden 1996). For plants introduced into areas where specialist herbivores are absent, this dilemma does not longer exists. The expectation is that in the invasive area levels of expensive digestibility reducers are decreased at the expense of cheap toxins, through natural selection, yielding an energy surplus that can be diverted to growth and reproduction. This evolutionary shift of quantitative defence to qualitative defence in the invasive area is called the *Shifting Defence Hypothesis* (SDH) (Muller-Scharer et al. 2004, Joshi and Vrieling 2005). In this thesis I will focus on testing the SDH and particularly I will focus on the predicted decrease in quantitative defences in the invasive area.

Evolution or preadaptation in invasive plant species

The potency of an introduced species to evolve depends on the genetic variation introduced in the new area. About fifty years ago it was already discussed how genetic architecture might impact the possibility of a species to maintain and spread in the new area (Baker and Stebbins 1965). However it lasted another 30 years before proper experimental studies were carried out. These studies of natural populations showed that evolutionary changes can happen rapidly in invasive species (Grant and Grant 1995, Losos et al. 1997, Reznick et al. 1997). Several studies are carried out on trait differences between native and invasive individuals (Siemann and Rogers 2001, Leger and Rice 2003). Apart from some studies (Grant and Grant 1995, Losos et al. 1997) most studies, like the study by Joshi and Vrieling (2005), compared random populations from the native area with those from the invasive area. They assumed that trait differences are based on evolutionary changes without taking preadaptation into account (Bossdorf et al. 2005, Abhilasha and Joshi 2009). Preadaption assumes the introduction of a (small) subset of genotypes from the native range from one or few populations with by coincidence "preadapted" traits into the invasive range (Dlugosch and Parker 2007). Observed trait differences between native and invasive individuals are in such case not due to adaptation by natural selection acting upon new mutations but by accidental introduction of genotypes which already possessed "adaptive" traits upon introduction. The chance that multiple introductions from different source populations all contain "preadapted" individuals is very small. For this reason preadaptation is only expected to occur when there is one or very few introduction(s).

To establish whether evolution or preadaptation took place in invasive individuals, ecological traits of the source population(s) need to be compared with ecological traits of the introduced population in a common environment (Bossdorf et al. 2005, Abhilasha and Joshi 2009). Evolution has taken place if the introduced and source population differ significantly in the ecological traits of interest. Unfortunately the source population(s) is (are) seldom known.

The room for evolutionary change can be increased if multiple introductions lead to new combinations of genotypes. It has been hypothesized that multiple introductions may lead to especially problematic invaders (Ellstrand and Schierenbeck 2000, Verhoeven et al. 2010). Recent research indicates that multiple introductions are quite common in invasive species (Williams et al. 2005, Chun et al. 2010, Lachmuth et al. 2010). Admixture of genotypes from different sources can lead to novel genotypes in invasive populations compared to native populations which might result in a fast evolutionary response to selection pressure (Hufbauer 2008, Prentis et al. 2008). Furthermore admixture can mask the inbreeding load (Verhoeven et al. 2010). In this thesis I will determine the source populations of an invasive species to determine if this species evolved upon introduction or that preadapted individuals were introduced. As a model system I will use *Jacobaea vulgaris*.

Common ragwort

Common ragwort, or *Jacobaea vulgaris*, formerly known as *Senecio jacobaea* (Pelser et al. 2004), belongs to the Asteraceae. It is a native Eurasian plant species whose native range extends from southern Norway into northern Spain and from Great Britain to the Ural mountains (Harper and Wood 1957). It has been introduced to New Zealand, Australia, North America and Canada where it is a pest species

(Harper and Wood 1957). This perennial monocarpic species has four distinct life history stages: seeds, seedlings, rosettes and flowering plants. Rosettes must achieve a minimum size before they can be vernalized (Wesselingh and Klinkhamer 1996), but under ideal conditions plants behave as strict biennials (van der Meijden and van der Waals-Kooi 1979). Common ragwort produces a large number of seeds which can survive for several years in the soil (van der Meijden and van der Waals-Kooi 1979) and are dispersed by wind. It is self-incompatible (Kirk et al. 2005) and pollinated by insects, mainly bees, wasps (hymenopteran) and flies (dipteran) (Harper and Wood 1957).

In Great Britain *J. vulgaris* is attacked by more than 70 specialist as well as generalist herbivores (Harper and Wood 1957). Especially the presence of specialist herbivores like the cinnabar moth (*Tyria jacobaeae*), the fleabeetle (*Longitarsus jacobaeae*) and the ragwort seed fly (*Botanophila seneciella*) can have a negative impact on the fitness of *J. vulgaris* (McEvoy and Coombs 1999). As a defence against attackers *J. vulgaris* produces pyrrolizidine alkaloids (PAs). PAs are deterrent against generalist herbivores whereas specialist herbivores are attracted to it and it can even function as oviposition and feeding stimulants (van Dam and Vrieling 1994, Macel and Vrieling 2003). It is reported for several specialist herbivores that they sequester PAs and use these as a defence against their predators (Eisner and Eisner 1991). PAs can be lethal to many vertebrates like cattle and horses, who can not distinguish toxic ragwort from non-toxic herbs in hay (Harper and Wood 1957). The cumulative storage of PAs in the liver leads to a sudden death in apparently healthy cows and horses. These PAs can even pose a threat to humans as it can be found in milk and honey (Hoogenboom et al. 2011), (Deinzer et al. 1977). They can reduce butterfat of cow's milk and can make honey bitter and off-color (Stegelmeier et al. 1999). Besides defence, another strategy of *J. vulgaris* to cope with defoliation of herbivores is the ability of fast re-growth after defoliation. After complete defoliation of above ground parts, individuals of *J. vulgaris* still have resources stored in their roots that can be used for fast re-growth (van der Meijden et al. 1988).

Ragwort has become invasive in North America, Australia and New Zealand. Around 1850 *J. vulgaris* was first spotted outside its native range on the east coast of Canada (Harris et al. 1971), followed by New Zealand (Thomson 1922) and Australia (Schmidl 1972) around 1874, and it was noted for the first time on the west coast of the USA (Gilkey 1957) in 1901.

Ragwort is most troublesome in pastures, waste areas and along roadsides because seed survival is related to the amount of vegetative cover (Poole 1940). For example, grazed pastures on Prince Edward Island in Canada had about eight times more *J. vulgaris* plants than ungrazed pastures. Infestations can result in significant livestock losses, decreased pasture yields till 50% and increased management costs (Jacobs 2009).The annual costs of common ragwort to Australia has been estimated at four million dollar, including production losses to the dairy and beef industries and the costs of control (McLaren 1997). Because of the big negative impact in the introduced areas, biocontrol agents like the cinnabar moth (*Tyria jacobaeae*), fleabeetle (*Longitarsus jacobaeae*), a plume moth (*Platptilia isodactylis*) and the ragwort seed fly (*Botanophila seneciella*) were introduced, however with mixed results (Julien et al. 1984, McEvoy et al. 1993).

In a common garden experiment on the invasiveness of *J. vulgaris*, Joshi and Vrieling (2005) (Joshi and Vrieling 2005) examined life history traits in relation to the EICA hypothesis and the SDH. Invasive individuals had a higher growth rate and reproduction compared to native individuals. Furthermore they were better protected against generalists, but less defended against specialists, had a lower ability for re-growth and produced more pyrrolizidine alkaloids. These outcomes are fully explained by the SDH and partly in line with the EICA hypothesis. It seems that within 200 years *J. vulgaris* has adapted to the invaded area by the evolution of some fitness related life history traits. These trait differences are beneficial under the novel selection conditions and therefore evolution could have occurred (Bossdorf et al. 2005). However, as indicated before, a hypothesis alternative to these assumed evolutionary changes in life history traits, is that invading individuals were preadapted and are a selection of the genotypes present in the native range. To make a distinction between evolution and preadaptation it is crucial to determine the source population(s).

Determining source populations

Reconstructing invasion histories to find source populations is generally a difficult task, as introduction events can occur over large temporal and spatial scales. However, genetic markers can help to identify source populations and can also give insight in the level of admixture by comparing the genetic diversity of introduced and native populations (Bossdorf et al. 2005, Lavergne and Molofsky 2007, Hufbauer and Sforza 2008). Often genetic markers from neutrally evolving areas of the genome are used because these will not be affected by natural selection (Marrs et al. 2008). In several studies polymorphic markers are used to trace source populations with different outcomes. In a study of Lachmuth 2010 et al (Lachmuth et al. 2010) the genetic structure and the source population of the invader *Senecio inaequidens* was investigated. They used nuclear microsatellites and they showed that invasive populations from *S. inaequidens* originated from multiple introductions. Furthermore with the help of historical data, different invasion routes of *S. inaequidens* were clarified. In another study, which also used nuclear microsatellites, the source population of the aggressive weed *Ambrosia artemisiifolia* was traced. It turned out that French invasive populations originated also from a mixture of sources (Chun et al. 2010). Research on the introduction history of the Brazilian peppertree (*Schinus terebinthifolius*), using nuclear microsatellites and chloroplast DNA (cpDNA), identified at least two independent introductions into Florida (Williams et al. 2005). In contrast, research on the invasive plant species *Macfadyena unguis-cati*¸using chloroplast microsatellites showed that this invader most probably originated from one single introduction out of the native area (Prentis et al. 2009). I will use AFLP markers and cpDNA markers to determine the source populations of *J. vulgaris*.

Research questions

In this thesis I will test the prediction of a decrease in costly quantitative defence products of invasive *J. vulgaris* individuals as expected by the SDH. In light of possible trade offs, I will investigate if this decrease has consequences for other life history traits. To get more insight about the generality of the SDH I conducted a literature study on several invasive plant species and investigated if the outcomes are supported by the SDH.

To investigate if trait differences between native and invasive individuals of *J. vulgaris* are driven by evolution or preadaptation I searched for the source population(s) of *J. vulgaris*. To get an indication if admixture played a role in the invasive character of this species I have unravelled the genetic structure of native and invasive populations. I have focussed on the following questions.

1) Has invasion led to a reduction in costly quantitative defence products and if so, what are the consequences of other fitness related traits?
2) Is the shifting defence hypothesis a general phenomenon in invasive plant species?

3) Which is/ are the source population(s) of invasive individuals of *J. vulgaris*?
4) Is the genetic diversity and differentiation rate of *J. vulgaris* lower in invasive populations compared to native populations and did admixture occur?
5) Are trait differences between native and invasive individuals of *J. vulgaris* driven by evolution or preadaptation?

Outline of this thesis

To answer the first question, morphological and physiological traits of native and invasive individuals are measured to investigate if a resource allocation of invasive individuals takes place from expensive quantitative defence as a response to the absence of specialists into life history traits that are beneficial in the new environment **(chapter 2).** In addition in **chapter 3** the second question is answered based on a literature study. A biogeographical approach is used to compare the amount of defence products in native and invasive individuals of the same species. The remaining three questions are answered in chapter 4, 5, and 6. In **chapter 4** an AFLP study is carried out to test for genetic bottlenecks in the invasive populations and to investigate the invasion pathways of *J. vulgaris* from Europe to New Zealand, Australia and North America. In the following chapter (**chapter 5)** polymorphic markers, microsatellites and single nucleotide polymorphisms (SNPs), on the chloroplast genome are developed by sequencing the complete chloroplast genomes of several native and invasive individuals of *J. vulgaris*. The microsatellites and SNPs of the chloroplast are used in **chapter 6** to trace the source population(s) and study the genetic structure of the native and invasive populations of *J. vulgaris*. **Chapter 7** summarizes the findings presented in this thesis and presents general conclusions.

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Chapter

2

Enemies lost: Changes in anatomy and physiology of the invasive plant Jacobaea vulgaris (Asteraceae)

Leonie Doorduin*, Andries Temme *, Thijs L. Pons §, Gerda Lamers#, Niels P.R. Anten$, Klaas Vrieling*

* Institute of Biology, Section Plant Ecology and Phytochemistry, Leiden University, PO Box 9505, 2300 RA Leiden, the Netherlands

§ Plant Ecophysiology, Institute of Environmental Biology, , Utrecht University, PP Box 800.84, 3508 TB Utrecht, the Netherlands

Institute of Biology, Section Microscopy Unit, Leiden University, PO Box 9505, 2300 RA Leiden, the Netherlands

$ Ecology and Biodiversity, Institute of Environmental Biology, Section, Utrecht University, 3508 TB Utrecht, the Netherlands

Introduction

One of the benefits for invading plant species in an invaded area is a reduced impact of specialist herbivores compared to the native area (Keane and Crawley 2002). Specialized herbivores are often held responsible for a high plant biomass loss. The absence of the selection pressure of specialist herbivores in the invasive area may lead to evolutionary changes in morphological, physiological and growth patterns of invasive plants (Feng et al. 2009).

The Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey and Notzold 1995), states that the release from specialist herbivores allows for an evolutionary change of invasive plants in energy allocation from defence to growth. Defence is often divided in two types of defences, quantitative defences (digestibility reducers) which are costly to produce and qualitative defences (toxins) which are cheaper to produce. Quantitative defences act against specialist as well as generalist herbivores. Qualitative defences act against generalist herbivores but specialist herbivores are often adapted to these defences and even can use these compounds as a cue to locate their host plant, and act as an oviposition and feeding stimulant (Bernays et al. 2003, Macel and Vrieling 2003). Qualitative defences have lower allocation costs and defend the plant against generalists, but simultaneously make the plant more vulnerable to adapted specialist herbivores. This dilemma is referred to as the specialist- generalist dilemma (van der Meijden 1996). In the invasive area, where specialists are absent, it is the best strategy for a plant to increase its cheap qualitative defence against generalist herbivores without having the side effect of attracting specialist herbivores and decreasing their quantitative defences. As a result of the changed allocation patterns to the different defences a net gain is achieved, by exchanging costly quantitative defences for cheap qualitative defences that can be allocated to growth (Doorduin and Vrieling 2011). This evolutionary shift of quantitative defence to qualitative defence in the invasive area is called the Shifting Defence Hypothesis (SDH) (Muller-Scharer et al. 2004, Joshi and Vrieling 2005). In a common environment without herbivores several invasive plant species indeed showed a more vigorous growth (Blair and Wolfe 2004, Lewis et al. 2006, Ridenour et al. 2008) and a higher level of qualitative defences (Joshi and Vrieling 2005, Lewis et al. 2006, Cano et al. 2009) compared to individuals in the native area.

Feng et al (2009) explained the vigorous growth of invaded plants as a consequence of an enhanced investment in photosynthesis at the cost of a reduced cell wall content both in mass and nitrogen allocation (Feng et al. 2009). Nitrogen (N) is one of the most important limiting resources for plant growth (Niinemets 2007) and most leaf N is allocated to chloroplasts, needed for photosynthesis (Evans 1989, Pons and Anten 2004). However, the primary plant cell wall consists for 5-10% of proteins (Loomis 1997) and therefore cell walls can be considered as an important N sink as well. An increased N allocation to cell walls is related to better defence against herbivores (Showalter 1993). In the absence of herbivores allocation of N may therefore shift from cell walls to photosynthesis. Feng et al. (2009) indeed found that *Ageratina adenophora* shrubs from the invasive area allocated 40-50% less proteins to cell walls and 13% more nitrogen to photosynthesis compared to native plants (Feng et al. 2009). The nitrogen Use Efficiency (PNUE) was also 20% higher, indicating more efficient use of nitrogen in photosynthesis. This N reallocation was coupled with a decrease in leaf mass per area (LMA) indicating poorer structural defences. In the native area leaf toughness can be beneficial because it possibly reduces palatability of *A. adenophora* to specialist herbivores whereas in the invasive area these specialists are not present. This selection for increased photosynthesis, albeit at the expense of defence, allows for a higher reproductive output in invasive populations. This allocation change is beneficial in the light of competition and dispersal and may have contributed to the invasive character of *A. adenophora*. Besides defence, another strategy to cope with herbivory by specialists is tolerance. This is the innate capacity of plants to reduce fitness loss in spite of tissue losses (van der Meijden et al. 1988). Fitness loss is often reduced by the ability of plants to regrow fast after defoliation (Rosenthal and Kotanen 1994).

We did a study on *Jacobaea vulgaris* to investigate if the invasive individuals have an increased photosynthetic capacity and a decreased allocation to quantitative structural defences as shown in *A. adenophora*. Furthermore we investigated whether in the invasive area, in the absence of specialists, regrowth capacity is lower in invasive *J. vulgaris* individuals compared to native individuals.

Jacobaea vulgaris (Asteraceae, syn. *Senecio jacobaea*) or common ragwort is a plant native to Europe and invasive in parts of Australia, New Zealand and North America. It contains pyrrolizidine alkaloids (PAs) which are toxic and can be lethal to cattle (Johnson 1978, Stegelmeier et al. 1999). In the invasive areas *J. vulgaris* is considered a weedy species because of its wide spread and distribution. Furthermore it received a pest status because infestations have resulted in significant livestock losses due to alkaloid poisoning and decreased pasture yields (Jakobs et al. 2004). Because of its weedy character, research is being devoted to discover how *J. vulgaris* has evolved into a pest species in the invasive areas (Willis et al. 2000, Joshi and Vrieling 2005, Stastny et al. 2005). A major difference in the invasive communities compared to the native community is the absence of specialist herbivores on *J. vulgaris*. The Cinnabar moth *Tyria jacobaeae* and the flea beetle *Longitarsus jacobaeae* are both specialists and absent in the invaded area, though they have been introduced between 30-40 years ago in some areas of North America, Canada and Australia to act as biological control (James et al. 1992, McEvoy et al. 1993, Ireson et al. 2000). In a common garden experiment Joshi and Vrieling showed that invasive individuals of *J. vulgaris* had more vegetative growth and had reached a 37% higher inflorescences dry weight compared to native individuals (Joshi and Vrieling 2005). Moreover, as predicted by the shifting defence hypothesis, plants from invasive *J. vulgaris* populations produced on average 90% more PAs (a qualitative defence) than plants from native areas (Joshi and Vrieling 2005) which was shown to result in a better defence against generalist herbivores. The leaf mass area (LMA) which is the inverted value of SLA, is often used as indicator of structural biomass (Reich et al. 1991) and is considered as an estimator of quantitative defences. Although we expect that native plants have thicker leaves and therefore a higher LMA, in the study of Joshi and Vrieling native and invasive individuals of *J. vulgaris* did not differ. The same holds true for leaf nitrogen content that was similar for invasive and native populations. Interestingly, regrowth ability of invasive individuals was decreased compared to that of native individuals by 12% (Joshi and Vrieling 2005).

The aim of this study is to investigate whether invasive *J. vulgaris* individuals increased their growth due to an increase of photosynthesis per leaf area (P_{max}), more efficient use of nitrogen in fotosynthesis (PNUE) and a decreased allocation to quantitative structural defences. Besides LMA we also investigated leaf structure, the amount of cell wall material and leaf toughness as estimation for structural biomass. As an indicator of regrowth capacity we measured the root to shoot ratio because fast regrowth is positively correlated with the root/shoot ratio (van der Meijden et al. 1988, van der Meijden et al. 1988, Iwasa and Kubo 1997).

Methods

Study species

J. vulgaris is a self-incompatible monocarpic perennial plant (Harper and Wood 1957, Kirk et al. 2005). It is a serious pest in Australia, New Zealand, the United States and Canada. *J. vulgaris* was first spotted outside its native distribution in the 1850s on the east coast of Canada (Bain 1991), around 1874 in New Zealand (Thomson 1922) and Australia (McLaren 1997) and in 1901 on the west coast of the USA (Gilkey 1957).

Plant material and growth conditions

Seeds were collected from 19 native populations in Europe and from 20 invasive populations in New Zealand, Australia and the USA (Table 1). Seeds were germinated in a petri dish with moistened filter paper and per population 5 seedlings each of a different maternal line were potted in 0.5 L pots with 5% potting soil, 95% sand mixture and 0.75g osmocote® (N:P:K:MgO 15:9:11:2.5). Plants were grown in a climate room for 17 weeks at 20°C, 70% humidity (day and night), 16 hours daylight with a light intensity of 113 umol PAR $m^{-2}s^{-1}$. They were watered when needed. After 10 weeks, 50 mL Pokon solution NPK 7-5-6 (8 mL/L) and Fe-EDTA of 3.2 g/L was given to the plants twice a week. Per population two plants were randomly picked to use in the analysis of photosynthesis and cell wall measurements (after 12 weeks), microscopy (after 14 weeks) and for toughness measurements (after 16 weeks). After 17 weeks all plants were harvested. For details of measurements see Table 1.

Physiology

Photosynthesis and chlorophyll content

Twelve weeks after planting the light saturated rate of photosynthesis per unit leaf area (Pmax), respiration (R), stomatal conductance ($g_{st)}$ and intercellular CO_2 concentration (C_i) were measured on the middlemost leaf of a plant using a LICOR 6400 at atmospheric CO_2 (ca. 380 µmol/mol in the leaf chamber), growth temperature and 1250 (µmol/s) PAR. After two minutes of incubation in the light, each leaf was measured and three minutes after switching the light off, dark respiration was measured. Gas exchange rates were corrected for dark respiration under the leaf chamber gasket according to Pons and Welchen (2002) (Pons and Welschen 2002). Pmax was also calculated for total shoot. First Pmax was calculated for dry shoot by Pmax (µmol m^{-2} s^{-1}) * % DM of the shoot (1). Then the surface of the dry shoot was calculated by dry mass shoot (g)/ leaf mass area (g m^2) (2). Finally, total µmol photosynthesis in the shoot was calculated by (1) * (2). The measured leaf sections (2x3 cm) were cut out and two 1 cm diameter leaf punches were removed (Fig. 1). After weighing, leaf sections, punches and leaf remainder were frozen in liquid nitrogen. The remainder of the dissected leaf part was used for analysis of C and N concentration in dry matter using an elemental analyser (Carlo Erba, Milan, Italy). Photosynthetic Nitrogen Use Efficiency (PNUE) was calculated by dividing Pmax (µmol CO2 m^{-2} s^{-1}) by N per leaf area, N_{LA} (mmol/m^2). Chlorophyll content was determined on the two punches using DMF (N,N-Dimethylformamide) according to Porra *et al.* (1989) (Porra et al. 1989). Total chlorophyll content in the dry shoot (mg) was calculated as: chlorophyll mg/g fresh * % DM in shoot.* shoot dry mass (g).

Leaf anatomy

Microscopy

Fourteen weeks after planting, coupes were cut from the middlemost leaf at the tip of the leaf (Fig. 1) using a hand microtome. Coupes were then stained using Propidium Iodide for 15 minutes. Propidium iodide stains DNA as well as cell wall material. Images were then scanned using a Zeiss Observer with a LSM 5 exciter scanhead confocal microscope at 545 nm. A full cross section of the leaf was obtained by tile scanning the specimen with a 40 x 1.2 NA Plan APO water immersion objective. This gave an image size of 321.43 µm x 482.14 µm with a resolution of 80 nm. per pixel. Measurements were made using ImageJ® 1.42q. Each measurement was made 5 times on different parts of the cross-section as outlined in Figure 2.

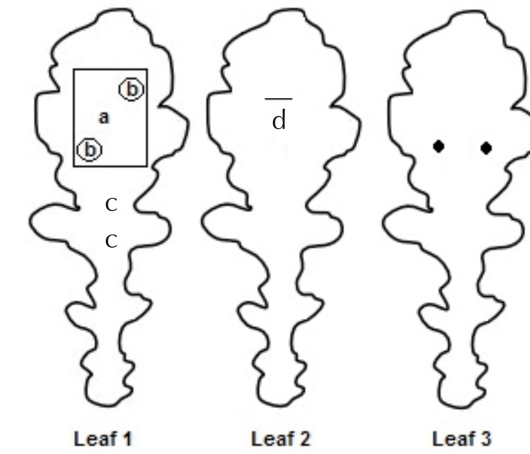

Fig.1 Parts of the leaf measured. **Leaf 1;** a: area measured for photosynthesis and for nitrogen analysis. b: Punches for chlorophyll determination. c: Punches for cell wall material analysis. **Leaf 2**; d: coupe location microscopy. **Leaf 3**; e: Toughness measurements.

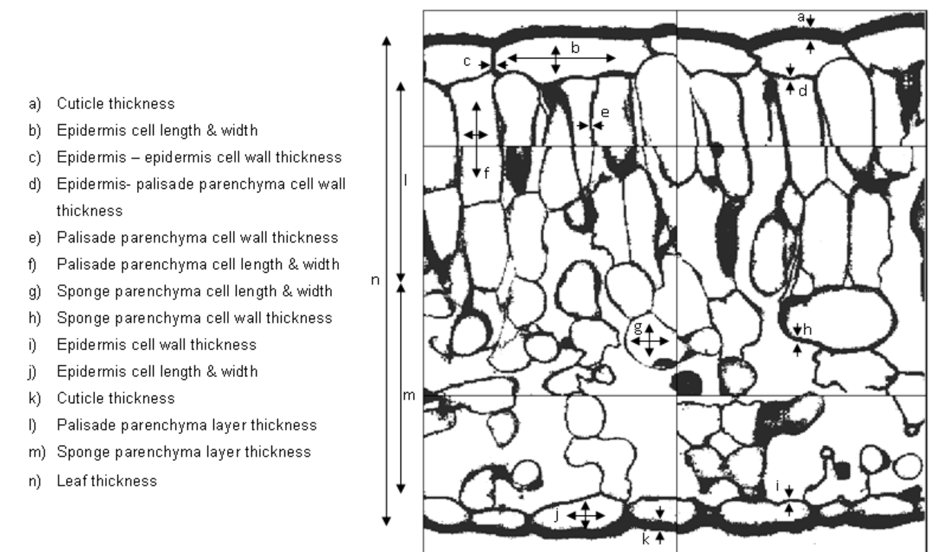

Fig 2. Measurements made on leaf cross section. Arrows indicate measurements made. Length is defined as top to bottom and width as side to side. This sample cross section is a cropped version of a coupe from Landsborough/ Haast New Zealand.

Toughness

After sixteen weeks of growth the then middlemost leaf was removed and used in toughness measurements (Fig. 1). Toughness was measured by using a punch and die method on an Instron 4000 after Onoda *et al.* (2008) (Onoda et al. 2008). A flat ended sharp-edged cylindrical steel punch and a steel die with a sharp-edged hole were used. The punch and die were installed into the machine. When the punch started to compress the leaf, a sharp increase in force was observed. Maximum force (N) was recorded just before the leaf fractured. Work (μ Joule) was recorded during the whole process and the total work to penetrate the leaf was calculated. Each leaf was measured twice and for analysis the average of two measurements was taken. Maximum force and work to puncture the leaf were calculated from a force-displacement curve (Aranwela et al. 1999).

Cell walls

Two one cm diameter punches were extracted from the same leaf used for the photosynthesis analysis (material after twelve weeks of growth). Cell wall material was extracted using the protein extraction protocol of Takashima *et al* (2004) (Takashima et al. 2004). Water soluble material and SDS soluble material was removed. The remaining cell wall material was oven dried at 60°C for 18 hours and weighed. Cell wall material extraction was replicated thrice on the same leaf. For statistical analysis, the average of three measurements was taken.

Growth

After 17 weeks all plants were harvested. Fresh mass of roots and shoots were determined. After oven drying at 60°C for a minimum of 48 hours, dry mass of shoots and roots was determined. From leaves that were used in physiological and morphological measurements, fresh and dry mass was determined and added to the total plant mass.

LMA

After 17 weeks, on the same day of total harvest, the fifth leaf was used to determine the area by using a portable area meter. After oven drying at 60°C for 48 hours the dry mass of the leaf was determined and used to define the leaf mass area (dry mass leaf (g)/ surface of leaf (m^2)).

Data analysis

As the main interest of this study was to find differences in invasive versus native areas, statistical analysis was performed by a nested ANOVA. When covariates were taken into account a nested ANCOVA was used. Individual plants were nested within population of origin which was nested within either the invasive or native area. Averages of invasive or native areas are based on estimated marginal means from the analysis. Normality of the residuals was checked with a Kolmogorov-Smirnov test and equality of the variances with a Levene's test. When variances were found to be significantly different, a Mann-Whitney U test was performed. The significance of the correlations between variables was tested using a Pearson correlation. All analyses were carried out using SPSS 18.0 (SPSS: An IBM Company).

Table 1. Origin of populations used in this study and number of plants measured. Growth (harvest): measurement of fresh mass, dry mass and LMA. (17 weeks) Microsc.:measurement of cell wall parameters (14 weeks). Photosyn.: photosynthetic measurements, Nitrogen and Carbon content (12 weeks). Chl.: measurement of chlorophyll content (12 weeks). Tough.:measurement of leaf toughness (16 weeks). CW: measurement of cell wall weight (12 weeks).

				Number of plants used in measurements					
Range	***Country***	***Location***	***Coordinates***	***Growth***	***Microsc.***	***Photosyn.***	***Chl.***	***Tough.***	***CW***
Invasive	*Australia*	Barramunga	*Lat38.33 Lon143.41*	4	2	2	2	2	1
		Beech forest	*Lat38.38 Lon143.33*	4	2	2	2	3	1
		Dairy Plains	*Lat41.34 Lon146.31*	4	1	2	1	1	1
		Franklin	*Lat43.05 Lon147.00*	5	2	2	2	2	1
		Mayberry	*Lat41.33 Lon146.18*	4	2	2	2	2	1
		Turton's Creek	*Lat38.33 Lon146.15*	5	2	3	2	1	1
	New Zealand	Craigieburn/Grey valley	*Lat39.25 Lon174.13*	4	2	1		2	1
		Landsborough/Haast	*Lat43.53 Lon169.02*	5	2	1	1	2	
		Maruia	*Lat42.11 Lon172.13*	2	2	2	2	2	1
		Opunake/Taranaki	*Lat39.25 Lon174.13*	5	2	2	2	2	1
		Soutland/New Zealand	*Lat45.28 Lon167.55*	1	1	1	1	1	1
		Whatipu	*Lat37.01 Lon174.31*	4	2	2	2	2	1
	USA	Basket Slough, Oregon	*Lat44.58 Lon123.19*	5	2	2	2	2	1
		C. spur/Montana	*Lat47.48 Lon111.35*	5	2	2	2	2	1
		Del Norte Clifornia	*Lat41.42 Lon123.57*	4	2	2	2	2	1
		Kootenai National Park, Montana	*Lat48.17 Lon114.53*	4	2	2	2	2	1
		No Bear/Oregon	*Lat43.00 Lon120.00*	5	2	2	2	2	1
		S. Cooper/Oregon	*Lat45.40 Lon122.50*	4	2	3	3	2	1
		Salem, Oregon	*Lat44.56 Lon123.02*	4	2	3	3	2	1
		Surprise Hill/Montana	*Lat48.15 Lon115.00*	4	1	2	1	1	1
Native	*Belgium*	Brussels	*Lat50.51 Lon04.25*	5	2	2	1	2	1
		Spa	*Lat50.29 Lon05.50*	4	2	2	2	2	1
	Denmark	Sundstrup	*Lat56.37 Lon18.30*	5	2	3	3	2	1
	England	Deal	*Lat51.13 Lon01.24*	4	2	1	2	2	1
	Finland	Kirkkonummi	*Lat26.15 Lon24.53*	5	1	2	2	1	1
	France	Mt. St. Michel	*Lat48.37 Lon01.32*	4	2	2	2	2	1
	Germany	Holzlarchen	*Lat47.53 Lon11.43*	4	2	1		2	1
		near Lubeck	*Lat54.05 Lon10.42*	3	2	2	2	2	1
	Hungary	Csokvaomany	*Lat48.10 Lon20.22*	4	2	1	1	2	1
	Ireland	near Caherdaniel	*Lat53.07 Lon8.02*	4	2	2	2	2	1
	Netherlands	Veluwe	*Lat52.19 Lon06.00*	5	2	2	2	2	1
		Wageningen	*Lat52.01 Lon05.34*	4	2	2	2	2	1
	Norway	Sor Trondelag/Malvik	*Lat60.33 Lon7.53*	5	2	2	2	2	1
	Poland	Near Warsaw	*Lat51.52 Lon19.25*	5	2	2	2	2	1
	Scotland	Dundee	*Lat56.29 Lon03.02*	2	2	1	1	2	1
	Spain	Porto de San Glorio	*Lat40.01 Lon3.37*	5	2	2	2	2	1
	Sweden	Lund	*Lat55.43 Lon13.13*	5	2	2	1	2	1
	Swiss	l'Himelette	*Lat47.07 Lon07.00*	5	2	2	2	2	1
		Rothenthurm	*Lat47.06 Lon08.040*	1	1	1	1	1	1

Results

Physiology

Maximum photosynthesis per leaf area (P_{max}) was 11.4 % lower for invasive *J. vulgaris* plants compared to native ones (Table 2). However Pmax of native and invasive individuals did not differ, if Pmax was calculated for the whole shoot (Table 2). Furthermore no significant difference was found in respiration, stomatal conductance and CO2 concentration in the intercellular spaces (Ci).

Leaf nitrogen content per area (N_{LA}) did not differ between native and invasive individuals of *J. vulgaris*. However the amount of nitrogen calculated as mmol/g dry weight (N_W) was higher in native individuals whereas invasive individuals contained significantly more carbon (Table 2). As a consequence the N:C ratio was significantly higher in native individuals. When the total amounts of N and C were calculated for the total shoot, invasive individuals contained significantly more carbon and also more nitrogen. The latter because they had larger shoots (Table 2). The PNUE, the Photosynthetic Nitrogen Use Efficiency, was not significantly different between *J. vulgaris* plants of both areas.

J. vulgaris from the invasive area contained on average 13.3 % less chlorophyll (mg g $freshmass^{-1}$) compared to individuals from the native area (Table 2). Nevertheless, the total amount of chlorophyll (mg) in the total shoot was higher in invasive individuals due to the their larger shoots (Table 2).

Both nitrogen (N_{LA}) and chlorophyll were correlated with Pmax. If nitrogen and chlorophyll were used as a covariate Pmax did not significantly differ between native and invasive individuals ($F_{1,34}$= 1,203, NS, for both covariates: $p<0.05$)

Leaf anatomy

Microscopy

Of the nineteen measurements of *J. vulgaris* leaf cross sections (see Figure 2) made on 73 individuals, only the lower epidermis cell wall was significantly thicker in invasive individuals compared to native individuals (Table 2).

Toughness

By using a punch and die method, maximum force and work required to puncture a leaf was measured. For invasive plants significantly more work was needed to puncture a leaf compared to native plants (Table 2). The LMA (leaf dry mass (g)/ leaf area (cm^2)) as indicator of the amount of leaf dry matter per area did not differ. However, the thickness of the lower epidermis was positively correlated with work to puncture a leaf (Pearson correlation r= 0,415, p=0.001) (Fig. 3).

Cell walls

The amount of cell wall material was not significantly different between *J. vulgaris* plants from the invasive and native area (Table 2). Leaf nitrogen (N_{LA}) content and leaf cell wall material were not significantly correlated, for both native and invasive *J. vulgaris* plants.

Table 2: Results of nested ANOVA's for physiological, morphological and mass measurements on native and invasive *J. vulgaris* plants grown under standardized conditions in a climate room. Mass refers to dry mass unless otherwise indicated. Df indicates the degrees of freedom of the ANOVA among the native and invasive area. F indicates F ratio, Z indicates the Z value of a Mann-Whitney(MW) test when the requirements of an ANOVA were not met. p indicates the significance level of the ANOVA. ns= not significant. P_{max} = light saturated rate of photosynthesis per unit leaf area, NLA= leaf nitrogen content, PNUE= photosynthetic nitrogen use efficiency %DM= percentage dry mass.

Physiology and chemistry

ANOVA	Native	Invasive	Df	F/Z	p
Pmax (µmol m^{-2} s^{-1})	15,8	14	1/36	7,674	<0.01
Pmax dry shoot (µmol g)	0,196	0,24	1/37	3,094	ns.
Respiration (µmol m^{-2} s^{-1})	1,18	1,35	1/ 37	0,559	ns.
Intercelulair CO2 (µmol mol^{-1})	248	270	1/37	0,274	ns.
Stomatal conductance (mol m^{-2} s^{-1})	0,261	0,281	1/37	0,937	ns.
NLA (mmol m^{-2})	0,102	0,091	1/36	0,197	n.s.
Leaf Nitrogen (mmol g^{-1}) (Nw)	3,05	2,56	1/36	13	**<0.01**
Leaf Nitrogen dry shoot (mmol)	17,29	19,87	1/37	4,939	**<0.05**
Leaf Carbon (mmol g^{-1}) (C)	33,6	34,2	1/36	5,605	**<0.05**
Leaf Carbon dry shoot (mmol)	207	269	1/37	12,293	**<0.01**
N/C Ratio	0,092	0,075	1/36	21,511	**<0.001**
PNUE (µmol CO2 mol N^{-1} s^{-1})	154,9	153,8	1/36	0,866	ns.
Chlorophyll a+b (mg g $freshmass^{-1}$)	1,65	1,43	1/33	10,665	**<0.01**
Chlorophyll a+b dry shoot (mg)	1,04	1,27	1/35	4,679	**<0.05**
Chlorophyll a/chlorphyl b	3,07	3,13	1/33	3,622	ns.
Leaf anatomy					
Microscopy (all measurements in µm)					
Upper cuticle thickness	3,4	3,42	1/37	0,607	ns.
Upper epidermis cell length	25,2	25,7	1/37	0,24	ns.
Upper epidermis cell width	54,2	49,5	1/37	1,144	ns.
Epidermis-epidermis cell wall thickness	1,44	1,43		-0,001	ns.[MW]
Epidermis-palisade parenchyma cell wall thickness	1,13	1,11	1/37	0,086	ns.
Palisade parenchyma cell length	71,3	70,1	1/37	0,113	ns.
Palisade parenchyma cell width	32,2	30,3	1/37	2,204	ns.
Palisade parenchyma cell wall thickness	0,87	0,93		-1,072	ns.[MW]
Sponge parenchyma cell length	27,4	27,6	1/37	0,002	ns.
Sponge parenchyma cell width	37,7	38,4	1/37	0,017	ns.
Sponge parenchyma cell wall thickness	0,89	0,89	1/37	0,005	ns.
Lower epidermis cell length	16,7	17,2	1/37	0,029	ns.
Lower epidermis cell width	30,8	32,3	1/37	0,386	ns.
Lower epidermis cell wall thickness	1,03	1,12	1/37	4,607	**<0.05**
Lower cuticle thickness	2,18	2,26		-0,451	ns.[MW]
Leaf thickness	277	268	1/37	0,682	ns.
Palisade parenchyma layer thickness (Pal)	117	112	1/37	0,898	ns.
Sponge parenchyma layer thickness (Spo)	122	121	1/37	0,06	ns.
Pal/Spo	1,00	0,95	1/37	0,357	ns.
Toughness					
Maximum Force (N)	0,90	0,90	1/37	0,001	ns.
Work (µjoule)	297	330	1/37	4,163	**<0.05**
Cell walls					
Cell wall material (g • m^{-2})	39,2	37,2	1/37	0,348	n.s.
Cell wall material (g • g^{-1})	0,29	0,28	1/37	0,536	n.s.
Growth					
Plant (g)	11,7	12,2	1/37	0,698	ns.
Plant %DM (g • g^{-1})	13,20%	12,70%	1/37	2,216	ns.
Shoot (g)	5,00	5,68	1/37	5,164	**<0.05**
Shoot %DM (g •g^{-1})	11,0%	11,3%	1/37	0,533	ns.
Root (g)	5,61	5,23	1/37	0,177	ns.
Root % DM (g •g^{-1})	17,20%	15,50%	1/37	7,092	**<0.01**
Ratio Root/Shoot	0,92	0,75	1/37	5,001	**<0.05**
LMA of the 5th leaf (g·m^{-2})	57,5	55,8	1/37	0,024	n.s.

Growth

Invasive *J. vulgaris* plants had a 12 % higher shoot dry mass than native plants (Table 2). No difference was found in root dry mass. As a result,invasive individuals had an 18.1 % lower root to shoot ratio compared to native individuals (Table 2). With an increase in plant mass, plants showed an increase in root to shoot ratio (r= 0,472 p=0.01) (Fig. 4). Shoot mass was negatively correlated with N_w (Pearson correlation r= -0,471 p< 0.01) (Fig. 5).

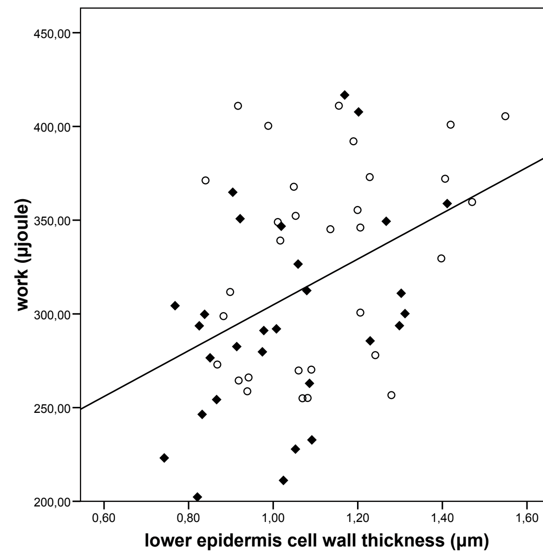

Fig. 3 Thickness of the lower epidermis cell wall measured in μm plotted against the work to puncture a leaf measured in μm. Native individuals are indicated with closed diamonds and invasive individuals are indicated with open circles.

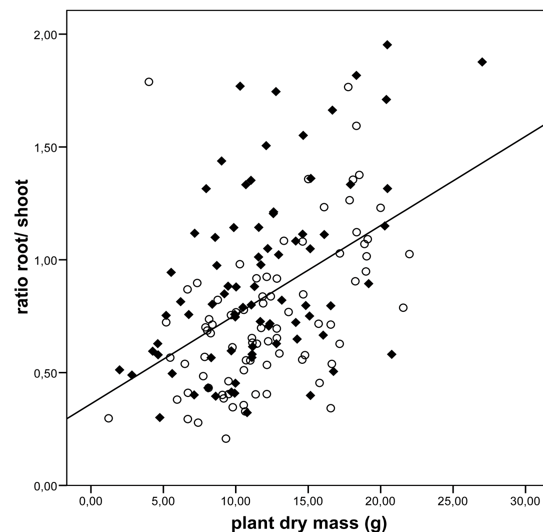

Fig. 4 Plant dry mass (g) plotted against the root to shoot ratio. Native individuals are indicated with closed diamonds and invasive individuals are indicated with open circles.

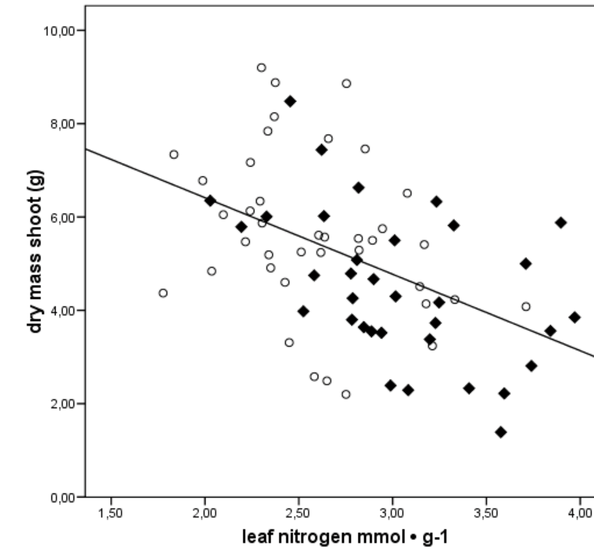

Fig. 5 Leaf nitrogen in mmol per gram dry mass plotted against dry mass of the shoot. Native individuals are indicated with closed diamonds and invasive individuals are indicated with open circles.

Discussion

In the study of Feng et al. (2009) invasive individuals of *A. adenophora* showed a change in nitrogen allocation from structural defences in cell walls into photosynthesis (Feng et al. 2009). According to this study and also to the EICA hypothesis we expected to find an increased maximum photosynthesis and higher photosynthetic efficiency and a decrease in leaf mass area (LMA), cell walls and regrowth capacity in invasive plants compared to native plants. In contrast we found that the maximum photosynthetic rate (Pmax) and chorophyll content per leaf area were significantly lower for invasive *J. vulgaris* plants. As expected, Pmax was influenced by the amount of chlorophyll and nitrogen (N_{LA}) (Jia and Gray 2004). PNUE did not differ between plants from the native and invasive area. Also, Pmax per total shoot did not differ between native and invasive individuals. Chlorophyll content for the total shoot is significantly higher in invasive individuals. Like chlorophyll, gram nitrogen in the total shoot was also significantly higher in invasive individuals. So, invasive plants accumulated more chlorophyll and leaf nitrogen although Pmax for the total shoot did not differ from native individuals. These data show that initially plant growth of invasive individuals was higher than that of native individuals. During the experiment nutrients in the pots became more limiting for the invasive plants due to a higher growth rate and as a result N_{LA} declined although the total amount of nitrogen in the shoots of invasive plants was still significantly higher due to the larger shoot (Fig. 5).

The shoot to root ratio was also influenced by leaf nitrogen. However, an ANCOVA with N_W as a covariate still showed a significantly higher shoot to root for invasive individuals. Although invasive plants

had a significantly higher shoot mass than native *J. vulgaris* plants, plant mass did not differ between native and invasive individuals. Invasive individuals had a slightly lower root mass albeit not significantly different. If nutrients become limiting plants alter there root to shoot ratio in the direction of larger roots. Invasive plants grew faster and therefore experienced nutrient limitations earlier than native plants. Larger plants started to adjust there root to shoot ratio to decreasing nutrient availability (Fig. 4) but still invasive plants maintained a lower root to shoot ratio than native plants. The data show that PNUE and Pmax of total shoot do not differ between native and invasive individuals as it differed in *A. adenophora* (Feng et al. 2009) but that a higher growth rate is obtained by a lower root to shoot ratio of invasive individuals. Regular complete defoliation by the cinnabar moth might select for higher root to shoot ratio in native individuals to be able to quickly regrow after defoliation (Van der Meijden et al. 1988).

Native individuals of *J. vulgaris* allocated more biomass to the roots compared to invasive plants, resulting in a 23 percent higher root to shoot ratio for native individuals compared to invasive individuals.

Only the lower epidermis cell wall showed a significant difference, with thicker cell walls for invasive individuals. Furthermore measurements on maximum work to penetrate a leaf showed also a higher value for invasive individuals of *J. vulgaris.* Although we did not find a significant correlation between leaf thickness and maximum work, we did find a significant positive correlation between thickness of the lower epidermis alone and maximum work (Fig. 3).

The lower epidermis may play a role to retain leaves moisture in dry climates. However following the climate classification of Köppen-Geiger individuals from the native and invasive areas belong in general to the same classification, namely a temperate climate without a dry season and with a warm summer (Peel et al. 2007).

LMA, which is an indication for the amount of leaf dry matter per area, did not differ between native and invasive plants. This is in line with the finding that the amount of cell wall material in a leaf did not differ between native and invasive plants of *J. vulgaris.*

In this study we did not find evidence for higher photosynthetic rates, leaf anatomy and allocation to cell walls. However an alternative strategy of native individuals to cope with herbivory is a high regrowth capacity. The amount of roots, which were found to be positively correlated with fast regrowth capacity (van der Meijden et al. 1988) was indeed higher for native individuals. Joshi and Vrieling (2005) already showed that plants from invasive areas had a 12% reduction in regrowth capacity (Joshi and Vrieling 2005). This is in line with a high herbivore pressure of specialists in the native area compared to the absence of specialist herbivores in the invasive area.

As *J. vulgaris* is not predated by specialised herbivores in its invasive range, regrowth capacity is not necessary to maintain thus mass can be re-allocated from roots to shoots that would otherwise stand a high risk of being eaten. Bigger shoots are known to be favourable in an environment with high levels of competition (Burns 2006).

These findings differ from the study of Feng et al 2009, where invasive success of *Ageratina adenophora* was ascribed to the allocation of leaf nitrogen (g/m2) from defence (cell walls) into photosynthesis (Feng et al. 2009). Although in both cases a release from herbivory seems to be an important factor for invasive success, reaction to this ecological change differs between both plant species.

In conclusion, in the native range of *J. vulgaris* there is heavy herbivory by specialist herbivores and plants that are introduced in a new area are released from this burden. Specialist herbivores in the native area have broken through the main lines of defence, so plants have to find other ways to cope with the damage done. For *J. vulgaris* this is not achieved by reducing palatability through costly investment in structural defence, but to invest in re-growth capacity which is detrimental to the fast growth rate. In the invasive range it is no longer necessary to maintain this potential and mass can be allocated into fast growth of shoots.

Acknowledgements

We are very thankful to T. Morley, J. Ireson, U. Schaffner, V. Vanparys, N. Agenbirk, S. Luijten, D. Ober, A. Balogh, M. Bartelheimer, L. Joosten, N. Sletvold- Hommelvik, P. Olejnicrak and S. Andersson for collecting seeds.

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A review of the phytochemical support for the shifting defence hypothesis

Leonie J. Doorduin* & Klaas Vrieling

PHYTOCHEMISTRY REVIEWS
Volume: 10 Issue: 1 Special Issue: SI Pages: 99-106
DOI: 10.1007/s11101-010-9195-8
Published: MAR 2011

Ecology and Phytochemistry
Institute of Biology
Leiden University
PO Box 9505
2300 RA Leiden
The Netherlands

Tel +31(071) 5275136
Fax +31(071) 5274900

* Corresponding author
Email: L.J.Doorduin@Biology.leidenuniv.nl

Abstract

Several theories have been developed to explain why invasive species are very successful and develop into pest species in their new area. The shifting defence hypothesis (SDH) argues that invasive plant species quickly evolve towards new defence levels in the invaded area because they lack their specialist herbivores but are still under attack by local (new) generalist herbivores. The SDH predicts that plants should increase their cheap, toxic defence compounds and lower their expensive digestibility reducing compounds. As a net result resources are saved that can be allocated to growth and reproduction giving these plants a competitive edge over the local plant species. We conducted a literature study to test whether general toxic defence compounds in the invaded area are increased and that digestibility reducing compounds are lowered. We specifically studied the levels of pyrrolizidine alkaloids, a toxin which is known for its beneficial and detrimental impact against specialists and generalists respectively. Digestibility reducers did not show a clear trend which might be due to the small number of studies and traits measured. The meta analysis showed that toxic compounds in general and pyrrolizidine alkaloid levels specifically, increased significantly in the invaded area, supporting the predictions of the SDH that a fast evolution takes place in the allocation towards defence.

Keywords: defence, EICA, invasion, PAs, SDH

Introduction

With an increase in human travel intensity over the past 300 years, many species have been introduced into new areas (Long 2003). The introduction of these species has often gone unnoticed, and many of these species have probably not survived. The species that do survive in their new habitats often have a marginal existence. However, a small number of species thrive. For example 21% of the North American flora consists of exotic species (Rejmanek 2000) but only 2% of these have developed into pests. These pest species can have economic consequences as well as severe impacts on the biodiversity and ecological networks in their new ranges. For instance, the introduction of goats on islands quickly led to deforestation of these islands (Long 2003), the introduction of the cane toad in Australia has been detrimental to local fauna (Easteal 1981), and the introduction of ragwort into Australia, New Zealand and North America has led to livestock poisoning (Craig et al. 1986, Coombs et al. 2004). In addition to causing economic and ecological losses, exotic species also offer opportunities to test ecological theories because biological introductions function as unplanned transplant experiments (Joshi and Vrieling 2005).

Here we will restrict our discussion to introduced plant species that have become successful enough to be designated as invasive pests. A number of theories have been proposed to explain the success of such plant species in their exotic ranges. We will focus on theories that are centered on plant release from natural enemies, and these theories directly or indirectly incorporate hypotheses regarding the chemical defences of invasive plants. The main theories that have been proposed are the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey and Nötzold 1995) and the Shifting Defence Hypothesis (SDH; Müller-Schärer et al. 2004, Joshi and Vrieling 2005). The EICA hypothesis is the evolutionary extension of the Enemy Release Hypothesis (ERH) (Keane and Crawley 2002). The ERH states that when plants are introduced into a new area, they leave their specialist herbivores behind and are therefore freed from detrimental herbivore pressure by these specialist herbivores. It is predicted that herbivory from local generalist herbivores is limited because newly introduced plants contain unknown, and therefore potent chemical defences to which local herbivores are not adapted (unless native relatives of the introduced plant species are present; Connor et al. 1980). This theory about chemical novelties is known as the novel weapons theory (Callaway and Ridenour 2004). Both the ERH and novel weapons theory do not predict per se a change in the chemistry of introduced plants in their exotic ranges. However, the EICA hypothesis predicts that an absence of specialist herbivores will cause plant defences against specialists to decline in exotic species over evolutionary time. The EICA hypothesis assumes that secondary metabolites act as chemical defences against specialists herbivores. It is known that many species vary genetically in composition and concentration of their secondary metabolites (Vrieling et al. 1993, Van Dam and Vrieling 1994, Arany et al. 2009). In the absence of specialist herbivores in the invasive area, selection favours plants that have lower concentrations of such compounds because these compounds are costly to produce; selection thus results in a decline in secondary metabolite concentrations over a number of generations (see Vrieling and van Wijk 1994 and review by Koricheva 2002). When plants reduce their investment in defence, they can allocate the freed resources to growth and reproduction, giving them a competitive edge over local plants. Many studies show that pest species show increased growth or reproduction compared to native individuals. The EICA therefore predicts an evolutionary change such that levels of chemical defence compounds are decreased in individuals in the invaded area compared to the individuals in the native area (Blossey and Nötzold 1995).

The SDH is a further extension of the EICA hypothesis. The SDH differentiates between defences based on their effectiveness against specialist and generalist herbivores, and couples this to types of defences (Müller-Schärer et al. 2004, Joshi and Vrieling 2005). Feeny (1976) and Rhoades and Cates (1976) developed the Apparency theory, which distinguishes between "quantitative" and "qualitative" defences in plants. Qualitative defences are toxins or deterrents against herbivores and occur in relative low quantities in plants. Quantitative defences are digestibility reducers and occur in higher concentrations. Toxins act mainly against unadapted generalist herbivores while specialist herbivores often are very well adapted to these compounds in their diet. An important class of toxins are the pyrrolizidine alkaloids (PAs), with more than 660 different structures indentified in over 600 plant species. About half of these PAs formed are toxic to livestock and wildlife and also to most insects. However, specialist herbivores use these compounds for their own benefit as cues to recognize their food plant, eg PAs acting as an oviposition stimulant (Mácel and Vrieling 2003) and as feeding stimulant (Bernays et al. 2004).

In addition, PAs and other compounds are sometimes sequestered for the defence of the herbivore itself (Eisner and Eisner 1991). In other cases PAs amongst others have become an essential part of the herbivore's sex pheromone system, or are used as a nuptial gift (Weller et al. 1999). Because PAs and other toxins occur in low concentrations (usually less than 1 percent of the dry weight), they are assumed to be a cheap defence. Digestibility reducers occur in higher concentrations and are more expensive for the plant to produce (Glawe et al. 2003) because costs of secondary metabolites increase with their concentration (Vrieling and van Wijk 1994). However, they are believed to be less easy to circumvent by specialist herbivores and generalist herbivores. Toxins therefore pose a dilemma for the plants in their native ranges, and this dilemma is referred to as the specialist-generalist dilemma (van der Meijden 1996). Increasing PA or toxin levels protects the plant against unadapted generalist herbivores, but simultaneously makes it more vulnerable to adapted specialist herbivores. PAs and toxins concentrations are therefore constrained by opposing selective forces from specialist herbivores and small allocation cost on one hand, and from herbivory by generalist herbivores on the other hand (Fig.1).

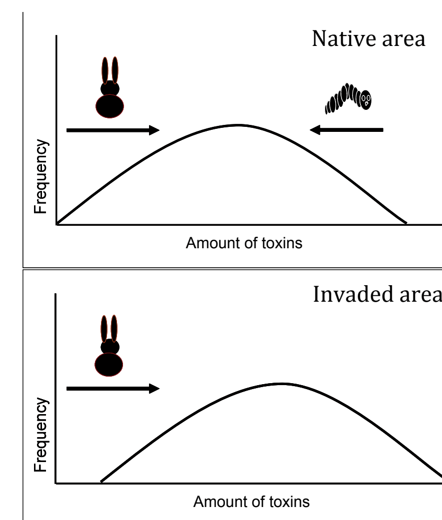

Fig. 1 Schematic overview of selection pressures of generalists and specialists in the native area and the invaded area. Under the influence of the selection pressure of the specialist herbivores in the invaded area the defence distribution has shifted to the right. Generalists are represented by a rabbit, specialists are represented by a caterpillar.

Digestibility reducers provide protection against both generalist and specialist herbivores but have a higher allocation cost (Glawe et al. 2003). The SDH comes into play for plants introduced into areas where their specialist herbivores are absent. Expensive digestibility reducer levels are decreased at the expense of cheap toxins, yielding a net allocation gain that can be diverted to growth and reproduction. The SDH therefore predicts that toxin concentrations will increase, digestibility reducer levels will decrease, and growth and reproduction will increase upon plant introduction into a new area. Fundamental to the EICA and the SDH is the assumption that rapid evolutionary change takes place upon plant introduction into the new area.

We searched the literature for studies in which defence levels were measured in common garden experiments in plants from both native and invasive areas to find evidence for increased levels of toxins and decreased levels of digestibility reducers in invaded areas.

Because PAs are toxins known for their beneficial impact on specialists and their detrimental impact on generalists, we expect differences in PA levels between the native and the invasive areas. As a sub study, PA levels from native and invasive plants measured in different studies were compared. Based on the SDH we expect increased levels of PAs in the invaded areas.

Material and methods

We used the ISI Web of Science to gather data for comparing defence levels between native and invasive individuals. The following keyword combinations were typed in to search for papers: invasive/ invasion AND defence/defense AND plant and invasive/ invasion AND common garden experiment. This search resulted in 398 papers. A first selection was made by reading the paper titles and abstracts. The majority of papers contained defence data from native or invasive individuals only; these papers were excluded. Moreover, several articles comprised data about allelopathy. The hypotheses and theories we wanted to test were not developed for allelopathic interactions and we therefore excluded these articles. Several papers could not be incorporated because they lacked quantitative data. After making this selection, we extended the literature search to the references in the articles that were dealing with our subject. With regard to digestibility reducers we included measurements of trichome density, toughness and dry matter content. These mechanical defence products were grouped with the digestibility reducers based on the study of Travers- Martin and Müller (2008). This study of matching plant defence syndromes showed that mechanical defence and digestibility reducers were clustered because the performance of specialists was the same for both defence mechanisms.

In some papers defence levels of chemical compounds were measured per genotype. For our analysis we averaged values over genotypes and populations. Units of measurement different between studies and could not be converted to standard measurement units in some cases.

Hedges et al. (1999) developed statistical tools for meta-analysis that can be used to compare ratios between different studies to estimate effect sizes. For each study effect sizes were calculated as L = ln(value of invasive plants/value of the native plants) = ln(value of the invasive plants) – ln(value of the native plants). Over all studies a weighted mean of L and confidence limits were calculated, taking into account sample sizes and standard errors within each study (Hedges et al. 1999). L values were returned to simple ratios by taking the antilog of the L value and calculating 95% confidence intervals. An antilog value of 1 therefore represents the situation that the level of defence in the native plants is exactly equal to the level of defence in the invasive plants. Antilog values larger than 1 indicate that the level of defences are higher in the invasive area compared to the native area. For the sub study on PAs, effect sizes were calculated in a similar way for PAs only.

For all studies except that of Willis et al. (1999), means and standard errors could be derived from the text. The study of Willis et al. (1999) was therefore not included although it supports the SDH.

Results

The literature yielded 15 publications in which plants from invaded and native areas were reared in a common garden set up, and in which toxins and/or digestibility reducers were measured (Table 1). In total 8 different toxins were measured in 9 different species yielding 13 comparisons. In 3 studies, comprising 4 data sets, PA levels were measured and these data were included in the sub study. We found 4 publications in which all data about digestibility reducers were available. Moreover, three other studies were found where 5 morphological traits such as dry matter content, trichome density or toughness were measured yielding 10 comparisons in total (Table 1). Antilog values of the weighted mean of L and confidence limits were 0.933 and 0.660-1.318 respectively (see also Figure 2). This is not in line with the expectation of the SDH that native individuals should have higher levels of digestibility reducers than invasive individuals. This meta- analysis therefore showed that digestibility reducers were not significantly decreased in plants from invaded areas as predicted by the SDH.

For toxins, antilog values of the weighted mean of L and confidence limits were 1.390 and 1.085-1.781 respectively (see also Figure 2). All values were above 1 which is in line with the expectation of the SDH that native individuals have lower levels of toxins than invasive individuals.

The meta-analysis therefore showed that toxins were significantly increased in plants from the invaded area as predicted by the SDH. For the PAs, antilog values of the weighted mean of L and confidence limits were even higher than for toxins overall (resp. 2.834 and 1.844- 4.354). This finding is in line with the SDH.

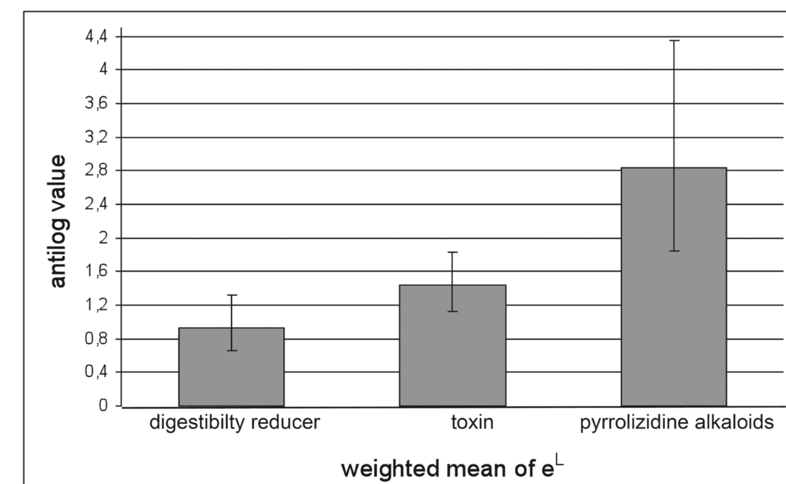

Fig. 2 To compare ratios between the studies L values were used to estimate effect sizes. Weighted means of L are calculated as ln (value of the invasive plants/value of the native plants). The x-axis indicates the antilog of the weighted means of L. Error bars indicate the antilogs of the 95% confidence limits of the antilog of L.

Table 1 Studies used for the analysis of toxins and digestibility reducers in native and invasive individuals. Studies used for the analysis on PA's alone are indicated with ^. In the P;R column the number of populations are given followed by the number of replicates. In the Area column, the region of origin is indicated where EU stands for Europe, AF for Africa, NA for North America, ME for Mexico, AU for Australia, NZ for New Zealand, CH for China, IN for India, SW for Sweden and ICE for Iceland. In the Conc.colum the concentration of defence compounds are indicated. Significance levels are indicated in the column Sig. with n.s. for not significant, * p<0.05, ** p<0.01 and *** p<0.001.

Toxins		Native			Invasive				
Species	**Compound**	**P;R**	**Area**	**Conc.**	**P;R.**	**Area**	**Conc.**	**Sig**	**Reference**
Alliaria petiolata	Sinigrin	3;30	EU	16.1 umol/g	3;30	NA	49.6 umol/g	**	(Lewis et al., 2006)
Senecio inaequidens^	Pyrrolizidine alkaloids	3;10	AF	0.00 ug/g	3;10	EU	0.81 ug/g	*	(Cano et al., 2009)
Senecio pterophorus^	Pyrrolizidine alkaloids	3;10	AF	0.24 ug/g	3;10	EU	1.04 ug/g	*	(Cano et al., 2009)
Senecio jacobaea^	Pyrrolizidine alkaloids	15;4	EU	2.03 ug/g	16;4	NA/AU/NZ	3.84 ug/g	***	(Joshi &Vrieling, 2005)
Hypericum perforatum	Hypericin	17;20	EU	0.27 mg/g	32;20	NA	0.2 mg/g	**	(Maron et al., 2004)
Hypericum perforatum	Hypericide	17;10	EU	28 mg/g	32;10	NA	22.8 mg/g	n.s.	(Maron et al., 2004)
Centaurea maculosa	Catechin	4;5	EU	24 ug/mL	11;5	NA	42 ug/mL	n.s.	(Ridenour et al., 2008)
Cynoglossum officinale^	Pyrrolizidine alkaloids	4;10	EU	0.07 mg/g	3;10	NA	0.068 mg/g	n.s.	(Eigenbrode et al., 2008)
Solidago gigantean	Sesquiterpenes	10;8	NA	1.36 mg/g	20;4	EU	1.25 mg/g	n.s.	(Hull-Sanders et al., 2007)
Solidago gigantean	Diterpenes	10;8	NA	1.20 mg/g	20;4	EU	1.04 mg/g	n.s.	(Hull-Sanders et al., 2007)
Lepidium draba	Total glucosinolates	11;5	EU	62.34 umol/g	10;5	NA	71.78 umol/g	n.s.	(Müller &Martens, 2005)
Lepidium draba	Total glucosinolates	11;5	EU	46.4 umol/g	10;5	NA	43.5 umol/g	n.s.	(Müller &Martens, 2005)
Alliaria petiolata	Total glucosinolates	7;10	EU	0.35 mg/g	7;10	NA	0.21 mg/g	n.s.	(Cipollini et al., 2005)
Digestibility reducer									
Silene latifolia	Trichomes	20;10	EU	84.5 no./3.2mm2	20;10	NA	88.5no./3.2mm2	n.s.	(Blair &Wolfe, 2004)
Silene latifolia	Trichomes	20;10	EU	114 no/3.2 mm2	20;10	NA	107 no/3.2 mm2	n.s.	(Blair &Wolfe, 2004)
Centaurea maculosa	Trichomes	22;5	EU	94 no./cm2	23;5	NA	135 no./cm2	**	(Ridenour et al., 2008)
Senecio jacobaea	Dry matter	8;7	EU	144 mg/g	14;7	NA/AU/NZ	122 mg/g	*	(Doorduin unpublished)
Alliaria petiolata	Trypsin inhibitors	7;10	EU	11.8 units/ g dw	4;10	NA	30.4 units/g dw	n.s.	(Cipollini et al., 2005)
Ageratina adenophora	Cell wall proteins.	5;10	ME	0.69 g/m2	10;10	CH/IN	0.39 g/m2	*	(Feng et al., 2009)
Fucus evanescens	Phlorotannin	3;10	SW	27.9 mg/g	3;10	ICE	57.1 mg/g	*	(Wikström et al., 2006)
Lythrum salicaria	Total phenolics	6;10	EU	4.8 mg/g	6;10	NA	3.3 mg/g	**	(Willis et al., 1999)
Sapium sebiferum	Tannins	1;7	CH	1.59% dr. wt.	3;8	NA	0.09 % dr. wt.	*	(Siemann and Rogers, 2001)

Discussion

As predicted by the shifting defence hypothesis (SDH), toxin concentrations were significantly higher in invasive individuals than in native individuals. Studies that were incorporated into this meta-analysis included a number of different chemical compounds such as alkaloids, terpenes and glucosinolates. Despite big differences in chemistry, a majority of the studies showed the same pattern. Because all studies were carried out in a common garden, native and invasive individuals were exposed to identical environmental conditions. For this reason differences in defence levels are evidence for evolutionary change (Bossdorf et al. 2005). Invasive plants evolved an energetically beneficial but effective defence strategy in response to the absence of specialists. The sub study on PA levels showed even a stronger pattern compared to the overall study of toxins, with concentrations significantly higher in invasive individuals.

However, in a study by Eigenbrode et al. (2008), no difference was found in the level of pyrrolizidine alkaloids between native and invasive individuals of *C. officinale*. Although herbivore pressure in the invaded area was not formally measured, it appeared that plants in this area experienced less herbivory compared to the native area. Because the production of defence compounds can be costly (Vrieling and van Wijk 1994, Koricheva 2002), the optimal defence theory poses that allocation to defence should be proportional to the risk of attack (Stamp 2003). If the herbivore pressure is (very) low in the invaded area, as in the above mentioned study, it could be more beneficial for a plant to save energy by not producing any defence products.

In a study by Hull-Sanders et al. (2007) no difference in concentration of diterpenes was found between native and invasive individuals of *S. gigantea*. A previous study showed that none of the invasive populations were infested by insects (Jakobs et al. 2004); therefore the optimal defence theory may also be responsible for this outcome. There is also some evidence that these compounds can reduce spore production by fungal pathogens (Biere et al. 2004) and therefore do not act solely as a defence against herbivores. Besides being beneficial traits, defence products can also lead to so called ecological costs (Strauss et al. 1999) such as increased susceptibility to other types of herbivores and pathogens, and deleterious effects on pollinators and herbivore predators and parasitoids. Besides herbivore defence, chemical compounds can therefore have multiple functions which can affect natural selection on chemical defences.

In a study by Maron et al. (2004), the level of hypericin was lower in invasive individuals compared to native individuals. In ongoing work no difference was found in resistance of native and invasive individuals against a specialist herbivore (Maron et al. 2004). It may be that selection in the native range by generalists has led to higher concentrations of hypericin in the native area.

Total concentration of glucosinolates was measured in leaves of the crucifer *L. draba*. Seedlings from the invaded range contained, as predicted by the SDH, a higher concentration of glucosinolates. In plants of three months old no difference was found. However, myrosinase activity was significantly higher in invasive individuals compared to native individuals. It is suggested that this product has even stronger adverse effects as a toxin for herbivores than glucosinolates themselves (Agrawal and Kurashige 2003) and may also attract parasitoids of herbivores (Bradburne and Mithen 2000). Moreover glucosinolates are also known for their inducibility. In a study on *A. petiolata*, invasive individuals contained reduced constitutive levels and increased induced levels of glucosinolates compared to native individuals (Cipollini et al. 2005). This may be a cost- saving strategy resulting from reduced selective pressure by herbivores (Koricheva et al. 2004).

The SDH also predicts a decrease in expensive digestibility reducing compounds of invasive individuals compared to native individuals. Our review of the literature did not find support for this prediction. However, most of the data consisted of morphological traits that have functions other than defence. Moreover, there can be morphological constraints for the production of defence chemistry. It is only possible to produce more terpenoids if there are more storage compartments such as resin ducts and glandular trichomes (Björkman et al. 1998). It is also known that trichomes have important functions in regulating leaf temperature and light reflection (Smith and Nobel 1977) and leaf evaporation (Brewer et al. 1991). One assumption of the SDH is that quantitative defence products are more expensive than qualitative defence products. However, this may depend on the environmental conditions of a plant. For example, leaf toughness is not necessarily expensive. Leaves can become tougher by increasing the thickness of the photosynthetic mesophyll (Read et al. 2009). In a sunny environment the costs of carbon gain due to internal self-shading are very small in relation to the increase of photosynthesis (Roderick et al. 1999). Under such conditions, toughening of leaves incurs no cost. These alternative benefits could also contribute to invasiveness and might be selected for in the invasive range. Therefore, the number of trichomes and leaf toughness are difficult to interpret in the light of quantitative defences. Besides having multiple functions within a particular species range, a chemical compound might also have different functions in native and invasive individuals.

Another strategy to cope with herbivory, which is not taken account by comparing defence compounds, is regrowth capacity. It has been argued that this strategy is especially beneficial for plants that suffer from high herbivory, such as that from specialists (van der Meijden et al. 2000). Instead of investing energy in defence, energy can be allocated to regrowth. Joshi and Vrieling (2005) indeed found evidence for this strategy. Invasive individuals without specialists had lower regrowth capacity compared to native individuals.

In conclusion, we found higher levels in invasive individuals for toxins in general and also specifically for PAs, which is in accordance with the SDH. Digestibility reducing products of native and invasive individuals did not differ. However, a smaller number of studies were available that addressed digestibility reducing defences, and a number of these defences are also known to be involved in other plant processes.

Acknowledgements

We are grateful to two anonymous reviewers for their helpful comments on the manuscript and for suggesting some papers that could be included in this study. Furthermore we want to thank B. Muton-Phillips and H. Kirk for revising the English.

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The lack of genetic bottleneck in invasive *Tansy ragwort* populations suggests multiple source populations.

L.J. Doorduin[a*], K. van den Hof[a,b], K. Vrieling[a] and J. Joshi[b,c]

BASIC AND APPLIED ECOLOGY
Volume: 11 Issue: 3 Pages: 244-250
DOI: 10.1016/j.baae.2009.12.007
Published: 2010

[a]Institute of Biology, Section Plant Ecology
Leiden University
Sylviusweg 72
233 BE Leiden, the Netherlands
[b]Institute of Environmental Sciences, University of Zurich
Winterthurerstrasse 190
8057 Zurich, Switzerland
[c]Institute of Biochemistry and Biology, Biodiversity Research/Systematic Botany
University of Potsdam, Maulbeerallee 1
14469 Potsdam, Germany
*Corresponding author. Tel.: +31715275117; fax: +310715274900.
E-mail address: l.j.doorduin@biology.leidenuniv.nl

Abstract

Jacobaea vulgaris (Asteraceae) is a species of Eurasian origin that has become a serious non-indigenous weed in Australia, New Zealand, and North America. We used neutral molecular markers to (1) test for genetic bottlenecks in invasive populations and (2) to investigate invasion pathways. It is for the first time that molecular markers were used to unravel the process of introduction in this species.

The genetic variation of 15 native populations from Europe and 16 invasive populations from Australia, New Zealand and North America was compared using Amplified Fragment Length Polymorphisms (AFLP's). An analysis of molecular variance showed that a significant part (10 %) of the total genetic variation between all individuals could be explained by native or invasive origin. Significant among-population differentiation was detected only in the native range, whereas populations from the invasive areas did not significantly differ from each other; nor did the Australian, New Zealand and North American regions differ within the invasive range. The result that native populations differed significantly from each other and that the amount of genetic variation, measured as the number of polymorphic bands, did not differ between the native and invasive area, strongly suggests that introductions from multiple source populations have occurred. The lack of differentiation between invasive regions suggests that either introductions may have occurred from the same native sources in all invasive regions or subsequent introductions took place from one into another invasive region and the same mix of genotypes was subsequently introduced into all invasive regions.

An assignment test showed that European populations from Ireland, the Netherlands and the United Kingdom most resembled the invasive populations.

Introduction

The spread of introduced species in new environments offers the unique opportunity to study the evolution and adaptation of organisms to a changing environment, which is a key issue in biology (Sakai *et al.* 2001). A number of non-indigenous species become serious pests in the new environment (Mack *et al.* 2000) whereas they are not dominant in their native range. The reason why these species only become a pest in the introduced area remains intensively debated (e.g. Elton 1958; Callaway & Maron, 2006; Mortenson & Mack 2006).

The introduction of a species into a new environment can have different outcomes related to genetic variation in the native and invasive areas. Genetic variation can decrease by founder effects and genetic bottlenecks (Dlugosch & Parker 2008). However, multiple introductions, hybridisation (Ellstrand & Schierenbeck 2000) and the release of epistatic genetic variation (Dlugosch & Parker 2008) can lead to an increase of genetic variation in the new area compared with the native area.

A number of studies show that if introductions occur independently from each other and do not stem from the same source population, large differences in genetic variation among regions in the invasive range can be expected (Ellstrand & Schierenbeck 2000; Lavergne & Molofsky 2007).

To study whether life-history and other traits did change upon becoming a pest in the invaded areas, it is necessary to compare the traits of the invasive populations with those of the source populations in the native area (Hierro, Maron & Callaway 2005). This, however, requires detailed information on the origin of the invasive populations.

In this study, we compared genetic variation, detected by neutral molecular markers (AFLPs), between and within native and invasive areas of *Jacobaea vulgaris,* (Tansy or Common Ragwort) *Asteraceae* (Pelser, Veldkamp & van der Meijden 2006) (syn. *Senecio jacobaea)*. *Jacobaea vulgaris* is a pest species in the invasive areas that is toxic to lifestock and humans caused by its pyrrolizidine alkaloids content (Witte, Ernst, Adam & Hartmann 1992). This monocarpic perennial has been introduced into New Zealand, Australia and North America. In those days, there was a merchandising route between the three invasive regions (Morison 1912*)* and introductions therefore, could also have occurred from one invasive region to the other.

In a previous study on the invasiveness of *J. vulgaris*, Joshi & Vrieling (2005) examined life-history traits, herbivory and chemical defence using common garden experiments. These experiments revealed that plants from invasive areas had a more vigorous growth and reproduction, were better protected against generalist herbivores, but less well defended against native specialist herbivores adapted to their main defence chemicals (Joshi & Vrieling 2005). Pyrrolizidine alkaloid (PA) concentration and composition varied considerably between populations from the native, but not from the invasive area.

In this study, we addressed the following questions: (1) Does the absolute amount of genetic variation differ between the native and invasive areas? (2) Is there genetic differentiation between (a) the native and invasive areas? (b) populations within the native and invasive areas, (c) the regions within the invasive area? (3) Can we identify the region in the native area which most likely represents the potential source population(s)? (4) Were multiple source populations introduced?

Methods

Study species

Jacobaea vulgaris, is a self-incompatible, allo-tetraploid, monocarpic perennial plant species (Harper & Wood 1957) that has become a serious pest in Australia, New Zealand, the United States and Canada. *J. vulgaris* was first recorded outside its native distribution area in the 1850s in Canada (Bain 1991), around 1874 in New Zealand (Poole & Cairns 1940) and Australia (McLaren, Ireson & Kwong 2000) and in 1901 on the west coast of the U.S.A. (Rice 2003**).**

We used the same set of *J. vulgaris* populations as studied by Joshi & Vrieling (2005) (Appendix A): 15 native populations (Europe) and 16 invasive populations (Australia, New Zealand and North America). From each population, seeds of 5-20 individuals (growing at least 2 m apart from each other) were collected. Seeds were germinated and grown in a climate-room at the Leiden University and leaf samples were taken from these plants.

AFLP analysis

DNA was extracted from 38 native and 44 invasive individuals. Since we were primarily interested in interpopulation differentiation across the native and invasive range, we chose to sample as many populations as possible at the expense of less individuals per population. In this way most of the genetic variation in the area is estimated (Barbosa et al. 2003). Finally we ended up with DNA from 1-4 offspring of different maternal genotypes per population. In the case of bulk samples, seeds were chosen at random from the sample (Appendix A). A fresh leaf was collected from each individual and stored at –80°C until DNA isolation with the Qiagen DNeasy plant extraction kit.

AFLP fingerprints (Vos *et al.* 1995) were generated following the protocol from Kirk, Macel, Klinkhamer & Vrieling (2004) using the AFLP core mix (Applied Biosytems) for PCR. A pre-selective PCR with one selective base pair (*Eco*RI + A and *Mse*I + C) was carried out followed by selective amplification using six primer combinations on the *Mse*I side: CAA, CAG, CCG, CGT, CTG and CTT. The *Eco*RI primer (*Eco*RI - ACA) was labelled with the fluorescent dye 5-FAM. PCR products were separated with an ABI Prism™ 310 capillary sequencer (Applied Biosystems, Rotkreuz, Switzerland) using Genescan ROX 500 as an internal standard. Electropherograms were scored using Genographer 1.6.0 (Benham, Jeung, Jasieniuk, Kanazin & Blake 1999). Fragments in the range of 100 to 500 base pairs were scored by two different people to test for repeatability. Fragments were only used for further analyses if the scoring differences were less than 5%. Repetition tests showed that the primers produced highly reproducible AFLP patterns.

Statistical analyses

Two populations of the native area were not used in the analyses because of only one individual (Rothenthurm) and because of missing values (Buggingen). So, all analyses were done on 34 native and 44 invasive individuals.

To test if fixation in the invasive area did occur, the percentage of polymorphic loci present in each population was calculated and analysed with an analysis of variance testing differences among populations in native and invasive areas.

To estimate the genetic differentiation between invasive and native areas and between populations within the native and invasive area, an analysis of molecular variance (AMOVA) was carried

out using Arlequin (Version 2.0; Schneider, Roessli & Excoffier 2000). Analogous to an analysis of variance, an AMOVA partitions the total genetic variance into a part that can be attributed to differences between population and differences within populations. The software package Geneclass 2 (Piry *et al*. 2004) was used for an assignment test (Waser & Strobeck 1998), determining the most likely source population among the native populations sampled. Missing values seriously influenced the results of the assignment analysis. To eliminate this effect the dataset was pruned by omitting two primer combinations (EcoRI + ACA – MseI + CTT; EcoRI + ACA – MseI + CGT) so that no missing values were present in the native populations. As a result, 23 loci remained in the dataset. Since AFLP is a dominant marker, the second allele of the phenotype "band present" was scored as missing in the input files. Geneclass calculated for each invasive individual the likelihood that it is related to each native population using the Bayesian method of Rannala and Mountain (1997). Subsequently for each invasive individual the likelihood mass was calculated as: likelihood of each invasive individual related to a particular native population/ sum of likelihoods for that invasive individual for all native populations. To see how each native population contributed to the likelihoodmass of individuals of the invasive area, for each native population the likelihood masses were summed over all invasive individuals. This yielded for each native population a sum of likelihood masses. To obtain a relative likelihood masses for each native population, the sum of likelihood masses per native population was divided by the sum of the sum of likelihood masses for all native populations. The same procedure was carried out separately for the three regions within the invasive range (New Zealand, Australia and North America). The percentage likelihood mass obtained gives a relative ranking among the native populations how well they fit to the invasive area or region.

Finally, the percentage of shared bands was calculated for every native population to each invasive region (Appendix B).

Results

AFLP analysis

In the range of 100 to 500 base pairs for the six primer combinations, 141 out of 197 bands (71.6%) were polymorphic. Of these bands, 39 were used for analysis because of their repeatability.

Amount of genetic variation

Polymorphic bands

All polymorphic bands found in the native area were also polymorphic within the invasive area indicating that the amount of neutral genetic variation did not differ between these areas. This suggests that the total amount of genetic variation among invasive populations was not reduced by severe bottlenecks and/or single introductions. Moreover, there was a significant correlation between both areas in the frequency of bands present at each locus (r= 0.643, n=39, $P< 0.01$).

Invasive areas did not differ from native areas in the percentage of polymorphic loci per population (39.61 ± 5.51 vs. 36.79 ± 4.15; $F_{1, 27} = 0.15$ $P>0.7$). Some polymorphic bands were absent in some regions (2 in North America, 1 in New Zealand and 5 in Australia). One polymorphic band (EcoRI + ACA – MseI + CTG, 232bp) present in 83% of the plants from the British Isles, was present in 89% of all invasive samples, while it was absent in all other European populations. All chosen loci were polymorphic at the level of the area for both the native and the invasive area. None of the native populations contained all bands present in an invasive region (Appendix B). Baldoyle (Ireland) showed the highest percentage of shared bands with the invasive regions (average of 67%). This indicates that 33% of the bands still originated from (an)other native population(s).

AMOVA analysis

Significant genetic differentiation between the native (European) and invasive populations was detected by an AMOVA analysis (Table 1). Ten percent of all genetic variation was among the invasive and native area and five percent of the total genetic variation was among populations within an area (Table 1). So, ten percent of the allelic variation between individuals could be explained by native or invasive origin. Variation among populations within an area was only five percent. The remaining variation could be ascribed to allelic variation within populations of the native and invasive area. When native and invasive populations and regions were analyzed separately, only native populations were significantly different from each other (Table 1). In contrast, no significant genetic differentiation between AFLP haplotypes was detected among different regions within the invasive range and populations within these regions (Table 1).

Assignment analysis

The percentage relative likelihood masses (Table 2) indicated that the populations from the Irish, UK and Dutch coast (Leiden) are the most likely source populations out of the 13 native populations used in this study. Interestingly, Baldoyle (Ireland) was the only native population with jacobine-type plants only, just as the invasive populations (see Appendix A), and had the highest likelihood mass (Table 2).

The pattern of the distribution of likelihood masses is largely congruent for the three invasive regions. The UK population shows a high likelihood mass for Australia and New Zealand.

Table 1. Analysis of Molecular Variances (AMOVA's) for native populations (Baldoyle, Leiden, Wales, Chereng, l'Himelette, Plombieres, Meijendel, Westervoort, Zlin, Warsaw, Darmstadt, Gotland and Brocherbeck) and all invasive populations of *Jacobaea vulgaris*.

The "all populations combined" AMOVA attributes the total genetic variance to the difference between native and invasive populations, differences among populations and variation within populations. In the "invasive population only" analysis, the invasive area is split up in the three regions respectively Australia, New Zealand and North America. (n= number of populations ** p < 0.01 ** p < 0.05).

Source of Variation	d.f.	Sum of Squares	Percentage of Variance explained
All populations combined (n=29)			
Native-vs.-invasive	1	41.14	10.55**
Among populations within native/invasive areas	27	216.66	5.21*
Within populations	49	337.25	84.24**
Total	77	595.05	
Native populations only (n=13)			
Among populations	12	105.63	13.26**
Within populations	21	132.17	86.74**
Total	33	237.79	
Invasive populations only (n=16)			
Among regions	2	17.34	1.45
Among populations within regions	13	93.69	-0.58
Within populations	28	205.08	99.14
Total	43	316.11	

Table 2 Percentage relative likelihood masses derived from the assignment test for invasive *Jacobaea vulgaris* individuals (see methods) for each invasive region. The percentages indicate how likely a native population is a source population relative to the other native populations for a particular region. For the detailed calculation see text.

	Percentage relative likelihood mass			
Native population	**North America**	**Australia**	**New Zealand**	**Invasive area**
Ireland (Baldoyle)	28.88	24.21	21.73	24.94
Netherlands (Leiden)	28.29	18.69	14.52	20.50
United Kingdom (Wales)	6.30	16.17	15.20	12.56
France (Chéreng)	10.80	14.02	2.62	9.15
Switzerland (l'Himelette)	8.32	9.46	7.86	8.55
France (Plombieres)	6.68	5.35	7.17	6.40
Netherlands (Meijendel)	5.15	3.23	6.34	4.91
Netherlands (Westervoort)	0.69	4.80	8.72	4.74
Czech Republic (Zlin)	1.74	0.79	9.96	4.16
Poland (Warsaw)	0.85	0.53	5.50	2.29
Germany (Darmstadt)	1.39	0.38	0.03	0.60
Sweden (Gotland)	0.69	1.99	0.02	0.90
Germany (Brochterbeck)	0.23	0.37	0.32	0.31

Discussion

Although it is generally assumed that genetic variation across introduced populations will increase/decrease compared to populations in native areas (e.g. Nei, Maruyama & Chakraborty 1975, Novak & Mack 1993), there is no indication of such a pattern in our study. All polymorphic bands present in the native populations were also present in the invasive area. So the amount of neutral genetic variation of individuals from the native area was similar to individuals of the invasive area. Among populations, differentiation was detected only in the native range, whereas no significant genetic differentiation between AFLP haplotypes was detected among invasive populations within regions and not even among the different regions. The absence of genetic differentiation between regions is surprising considering the large geographical distance. Because of the small sample sizes it is possible that differences between populations in the invasive range were not detected.

Different scenarios of the route of introduction can explain these findings: (1) a single introduction from one population in Europe representing all genetic variation of native populations used in this study into different regions in the invasive area or into one invasive region followed by subsequent introductions to the other regions (Fig. 1A) However, the existence of one European population representing all the genetic variation of all European populations is very unlikely. (2) Introductions from different native populations, together representing all the genetic variation of native populations used in this study into all different regions in the invasive area or into one invasive region followed by subsequent introductions to the other regions (Fig. 1B). We consider the second scenario more likely because there is a very little chance that the same native populations were introduced independently to all three invasive regions.

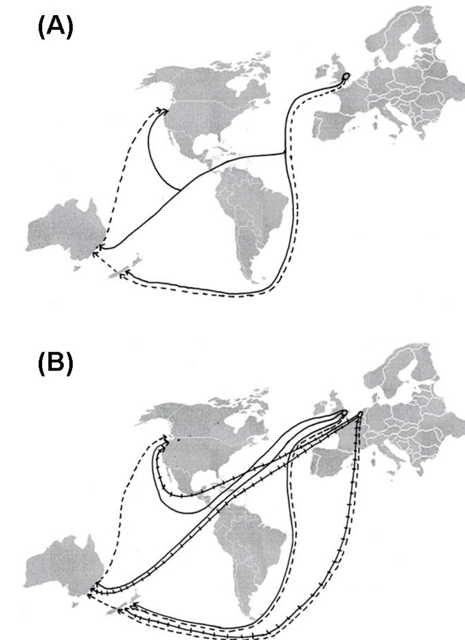

Fig. 1. Different scenarios of the route of introduction from native European *Jacobaea vulgaris* individuals to invasive regions Australia, New Zealand and North America.

(A) A single introduction from one population in Europe into all different regions in the invasive area (solid lines) or into one invasive region followed by subsequent introductions to the other regions (dashed lines)

(B) Introductions from different populations in Europe into all different regions in the invasive area. One introduction from a European population is indicated with solid lines the introduction from another European population is indicated with solid lines with strokes. Because of clarity, the example is given for only two European introductions. Dashed lines indicate the invasion of different European populations into one invasive region followed by subsequent introductions to the other regions.

Joshi & Vrieling (2005) analyzed pyrrolizidine alkaloid (PA) patterns in native and invasive populations and only found populations of the jacobine-chemotype in the invasive range. The bouquet of PA's from *J. vulgaris* plants from Baldoyle (Ireland) was most similar to the PA composition pattern found in the invasive range. In our study, the assignment test indicated that out of the 13 populations used for this study, Baldoyle (Ireland), Wales (U.K) or Leiden (The Netherlands) were the populations with the highest genetic similarity to *J. vulgaris* populations. It should be kept in mind that the exact source population(s) cannot be pinpointed due to the limited sample size in the analysis. However it suggests that if multiple source populations were introduced, populations from Ireland, the UK and the Netherlands are the most likely source population(s) out of the European populations analyzed.

In conclusion, the present study shows that the invasion of Australia, New Zealand, and North America by *Jacobaea vulgaris* did not involve strong bottleneck events. AFLPs identify populations from the United Kingdom, Ireland and The Netherlands, as putative source populations. The homogeneity of the genetic variation between populations in the invasive area suggests a common origin.

Acknowledgements

We thank S. Anderson, M. Armstrong, N. de Boer, R. Cranston, H. van Dijk, T. Morley, K. Potter, K. Puliafico, U. Schaffner, P. Syrett, C. Winks, and the Botanical Gardens of Berlin, Dijon, Dublin, Olomouc and Warsaw for collecting seeds, P.B. Pelser for valuable comments on the manuscript and E. Bitume for revising the English. We are grateful to three anonymous reviewers for their helpful comments on the manuscript. The competent help and support of René Husi with the AFLP analyses is gratefully acknowledged. The Swiss National Science Foundation (Project to J. Joshi), the Socrates/Erasmus exchange program and the Funke Foundation for Experimental Plant Sciences (Project to K. van den Hof) funded this project.

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Appendices

Appendix A Sampled *Jacobaea vulgaris* populations, the number of plants used for the AFLP analysis from each population, if plants are of the jacobine or erucifoline chemotype (JAC/ERU), and the type of seed sample available. Bulk samples are printed in bold face.

Country	Location	Latitude/ longitude	Nr. of samples	Jacobine / erucifoline
European populations				
Sweden	Gotland	N 57° E 18°	3	JAC + ERU
Ireland	**Baldoyle**	**N 53° W 6°**	**3**	**JAC**
Poland	**Warsaw**	**N 52° E 21°**	**2**	**ERU**
The Netherlands	Meijendel	N 52° E 4°	2	JAC + ERU
The Netherlands	Leiden	N 52° W 5°	3	JAC + ERU
Germany	**Brochterbec**k	**N 52° E 4°**	**3**	**ERU**
UK	Wales	N 51° E 7°	3	JAC+ ERU
The Netherlands	Westervoort	N 51° E 5°	2	JAC+ ERU
France	Chéreng	N 50° E 2°	3	JAC + ERU
France	**Plombieres (Dijon)**	**N 47° E 4°**	**2**	**ERU**
Germany	**Darmstadt**	**N 49° E 8°**	**2**	**ERU**
Czech Republic	**Zlin**	**N 49° E 18°**	**3**	**ERU**
Germany	Buggingen	N 48° E 8°	3	ERU
Switzerland	Rothenturm	N 47° E 8°	1	ERU
Switzerland	L'Himelette	N 47° E 7°	3	ERU
Invasive Populations				
Canada	**Abbotsford (BC)**	**N 49° W 122°**	**2**	**JAC**
Canada	**Yarrow (BC)**	**N 49° W 122°**	**2**	**JAC**
USA	Island Lake Road (MT)	N 48° W 114°	3	JAC
USA	Surprise Hill-Sylvia Lake (MT)	N 48° W 114°	2	JAC
USA	Silvertown (OR)	N 45° W 122°	4	JAC
USA	Larch Slope (OR)	N 45° W 121°	3	JAC
USA	South Cooper (OR)	N 45° W 121°	3	JAC
USA	Indian Creek Road (OR)	N 44° W 122°	3	JAC
New Zealand	Waikato, Hamilton (North Island)	S 48° E 173°	3	JAC + ERU
New Zealand	Marlborough Sounds (South Island)	S 41° E 170°	3	JAC
New Zealand	Marble Hill (South Island)	S 42° E 172°	2	JAC
New Zealand	Inchbonnie (South Island)	S 42° E 171°	3	JAC
Australia	Southern Tasmania	S 43° E 147°	3	JAC
Australia	Northern Tasmania	S 41° E 146°	2	JAC
Australia	Mornigton Peninsula (Victoria)	S 38° E144°	3	JAC
Australia	**Snake island (Victoria)**	**S 38° E145°**	**3**	**JAC**

Appendix B Similarity matrix with percentage of bands that each native population of *Jacobaea vulgaris* shares with all populations in an invasive region.

		Regions	
Native population	**North America**	**Australia**	**New Zealand**
Ireland (Baldoyle)	69.23	69.23	66.67
Netherlands (Leiden)	43.59	46.15	41.03
United Kingdom (Wales)	34.78	34.78	30.43
France (Chereng)	44.83	44.83	44.83
Switzerland (l'Himelette)	48.72	51.28	48.72
France (Plombieres)	25.64	28.21	25.64
Netherlands (Meijendel)	38.24	41.18	35.29
Netherlands (Westervoort)	44.44	47.22	44.44
Czech Republic (Zlin)	64.10	66.67	58.97
Poland (Warsaw)	12.90	16.13	12.90
Germany (Darmstadt)	38.46	41.03	35.90
Sweden (Gotland)	33.33	33.33	33.33
Germany (Brochterbeck)	48.72	51.28	48.72

The complete chloroplast genome of 17 individuals of pest species *Jacobaea vulgaris*: SNPs, microsatellites and barcoding markers for population and phylogenetic studies

Running title: Complete chloroplast genome of *Jacobaea vulgaris*

Leonie Doorduin,* Barbara Gravendeel,*+ Youri Lammers,* Yavuz Ariyurek,§
Thomas Chin-A-Woeng¶ and Klaas Vrieling*

DNA RESEARCH
Volume: 18 Issue: 2 Pages: 93-105
DOI: 10.1093/dnares/dsr002
Published: APR 2011

* Institute of Biology, Section Plant Ecology and Phytochemistry
Leiden University
PO Box 9505, 2300 RA Leiden, The Netherlands
+NCB Naturalis - NHN Leiden University
Einsteinweg 2, 2333 CC Leiden, The Netherlands
§ Leiden Genome Technology Center, Human and Clinical Genetics
Leiden University Medical Center
PO box 9600, 2300RC Leiden, The Netherlands
¶ ServiceXS, Plesmanlaan 1d, 2333 BZ Leiden, The Netherlands
Corresponding author: Leonie Doorduin, Tel.: +31715275117, fax: +310715274900.
E-mail address: l.j.doorduin@biology.leidenuniv.nl
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Abstract

Invasive individuals from the pest species *Jacobaea vulgaris* show different allocation patterns in defence and growth compared to native individuals. To examine if these changes are caused by fast evolution, it is necessary to identify native source populations and compare these with invasive populations. For this purpose we are in need of intraspecific polymorphic markers. We therefore sequenced the complete chloroplast genomes of 12 native and 5 invasive individuals of *J. vulgaris* with next generation sequencing and discovered Single Nucleotide Polymorphisms (SNPs) and microsatellites. This is the first study in which the chloroplast genome of that many individuals within a single species was sequenced. Thirty two SNPs and 34 microsatellite regions were found. For none of the individuals differences were found between the Inverted Repeats. Furthermore, being the first chloroplast genome sequenced in the Senecioneae clade, we compared it with four other members of the Asteraceae family to identify new regions for phylogentic inference within this clade and also within the Asteraceae family. Five markers (*ndhC-trnV, ndhC-atpE, rps18-rpl20, clpP* and *psbM-trnD*) contained parsimony-informative characters higher than two percent. Finally we compared two procedures of preparing chloroplast DNA for next generation sequencing.

Keywords: phylogenetic markers, *Jacobaea vulgaris*, Asteraceae, cpDNA polymorphisms, inverted repeat

Introduction

Comprising one-tenth of all flowering plants and containing over 20 000 species, the Asteraceae are one of the largest vascular plant families. With the exception of Antarctica, the Asteraceae are distributed on all continents. Species in this family are extremely various in secondary chemistry, inflorescence morphology and chromosome numbers. This huge variation provides great opportunities to acquire insight into the diversification process in this family, which began 42-36 million years ago. The Asteraceae are not only interesting because of their phenotypic and species diversity, but this family also includes members of economically important food crops, herbal species, ornamentals, and plants for the cut flower industry. Other members such as *Jacobaea vulgaris*, *Senecio vulgaris* and *Taraxacum officinale* are weedy and have an economical and ecological impact.

We sequenced the complete chloroplast genome of *J. vulgaris* with next generation sequencing techniques to find new genetic markers that are phylogenetically informative and to discover intraspecific polymorphic markers for population studies. The conservative structure of the chloroplast genome makes it easy to compare with other members of the Asteraceae family. In a recent study of Panero and Funk (2008), 12 major lineages of Asteraceae were found with Bayesian and Maximum Parsimony methods by combining ten chloroplast loci from 108 taxa. Within the subfamily Asteroideae, strong statistical support was found for tribal relationships except for the Senecioneae tribe. In the Bayesian analysis, this tribe was unresolved and in the Maximum Parsimony analysis it was placed as a sister group to Calenduleae without strong statistical support (52% BS). In other studies of Pelser (2007, 2010) a phylogenetic analysis of the nuclear ribosomal internal spacers (nrITS) and external spacer (ETS) and five chloroplast loci was done to clarify intergeneric relationships within Senecioneae and to delimitate the genus *Senecio*. Although these phylogenies gave more insight, they still lacked strong statistical support and resolution.

No chloroplast genome has been previously sequenced from any species in the Senecioneae clade, and the chloroplast genome sequence of *J. vulgaris* can yield more information about variation within this clade, as well as between clades of the Asteroideae subfamily. In this study the chloroplast genome of *J. vulgaris* (tribe Jacobaea) was compared with *Guizotia abyssinica, Helianthus annuus*, *Parthenium argentatum* (all belonging to tribe Heliantheae) and *Lactuca sativa* (tribe Lactuceae). To guide future phylogenetic studies within the Asteraceae family, we identified new phylogenetically informative chloroplast markers by finding differences within and between genome organization.

Jacobaea vulgaris is a troublesome weed that belongs to the Asteraceae family and is native to Europe and western Asia, ranging from Norway through Turkey, and from Great Britain to Siberia. It was first reported in the 1850s in Canada, in 1875 in New Zealand and shortly thereafter in Australia and in 1900 at the west coast of North America. In introduced areas *J. vulgaris* is a pest species, outcompeting local plants and containing pyrrolizidine alkaloids which are toxic to herbivores. Control is difficult, since the lifecycle can vary from annual to short- lived perennial, depending on the genotype. Moreover, seeds remain viable in the soil for several years. *Jacobaea vulgaris* causes four million dollar losses annually to cattle poisoning and control in Australia alone.

Joshi and Vrieling (2005) compared *J. vulgaris* plants from the invasive areas with plants from the native area and found that invasive individuals contained higher pyrrolizidine alkaloid levels, have a 30% higher reproductive effort, are more susceptible to attack by specialist herbivores and less susceptible to generalist herbivores. These results suggest that selection pressures in the invasive area shaped the different allocation patterns in *J. vulgaris* in the invasive areas within 70 generations. However it is possible that introduced populations were derived from native European populations that happened to express pyrrolizidine alkaloid and allocation patterns that are similar to those currently observed in invasive ranges.

To exclude the null hypothesis that these patterns are observed as a result of genetic drift rather than natural selection, native source populations need to be identified and compared to invasive populations. Source populations can be pinpointed by using neutral molecular markers such as AFLPs. A previous study on *J vulgaris,* based on nuclear AFLP data, did not show a difference in the amount of variation between native and invasive individuals. These findings suggest that introductions from multiple source populations have occurred. Other neutral markers are Single Nucleotide Polymorphisms (SNPs) and microsatellite markers in the chloroplast genome.

Next generation sequencing can produce DNA sequences cheaply and quickly, facilitating the rapid sequencing of nuclear and organellar genomes. Chloroplasts genomes are known for their conservative rates of evolution. With an average size of 150 kb, chloroplast genomes are sufficiently large to find differences between and within species. The absence of recombination and maternal transmission of the chloroplast genome (limiting gene flow to seed dispersal only) makes cpDNA markers useful for tracing source population(s).

In this study, we sequenced the chloroplast genome of 17 *J. vulgaris* individuals by using the Illumina Genome analyzer platform. This is the first study sequencing multiple individuals of the same species with next generation sequencing. Multiple individuals were sequenced to reveal intra-specific variation (SNPs and microsatellite loci). Finally, we compared two different procedures of preparation for sequencing the chloroplast genome, namely direct extraction of the chloroplast DNA and amplifying the cpDNA with long range PCR.

Materials and Methods

Extraction of chloroplasts and isolation of DNA from chloroplasts

By using the chloroplast extraction kit of Sigma- Aldrich [CP-ISO] and following the manufacturer's protocol, chloroplasts from sample nr. 17 (see Table 1) were isolated out of 30 g of fresh leaf material. To remove unwanted whole cells and cell wall debris, the blended leaf material with the chloroplast isolation buffer was centrifuged. To separate the intact from the broken chloroplasts a 40 % percoll layer was used. Before DNA extraction, the intact chloroplasts were treated with ST buffer (400 mM sucrose, 50 mM Tris pH 7.8, 0.1% bovine serum albumin) with a final concentration of 25 µg/mL DNAse-1 (Sigma Aldrich) per gram of leaf material to digest DNA outside the intact chloroplasts. After

centrifuging, the chloroplast pellet was resuspended in a TEN buffer (100 mM Tris pH 7.2, 50 mM EDTA, 100 mM NaCl, 0.2% ß-mercaptoethanol). To extract the DNA from the chloroplasts, the chloroplasts were lysed with 1% sodium dodecyl sulfate followed by a phenol/chloroform step to remove proteins. The DNA was precipitated overnight with1/10 vol. of 5M ammonium acetate and 1 vol. of isopropanol. After centrifuging, the pellet was washed with 70% ethanol and re-dissolved in TE buffer (1M Tris HCl pH=8.0, 0.5M EDTA).

Table 1. Geographical information, percentage of the chloroplast genome sequenced, method used for preparing the template for Illumina sequencing, lane number on the Illumina platform and reads obtained of the 17 individuals of *Jacobaea vulgaris* that were sequenced.

Sample	Country	Location	Latitude/ longitude	% cp genome sequenced	Template sequencing	Illumina lane
1	New Zealand	Haast (South Island)	S 43° E 169°	89.9	Long range PCR	2 (776)
2	Ireland	Caherdaniel	N 51° W 10°	88.5	Long range PCR	2 (545)
3	Norway	Malvik	N 63° E 10°	83.4	Long range PCR	2 (543)
4	Canada	Cardigan	N 46° W 62°	89.8	Long range PCR	2 (838)
5	UK	Padstow	N 50° W 4°	98.3	Long range PCR	2 (1043)
6	Poland	Warsaw	N 52° E 18°	94.3	Long range PCR	2 (650)
7	Spain	Covadonga	N 43° W 04°	91.5	Long range PCR	2 (457)
8	France	Perrogney	N 47° E 05°	89.9	Long range PCR	2 (558)
9	Hungary	Lénárddaróc	N 48° E 20°	86.7	Long range PCR	2 (80)
10	The Netherlands	Ameland	N 53° E 05°	88.6	Long range PCR	2 (468)
11	Australia	Barramonga	S 38° E 143°	90.6	Long range PCR	2 (680)
12	Australia	Franklin (Tasmania)	S 43° E 147°	91.8	Long range PCR	2 (465)
13	UK	Portsmouth	N 50° W 01°	98.9	Long range PCR	2 (1102)
14	Sweden	Kapellskär	N 59° E 53°	99.9	Long range PCR	3 (11 084)
15	New Zealand	Opunake (North Island)	S 39° E 173°	94.7	Long range PCR	2 (691)
16	Germany	Halle	N 51° E 11°	98.7	Long range PCR	2 (805)
17	Spain	Covadonga	N 43° W 04°	99.9	chloroplast DNA extract	1 (18 646)*

Numbers given in parenthesis are the number of single-end reads x 1000. * paired-end reads.

Total DNA extraction

Total DNA extractions from samples 1 to 16 of *J. vulgaris* (Table 1) were carried out on five leaf punches of 1cm diameter each, using the CTAB extraction protocol of Doyle and Doyle (1987).

Long range PCR

To develop primers for long range PCR, the sequences of *Helianthus annuus* (NC007977), *Lactuca sativa* (DQ383816) and *Guizotia abyssinica* (EU549769) were aligned with BioEdit. With the aid of this alignment and the annotation of *H. annuus*, primers were designed in conserved regions of genes. A total of 18 primer pairs was designed by Primer3 software, that collectively amplified the total chloroplast genome of *J. vulgaris* with overlapping fragments resulting in amplicons between 5808 and 11 110 base pairs (see supplementary Table 1 for primer sequences). For amplification, the Takara La Taq kit (Takara bio inc., Otsu, Shiga, Japan) was used. PCR was carried out in a total volume of 20 µL containing 8-80 ng DNA, 2.5 mM $MgCl_2$, 2.5 mM of each dNTP, 0.7 µM of each primer and 1 unit *Taq* DNA polymerase. The PCR cycling conditions were as follows: 1 min at 94ºC; 30 cycles of 10 s at 98 ºC and 12 min at 69 ºC; followed by 10 min at 72 ºC. PCR products were loaded on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light to check for amplification. If the PCR products contained more than 1 band, the total product was always loaded on a 1% agarose gel and bands of the right size were cut out of the gel. To extract and purify the DNA fragments from the gel, the Wizard SV gel and PCR Clean-Up System of Promega was used. All cleaned PCR products were run on a gel to estimate the amount of product and in addition the amount of DNA was quantified with an ND-1000 spectrophotometer (Nanodrop Technologies). All 18 amplicons for each individual sample were pooled in equal molar ratios containing roughly 200- 300 nanogram of DNA resulting in 16 pooled samples of 75 µL each.

Sequencing

For sequencing of the cpDNA, three lanes on an Illumina sequencer (Illumina 1G/Solexa, Illumina Inc., San Diego, CA) were used. Sequencing was carried out at the Leiden Genome Technology Center. In the first lane, the DNA isolated from the chloroplasts of sample 17 was run with paired-end reads of 32 basepairs. In the second lane the pooled long range PCR products of samples 1 to 13, 15 and 16 were run and in the third lane sample14. Both were single-end runs of 35 basepairs (Table 1). Sample 14 was run in a separate lane because of its low DNA concentration. Preparation of all products was done following the protocol of Illumina kits with minor modifications. For sample 17, DNA was fragmented by a nebulizer using 32 psi N_2 for 6 minutes. After purification, the DNA was eluted in 15µL elution buffer. The samples were blunt-ended with T4 DNA polymerase, Klenow polymerase and T4 polynucleotide kinase. After purification, an A-residue was added to the 3-end of the DNA fragments using Klenow fragment (3'to 5'exo minus). Purification was done with a Qiagen MinElute column. Adapters of the paired-end adapter oligo mix were ligated to the DNA fragments. After purification with a Qiagen MinElute column, adapter-ligated DNAs in the range of 200-250bp were size selected using agarose electrophoresis. Products were isolated from the gel using a QIAquick Gel Extraction Kit and after purification a PCR was done.

For samples 1 to 16 (Table 1), sonication with a bioruptor was used to fragment the DNA. This machine was placed in a room at 4°C and was kept cool by adding ice. For a total of 15 minutes, the machine was set on 30 seconds active and 30 seconds inactive. This sonication step was repeated four times. All other steps were the same as done for sample 17 except for the PCR step. Unique index tags of six bases provided in the Multiplexing Sample Preparation Oligonucleotide kit were added in the PCR step to discriminate between the 16 samples. The amplified libraries were quantified by lab-on-a-chip (Agilent Technologies) followed by equimolar mixing of 10 nM per sample. Cluster generation was performed after applying 6 pM of each sample to the individual lanes of the Illumina flow cell and sequencing was carried out on the Illumina Genome Analyzer according to the manufacturer's instructions. Image analysis and base-calling were performed using the Illumina Pipeline 1.3.2, where sequence tags were obtained after purity filtering. This was followed by an alignment using MAQ.

Data filtering and genome assembly

Sample 17 from the first lane was used to assemble a draft cp genome of *J. vulgaris*. The software package MAQ v0.5.0 was used to map all quality-filtered paired reads of the first run against the chloroplast genome of *H. annuus*. To solve gaps in this consensus sequence, a *de novo* assembly was done with the same data using the software package Velvet v 0.6 (parameters: hashlength = 21), which produced 37 747 contigs. To find contigs with homology to the reference, these contigs were aligned to the *H. annuus* reference sequence with the program Mummer v3.0. The contigs having homology to the reference were extended by using the original reads with Velvet. These extended contigs were aligned to the reference of *H. annuus* with Mummer once again, and the contigs which assembled properly were saved. These final contigs were aligned against the consensus sequence; as a result some of the gaps in the consensus were solved. A new MAQ alignment was performed, mapping all the Illumina reads against the last consensus sequence made, to produce the draft sequence.

Bridging the gaps that where still in the draft sequence

The draft sequence still contained 23 gaps with an average gap length of 394 base pairs. Gaps were bridged by adding the data from the runs of the cpDNA amplified by long range PCR of 16 individuals. These data were used in Velvet to produce a *de novo* sequence (parameters: hashlength = 21, short fastq reads). The resulting *de novo* contigs were aligned against the draft sequence in Blast's bl2seq multiple sequence aligner. In this way, five gaps with a total of 1822 basepairs were bridged. The last 18 gaps were bridged by developing primers around the gaps, and traditional Sanger sequencing to yield the final complete cp genome.

Annotation

The program DOGMA was used for annotating all genes and to identify rRNAs and tRNAs. A circular cp genome map (Fig. 1) was drawn using the program GenomeVx.

Comparison of the chloroplast DNA of J. vulgaris with other Asteraceae genomes analysed

A total of 22 conserved protein-coding genes from five species, extracted from all available complete chloroplast genomes from Asteraceae deposited at NCBI GenBank (*Helianthus annuus*, NC007977; *Lactuca Sativa*, DQ383816; *Parthenium argentatum*, GU120098; *Guizotia abyssinica*, EU549769 and *Jacobaea vulgaris,* HQ234669, were aligned using the pairwise automatic alignment tool in MacClade 4.06 with further adjustment by hand. To get insight in the informative character of the selected protein-coding genes Maximum Parsimony analyses were run on the individual alignments comprising a total of 33 669 basepairs (bp) with PAUP* 4.0b10 using heuristic search, random addition with 100 replicates, and TBR swapping. The relative robustness for clades found in all single Most Parsimonious Trees (MPTs) was assessed by performing 1000 replicates of bootstrapping using fast, stepwise additions, TBR branch-swapping with 10 random taxon additions per replicate, MULTREES on, and holding 100 trees per replicate. We also calculated tree lengths and CI and RI values measuring the extent of homoplasy.

Detection of polymorphic loci

For visualizing the output of all reads, Mapview was used. This program visualizes all reads that are mapped against the reference genome. Furthermore it can produce a SNP list. The final assembled cp genome was used as a reference. To find SNPs, genomes of individuals 1-17 were used. SNPs were only added to the list if at a least one individual that varied from the reference genome had a coverage of at least 30 reads traversing that particular nucleotide and only when SNPs were located outside A and T polymer regions. Potential microsatellite regions were tracked by looking for 10 or more repeats of A and T nucleotides.

Results and discussion

Construction of the chloroplast genome of *J. vulgaris*

The chloroplast genome of *J. vulgaris* is 150 686 bp in length. The genome contains two inverted repeat (IR) regions of 24 777 bp each. The inverted repeats are separated by a large and small single-copy region (LSC and SSC) of 82 855 and 18 277 bp, respectively. The genome comprises 81 protein-coding genes of which 7 are located in the IRs. *Ycf1* lies partly in the inverted repeat and the single copy region. The four rRNA genes are all located in the IR. There are 29 unique tRNA genes. Twenty two tRNA genes are located in the single copy region whereas the others are located in the IR (Fig. 1).

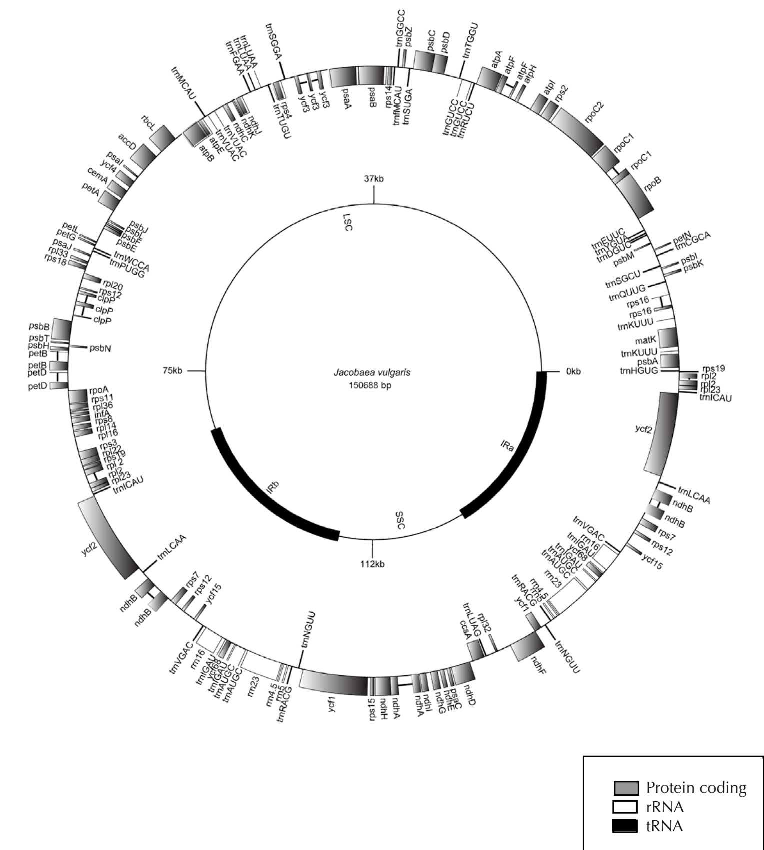

Fig. 1 Representive map of the chloroplast genome of *Jacobaea vulgaris* (Genbank Accession HQ234669).

The single lane on Illumina yielded sufficient reads to map more than 99.9 % of the complete cp genome of *J. vulgaris*. For the pooled individuals, on average 92% of the whole genome was mapped. There was a highly significant correlation between the number of reads and percentage of the genome mapped (Fig. 2). From the figure it is estimated that approximately 1 300 000 single-end Illumina reads of 32 basepair are needed to reach a mapping percentage higher than 99.9% of the cp genome of *J. vulgaris*.

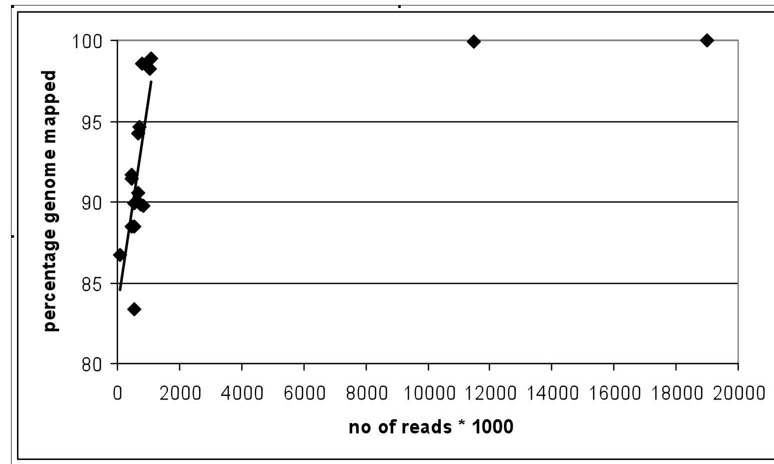

Fig. 2 Number of Illumina sequencing reads plotted against percentage of the chloroplast genome mapped for 17 individuals of *J. vulgaris*. There is a positive relationship between percentage genome mapped and nr of reads if the two points with complete mapping are excluded (r=71, n=15, p<0.001).y = 0,0126x + 83,544

Comparison of the sequencing success of cpDNA extracted from chloroplasts with amplified cpDNA using long range PCR

For the first lane with cpDNA extracted from isolated chloroplasts, a paired-end run was carried out on the Illumina platform, yielding 582 Mb of sequence with a read length of 32 bp. Of all reads, only 2.1% (391 604 reads) mapped against the chloroplast genome of *H. annuus*. The obtained reads covered 99.9% of the cp genome of *J. vulgaris* (Table 1, Fig.3). The average coverage was 83 with a coefficient of variation of 0.34 (Fig. 4).

For the other two lanes, containing long range PCR products of 15 individuals in one lane and the long range PCR products of one individual in a separate lane, a single-end run was carried out on the Illumina platform. This run yielded reads of 35 basepairs resulting in 339 and 388 Mb of sequence, respectively. For both lanes more than 99.9% of the reads (96 894 177 and 11 075 400 resp.) mapped against the chloroplast genome of *H. annuus*. In both lanes the reads obtained covered more than 99.9% of the cp genome of *J. vulgaris* (Table 1, Fig. 3). The average coverage obtained for both lanes combined was 4920 x. Average coverage varied largely between primer pairs, with average coverage ranging from 542 x for the lowest to 19755 x for the highest primer pair (Fig.4). The average coefficient of variation of coverage within primer pairs, averaged over all primer pairs, is 1.04 (Fig. 4). In summary, the variation was three times higher than that obtained with the direct cpDNA extraction.

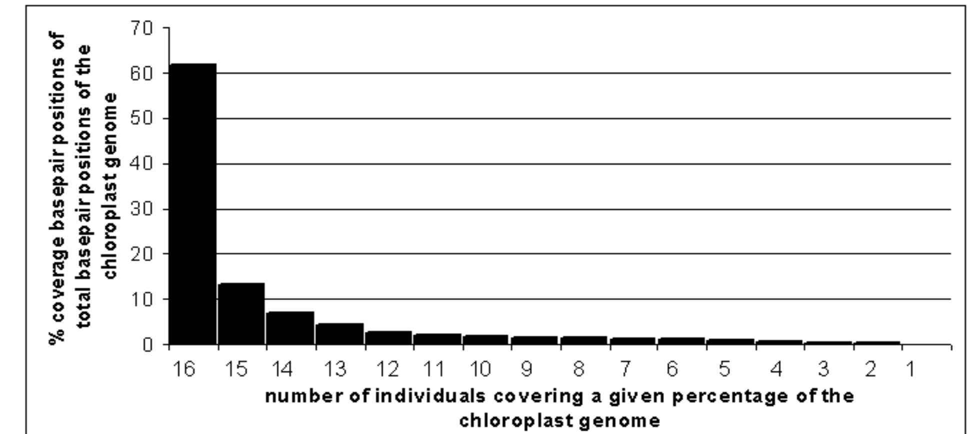

Fig. 3 The column labelled with 1 indicates the number of base pair positions in percentages of total basepair positions that was only covered once in any of these 16 individuals. So, 17 basepairs in the genome were covered by only one individual (less than 0.02 %). To the other extreme the column labelled with 16 indicates the number of base pair positions in percentages of total basepair positions that was covered in all 16 individuals. So, 92804 basepairs were covered by all individuals (more than 60 %).

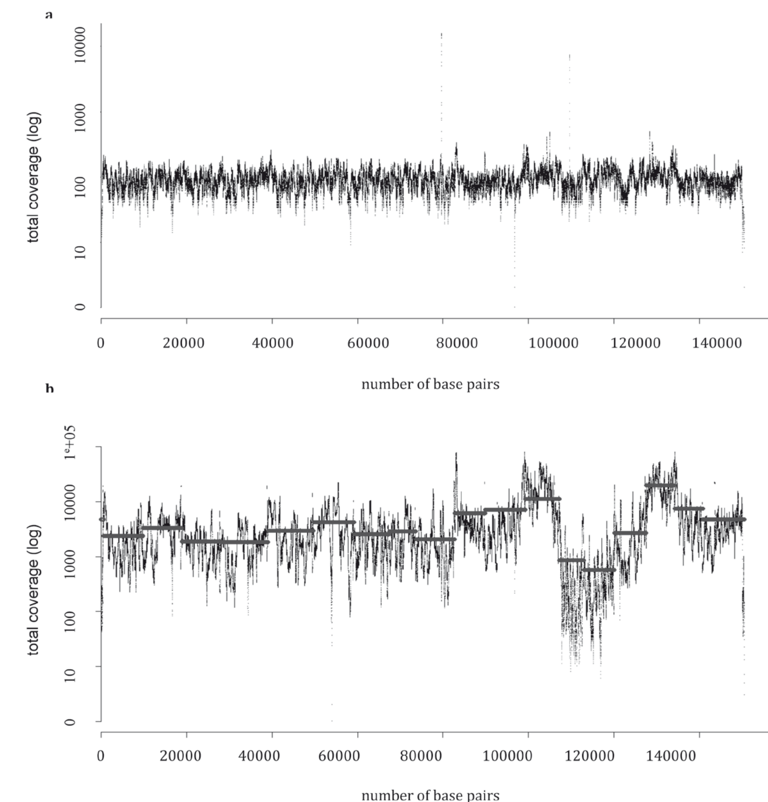

Fig. 4A Whole chloroplast genome coverage plotted for individual 17 of *Jacobaea vulgaris,* of which DNA was obtained by using the chloroplast extraction method. Average coverage = 83 bases per bp and coefficient of variation= 0.34

Fig. 4B Whole chloroplast genome coverage plotted for 16 individuals of *Jacobaea vulgaris* run in two lanes total of which DNA was obtained by using the long range PCR method. The 19 lines plotted indicate the average coverage of every primer. Average coverage= 4920 bases per bp and coefficient of variation= 1.04.

However, extraction of chloroplasts and subsequent extraction of DNA from these chloroplasts was not very efficient for sequencing the complete chloroplast genome. The cpDNA extract still contained around 98% of non cpDNA. The low efficiency of the chloroplast extraction method might be due to the fact that (nuclear) DNA sticks to the surface of the chloroplast or to a shortage of DNAases to remove DNA in the intact chloroplast solution. Furthermore the low efficiency can be caused by poor lysis of the chloroplasts. In contrast, the cpDNA amplified with long range PCR contained less than 1% non cpDNA. Apparently, long range PCR worked very efficiently in *J. vulgaris* and results were much better than the results obtained with the same method for *Pinus* cpDNA sequencing where non cpDNA ranged from 19 to 24%.

Although the number of cpDNA reads obtained with the chloroplast extraction method was far lower than that obtained with long range PCR methods, the variation in coverage over the total chloroplast genome was approximately 3 times lower (Fig 4). Moreover, the variation in coverage of the long range PCR products was primer dependent (see Fig. 4B). Despite the higher variation in coverage, using long range PCR products as templates for Illumina sequencing was far more efficient than using cpDNA directly. Moreover, the cpDNA extraction method proved to be cumbersome because we needed 30 grams of fresh material per individual.

Comparison of the chloroplast DNA of J. vulgaris with other Asteraceae genomes analysed

When comparing the full chloroplast genome of *J. vulgaris* with all complete Asteraceae chloroplast genomes (including those from *Guizotia abyssinica, Helianthus annuus, Lactuca sativa* and *Parthenium argentatum*), a few regions (*trnS-trnC* and *trnE-rpoB*) could not be aligned because these regions were absent in *Parthenium argentatum*, and most other regions showed almost no sequence divergence. Regions that could be aligned and that showed moderate sequence divergence between these five species are listed in Table 4. Five markers (*ndhC-trnV, ndhC-atpE, rps18-rpl20, clpP* and *psbM-trnD*) contained parsimony-informative characters higher than two percent and contained equally high phylogenetic information when compared with other phylogenetic markers that are frequently applied among Asteraceae species such as *trnL-trnF* (6.9%), *trnH-psbA* (1.7%), *rbcL* (1.4%), *rps16* (0.5%) and *ndhF* (0.4%). In Figure 5, the corresponding single MPTs are depicted.

In a former comparison with *H. annuus* against *L. sativa* and with *H. annuus* against *G. abyssinica*, the regions *ndhC-trnV* and *clpP* were already identified as divergent regions within the Asteraceae. CI indexes of the newly discovered phylogenetic markers, indicating homoplasy, of the newly discovered markers were all in the same range as the commonly used markers except for *ycf3-trnS* and *cemA*, which had slightly lower values. RI values ranged from 0.52 to 0.83 for the commonly used markers and from 0.50 to 1.00 for the newly discovered markers.

Analysis of all 22 regions combined resulted in a congruent topology with high support for all internal nodes. Gene trees can be incongruent with species trees when evolution of genes and species did not occur congruently. Gene trees of five regions *(trnL-trnF, clpP, psbM-trnD, rps8-rps14* and *rps15*) were found to be incongruent with the generally inferred species tree of the Asteraceae species analysed (Table 4; Fig. 5).

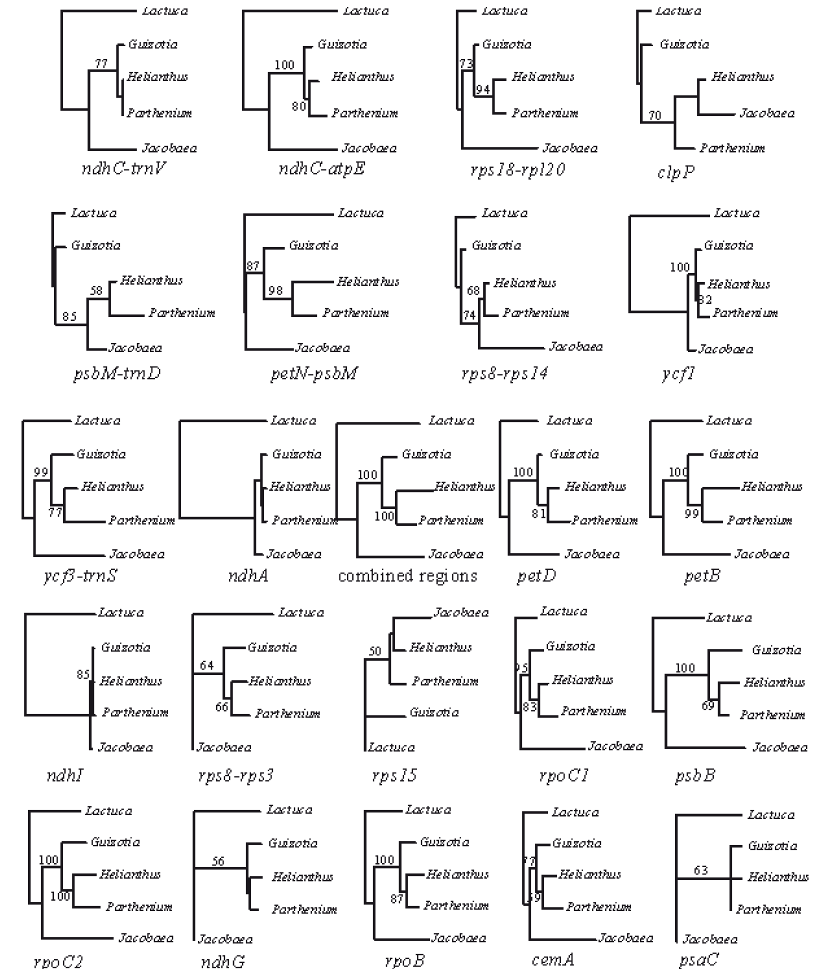

Fig. 5 Phylograms derived from Maximum Parsimony (MP) analysis of alignments of DNA sequences of 5 different Asteraceae species of a total of 27 individual chloroplast regions indicated below the trees. The phylogram called "Combined regions" in the middle is derived from MP analysis of all 27 regions together.

With a length of 150 686 base pairs, *J. vulgaris* has the smallest chloroplast genome compared to the four other Asteraceae cp genomes sequenced so far. The length is 2215 basepairs less than the largest cp genome of *Parthenium argentatum*. The genome is identical in gene content to *Helianthus annuus* and *Lactuca sativa* and differs in gene number with *Guizotia abyssinica* (which has 1 gene less) and *Parthenium argentatum* (which has 4 genes more). Although the similarity in gene content was high, few non-coding regions showed a high sequence divergence between the five Asteraceae species. A number of regions showing sequence divergence between these species contained a high phylogenetic content compared with the standardly applied phylogenetic markers used in the Asteraceae. Those regions seem promising for development of universal primers to further investigate clades in molecular phylogenies of Asteraceae hitherto unresolved. Furthermore, many of these regions are not yet used in angiosperm molecular phylogenetic studies and seem worthwhile to investigate further.

Table 4. Promising regions identified for molecular phylogenetic studies of Asteraceae by comparison of the full chloroplast genomes of *Guizotia abyssinica, Helianthus annuus, Jacobaea vulgaris, Lactuca sativa* and *Parthenium argenatum*.

Region	Length (bp)	Tree length	CI	RI length	Pars. inf. char. (%)	Topologies gene vs. species tree
trnL-trnF[1]	360	100	0.91	0.64	6.9	incongruent
ndhC-trnV	1189	520	0.89	0.88	4	congruent
ndhC-atpE	2376	665	0.96	0.75	3.5	congruent
rps18-rpl20	282	50	0.96	0.78	3	congruent
clpP	889	181	0.97	0.79	2.6	incongruent
psbM-trnD	800	114	0.92	0.55	2.5	incongruent
petN-psbM	569	92	0.97	0.83	2	congruent
rps8-rps14	219	29	0.96	0.75	2	incongruent
ycf1	5811	878	0.94	0.59	2	congruent
ycf3-trnS	1075	232	0.76	0.67	2	congruent
combined regions	40449	7719	0.97	0.62	1.8	congruent
ndhA	2317	208	0.94	0.70	1.7	congruent
trnH-psbA[1]	1571	172	0.92	0.52	1.7	congruent
petD	1266	108	0.97	0.86	1.6	congruent
rbcL[1]	1458	96	0.95	0.76	1.4	congruent
petB	1490	115	0.96	0.75	1.3	congruent
ndhI	547	241	0.95	0.83	1	congruent
rps8-rps3	2451	262	0.94	0.50	1	congruent
rps15	338	27	0.93	0.50	1	incongruent
rpoC1	780	82	0.97	0.80	1	congruent
psbB	1561	78	0.99	0.93	0.8	congruent
rpoC2	4609	260	0.97	0.81	0.8	congruent
ndhG	540	31	1.00	1.00	0.7	congruent
rpoB	3606	133	0.97	0.83	0.6	congruent
rps16[1]	1159	101	0.99	0.83	0.5	congruent
cemA	690	47	0.80	0.75	0.4	congruent
psaC	264	10	1.00	1.00	0.4	congruent
ndhF[1]	2232	156	0.98	0.67	0.4	congruent

The consistency index (CI) and retention index (RI) were calculated with autapomorphic characters excluded.
[1] Commonly used phylogenetic markers included for comparison

Detection of polymorphic loci

Single Nucleotide Polymorphisms (SNPs)

The 17 individuals of *J. vulgaris* yielded a total of 32 SNPs (Table 2A), which is on average 1 SNP per 4705 bp. In 66% of the cases a SNP allele was found only in a single individual. Fifty nine percent of the SNP polymorphisms where substitutions from a purine to a pyrimidine or vice versa. No SNPs were found in tRNA's (Table 3). Within the single copy region (LSC and SSC) SNPs were almost equally divided over coding DNA (tRNA + exons+ genic) (13) and intergenic spacers and introns (19). However, in the coding DNA,on average 1 SNP every 4573 bp was found compared to 1 SNP on average for every 2780 bp in intergenic and introns spacers (Table 3). Within the genes 2 SNPs were located in introns, this is on average 1 SNP per 3439 bp compared to 1 SNP per 4811 bp located in coding gene sequences (genes + exons) (Table 3). Of the 13 SNPs found in coding DNA, 3 resulted in non-synonymous substitutions (Table 2A).

Table 2. List of positions and variants of single nucleotide polymorphisms and microsatellites in the chloroplast genome of *Jacobaea vulgaris*.

A) SNP positions, alleles with the most occurring allele first, frequency of the least occurring allele in 17 individuals of the cp genome of *J. vulgaris* and region and locus of these SNPs.

Position	Alleles	Freq.	Region	Locus	Position	Alleles	Freq.	Region	Locus
165	T/A	0.13	Intergenic	*trnH-GUG/ psbA*	61 436	C/T	0.31	Genic	*petA*
4032	**A/C**	**0.06**	**Intergenic**	***matK/ trnK-UUU***	65 579	G/C	0.06	Intergenic	*trnP-UGG/ psaJ*
5555	**A/T**	**0.13**	**Intron**	***rps16***	66 056	T/G	0.19	Intergenic	*psaJ/ rpl33*
7837	**A/C**	**0.06**	**Intergenic**	***psbK/ psbL***	67 055	G/A	0.25	Intergenic	*Rps18/ rpl20*
11 353	**C/A**	**0.06**	**Intergenic**	***trnY-GUA /trnE-UUC***	67 963	T/C	0.69	Intergenic	*Rpl20/ rps12*
18 287	**A/C**	**0.13**	**Exon**	***rpoC1***	69 567**	T/C	0.06	Exon	*clpP*
22 648	C/T	0.06	Genic	*rpoC2*	70 234	T/G	0.06	Intron	*clpP*
24 906	T/G	0.38	Intergenic	*atpI- atpH*	92 417*	C/T	0.06	Intergenic	*trnL-CAA/ ndhB*
31 299	C/A	0.06	Intergenic	*trnT-GGU/ psbD*	97 496*	C/A	0.06	Intergenic	*Rps7/ ycf15*
39 790	A/G	0.44	Genic	*psaA*	106 663*	T/G	0.06	Intergenic	*trnR-ACG/trnN-GUU*
39 829	G/A	0.13	Genic	*psaA*	106 664*	C/A	0.06	Intergenic	*trnR-ACG/ trnN-GUU*
43 765	C/T	0.06	Intergenic	*Ycf3/ trnS- GCA*	108 200**	G/C	0.25	Genic	*Ycf1*
47 181	G/C	0.06	Intergenic	*trnL-UAA/ trnF-GAA*	**118 779**	**C/G**	**0.06**	**Genic**	***ndhD***
49 751	C/T	0.06	Genic	*ndhC*	123 423	A/C	0.06	Intergenic	*Rpl32/ ndhF*
53 025	G/A	0.06	Genic	*atpB*	124 027	C/T	0.06	Genic	*ndhF*
60 245	**C/T**	**0.06**	**Genic**	***cemA***	124 035**	C/T	0.06	Genic	*ndhF*

SNPs that were tested for multiple individuals with high resolution melting are indicated by bold typeface. SNPs located in the Inverted Repeat are indicated with *. Non-synonymous substitutions are indicated with **.

B) Potential microsatellite loci, repeat, repeat length in the consensus chloroplast sequence and the region and locus of these repeats in the cp genome of *J. vulgaris*.

position of repeat	Repeat	Repeat length of consensus	Region	Locus
6705	**A**	**11**	**Intergenic**	***rps16/ trnQ- UUG***
12459	**T**	**14**	**Intergenic**	***trnE-UUC/ rpoB***
13143	A	10	Genic	*rpoB*
16413	T	10	Intron	*rpoC1*
17759	A	10	Exon	*rpoC1*
18185	A	10	Exon	*rpoC1*
24848	**A**	**17**	**Intergenic**	***atpL/ atpH***
27760	**T**	**15**	**Intergenic**	***atpF/ atpA***
27776	A	11	Intergenic	*atpF/ atpA*
34901	A	10	Intergenic	*trnS- UGA/ psbZ*
41459	**T**	**10**	**Intergenic**	***psaA- ycf3***
41471	A	13	Intergenic	*psaA- ycf3*
46228	A	14	Intergenic	*trnT-UGU/ trnL-UAA*
49996	G	11	Intergenic	*ndhC/ trnV-UAC*
53630	A	10	Intergenic	*atpB/ rbcL*
54013	**T**	**18**	**Intergenic**	***atpB/ rbcL***
58662	T	10	Intergenic	*psaL/ ycf4*
64247	A	11	Intergenic	*psbIE/ petL*
69969	A	11	Intron	*clpP*
70312	A	10	Intron	*clpP*
72916	A	11	Genic/Intergenic	*psbT/ psbN*
74047	A	11	Intron	*petB*
76775	**T**	**17**	**Genic**	***rpoA***
79191	T	13	Intergenic	*rps8/ rpl14*
79774	**A**	**10**	**Intergenic**	***rpl14/ rpl16***
81396	T	10	Intergenic	*rpl16/ rps3*
82909*	T	10	Intergenic	*rps19/ rpl2*
109743	**A**	**10**	**Genic**	***ycf1***
112000	A	11	Genic	*ycf1*
114539	T	10	Intron	*ndhA*
121458	A	11	Intergenic	*ccsA/ trnL- UAG*
121889	T	11	Intergenic	*trnL- UAG/ rpl32*
123661	A	10	Intergenic	*rpl32/ ndhF*
150626*	A	10	Intergenic	*rpl2/ tnH-GUG*

Microsatellites that were tested for polymorphisms in multiple individuals are indicated by bold typeface. Microsatellites located in the Inverted Repeat are indicated with *.

Table 3. Summary of number of basepairs, number of SNPs, number of basepairs per SNP, number of microsatellite regions and number of basepairs per microsatellite region.

	nr of bp		nr of SNPs		nr of bp/nr of SNPs		nr of ms		nr of bp/nr of ms	
	SCR	IR's	SCR	IR's	SCR	IR	SCR	IR's	SCR	IR
non coding DNA	41 688	8574	16	4	2606	2144	28	1	1489	8574
coding DNA	59 445	11 688	12	0	4954		5	0	11 889	
rRNA	0	4515	0	0			0	0		
non coding gene	6877	1339	2	0	3439		5	0	1375	
coding gene	57 733	11 174	12	0	4811		6	0	9622	

Reads derived from the inverted repeats are distributed randomly to IRa or IRb by the assembly software. However if IRa is different from IRb by an indel or SNP this would be observed as a polymorphism within an individual. That was however never observed as we specifically checked for this. In the one case where we found that the sequence of the inverted repeat of individual 11 was deviating from other individuals for 4 positions, these positions within individual 11 were fully homozygous in both IRa and IRb. All SNPs found in the Inverted Repeats, 2 x 4 in total, were located in the intergenic spacers of individual 11. The 4 SNPs found in indivual 11 in IRa were found in exactly the same place and the same mutation as in IRb. This suggests "concerted evolution" or gene conversion for the inverted repeat region. On average 1 SNP every 1808 bp was found in the intergenic spacers in the IR. For a subset of 11 SNPs, primers were developed (Table 2A) and several individuals were genotyped using high resolution melting. For all these individuals, the SNP polymorphisms were confirmed.

The number of SNPs that were found in this study might be slightly underestimated because the whole cp genome was not mapped with sufficient coverage to detect all SNPs in the 17 individuals analysed. Although the number of synonymous substitutions in chloroplast genes is on average at least three times lower than that of nuclear genes [24], we still found SNPs using chloroplast genomes of 17 individuals of *J. vulgaris* originating from different populations. We found that SNPs were 1.8 times more frequent in intergenic spacers and introns as compared to DNA coding genes. These findings are in line with the assumption that coding DNA generally evolves more slowly than non-coding regions.

The result that individual 11 has 4 SNPs in both inverted repeat regions suggest that a mechanism is present that provides simultaneous mutations in both IRa and IRb. In all 17 individuals the sequences of IRa and IRb did not differ from each other by a single base. The gene *Ycf*1 starts at the end of IRb and extends into SSR, to yield the full *Ycf*1 sequence. In IRa the *Ycf*1 gene starts but is not extended into SSR yielding a non-functional sequence. It suggests that there is a selective force that prevents that the inverted repeat regions start to deviate from each other even when all the mutations are located in the intergenic spacer or non-functional genes. As a consequence the inverted repeats may contribute to the structural stability of the cp genome. Two plant groups, legumes and conifers, lost their inverted repeat and comparative sequence studies showed that these chloroplasts experienced a four fold increase in silent substitutions compared with chloroplasts containing the inverted repeat.

Microsatellites

A total of 34 microsatellite regions were found with A/T repeats longer than 9 repeats, which is 1 microsatellite per 4432 basepairs. Only one microsatellite region was found with 11 G repeats and no repeats of 10 or more Cs were found in the chloroplast genome of *J. vulgaris*.

Within the single copy region 5.6 times as much microsatellite regions were found in intergenic spacers and introns compared to coding DNA (28 against 5 respectively). No microsatellites were found in the tRNA and rRNA. We found on average one microsatellite region every 1489 bp in intergenic spacers and introns against 1 out of 11 889 bp in coding DNA (Table 3). Within the genes microsatellite regions were almost equally divided over exons and genes (6) and introns (5). This is on average 1 out of 1375 bp for introns against 1 out of 96 222 bp for exons and genes (Table 3). This is not in accordance with the data of SNPs where the number of SNPs per bp was relatively almost the same for exons+ genes and introns. An insertion or a deletion in an exon or gene will lead to a frame shift and therefore likely leads to a non-functional protein. Both microsatellite regions and SNPs occur less in DNA coding regions (exons +genes +tRNA) compared to non-coding regions (intergenic spacers + intron). However, this difference is more marked for microsatellite regions than SNPs.

Of the 34 microsatellite regions, only one was located on IRb in an intergenic spacer. This is surprising because concerted evolution, as earlier suggested, should lead to exact sequence duplication in IRa compared to IRb, and therefore both Inverted Repeats should contain the same number of nucleotide repeats. Indeed we found a microsatellite region at the same place on both IRs, but this repeat was only 8 basepairs on IRa and is therefore not included in Table 2B. For 10 repeat regions, primers were developed and multiple individuals from different populations were genotyped (Table 2B). Optimization failed for one primer pair, but the other 9 regions were amplified and they were all polymorphic. We tested 93 *J. vulgaris* individuals in total and found that all were polymorphic with the number of alleles per locus varying from two to six with an average of 3.3 alleles per locus.

The number of microsatellite regions is promising for investigating allele frequencies in populations and eventually, together with the SNP data, tracing the source population(s) of non-native *J. vulgaris*. The number of variable microsatellites might be higher since we arbitrarily decided to include only mononucleotide repeats that were at least 10 base pairs long. We found that potential microsatellite regions were 4.7 times more located in intergenic regions and introns compared to coding regions. Because SNPs were only 1.8 times more located in intergenic regions and introns compared to coding regions, we conclude that, point mutations are more frequent in coding DNA than indels leading to frame shifts immediately. Although the location of potential microsatellite loci is certain, the repeat length is an approximation. During long range PCR and PCR steps in the sample preparation steps for the Illumina platform indels can occur in microsatellite loci, leading to less or more repeats. Consequently the Illumina reads for microsatellite loci differed, making it hard to deduce the repeat length. This could also be the explanation for finding a difference in repeat length of a potential microsatellite locus between the IRs.

In conclusion, we found promising regions for development of universal primers that can be used for further investigation of clades in molecular phylogenies of Asteraceae. Considering the number of SNPs and microsatellites found in this study, we recommend screening of the complete chloroplast genome to

find differences within a species. Despite the higher variation in coverage, using long range PCR products as templates for Illumina sequencing, seemed to be far more efficient than using cpDNA directly.

Acknowledgements

We thank T. Morley, J. Ireson, H. Auge, M. Bartelheimer, U. Schaffner, P. Olejnicrak, A. Balogh, E. van der Meijden and N. Sletvold for collecting seeds. Furthermore we would like to acknowledge P. Pelser for sharing information about phylogenetic markers currently employed within the Senecioninae. At last we are very thankful to H. Kirk for revising the English.

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Supporting Information

Table S1
Primers used for amplifying the chloroplast genome of *Jacobaea vulgaris* with long range PCR.

Primer name	Start position (bp) in cp genome *J. vulgaris*	Primer sequence (5'> 3')	Tm in C
1F	400	GAG CAT TAC GTT CAT GCA TAA CTT CCA TAC CAA GG	72.8
1R	9794	TCC ACT TCT TCC CCA TAC TAC GAG TGA AAG AGA AA	72.1
2 F	9287	GAC TGC AAA TCC TTT TTC CCC AGT TCA AAT C	71.6
2 R	18772	CGG TAG CTT GTC GGA AAC CCA GAG TCT TTA CT	72.2
3 F	18683	AAA GTG ATA GAC GGA ACT GCC ATG AAA CGA CT	71.9
3 R	27901	TGA ATA CGA GCA ATG CCG TCA CCT ACT TGA	73.3
4 F	27822	CCG ACG AAA TTA GTA ATA TTA TCC GCG AAC GTA	70.1
4 R	38959	TTT TCC TAG GTG CTC ATT TTG TAT GGG CTT TT	70.4
5 F	38857	CGG CTG GGT AGC AGG AGC AAC TTT TAA TTT AT	701.4
5 R	49604	ACG GTT TTT CTT TAT CCA TGG GCA ATG AGT TT	71.7
6 F	49517	CCC TTT CGC CAT GCA TAA ACT AAA CCA ACA AT	72.5
6 R	59127	CCA AGA TAA CTC GAG GTT CCA ACC AAC AAG AA	72.0
7 F	59072	TCG AAA AAC AGG TAA TTT CTG CTG GGC TGT TA	72.0
7 R	67422	AAT CAA TTT TCG TCG TTT GTG GAT TAC TCG AA	70.5
8 F	67238	GCG GTG GAT TCC TTT CAA CTT ACT TCT TTT ATG A	70.7
8 R	73610	CCA TCT CTA TCA AGA TGA CAG ACC CAT TCT CTG	70.7
9 F	73524	TTG GTA GTT CGA CCG TGG AAT TTC TTT GTT TC	71.7
9 R	82677	GGA TCG TAT GGT AGG ACA CAA ATT GGG AGA AT	71.0
10 F	82624	GCG AGA TCT ATT ATC GCT TTT TGC ATG TCC TC	71.6
10 R	89850	CCT TGA GAC TTG TTA TCC AGG AAC TTG TTC AGA	70.2
11 F	89724	CTT TTC GCT CCG CTT AGC CTT ATC CCT CTC TA	72.6
11 R	98855	CTC CCA AGG GCA GGT TCT TAC GCG TTA CTC	74.1
12 F	98754	GAA TCT CAT GGA GAG TTC GAT CCT GGC TCA	73.3
12 R	107188	TCC ATA ACG TGA GCT CGG AGA AGG AAG AGA TA	72.1
13 F	107123	TCA ATT CGG TCG TTG TGG TCG GAC TCT ATT AT	72.2
13 R	112952	TGC TCT GGC CCA TTT ACA GTT ATT GCT TCT GT	72.4
14 F	112751	ATG ATA GTC AAT ATG GGA CCT CAC CAC CCA TC	71.9
14 R	120095	ATA GAA ACA AAT AAC TCG CGC GGT CCA GAA TC	72.4
15 F	120036	TGT CAC GGC AGA TGT TCT ATG GAT ACA AGT TAT TT	70.31
15 R	127553	ACC CCA ATT ATG ACA TCC CTT CTC TCC CAC TT	73.0
16 F	127382	TTC GTA GCC ACG TGC TCT AAT CCT CTG AGC TA	72.8
16 R	134278	TAA TAC AGA GGA TGC AAG CGT TAT CCG GAA TG	72.3
17 F	134174	CCC TAC CGT ACT CCA GCT TGG TAG TTT CCA C	71.8
17 R	140762	GGG CGG AAC AGA TCT ACT AAT TCT TTG ATT CCA	71.7
18 F	140596	AAG ATC CCC TTT AAG ATC AAA CAA TTC CAT CG	70.2
18 R	433	CAC TTG GGC TGA TAT CAT TAA CCG TGC TAA CC	71.9

Chapter

6

Multiple introductions of the invasive species *Jacobaea vulgaris* and a reduced genetic diversity in its invasive area

Leonie Doorduin*, Jelle Zandveld*, Rolf Vossen§, Peter Klinkhamer*, Rene Glas* and Klaas Vrieling*,

* Institute of Biology, Section Plant Ecology and Phytochemistry, Leiden University, PO Box 9505, 2300 RA Leiden, the Netherlands

§ Leiden Genome Technology Center, Human and Clinical Genetics, Leiden University Medical Center, PO box 9600, 2300 RC Leiden, the Netherlands

Corresponding author: Leonie Doorduin, Tel.: +31715275117, fax: +310715274900
E-mail address: leoniedoorduin@hotmail.com and K.vrieling@biology.leidenuniv.nl

Introduction

Species have always invaded new areas. However, the rate and spatial scale has changed tremendously since the beginning of the industrial revolution 300 years ago (CS Elton, 1958). Human-assisted species invasion is considered a key threat to native biodiversity (DS Wilcove ,D Rothstein ,J Dubow *et al.*, 1998) because invasive species can alter species distributions, community structure and ecosystem processes. In turn these effects on biodiversity can lead to high economic costs (RI Carruthers, 2003). In the United States non-indigenous crop weeds cause an estimated reduction of 12% in crop yields. In economic terms this represents about 33 billion dollars in lost crop production annually (D Pimentel ,R Zuniga ,D Morrison, 2005).

Recently adaptive evolution has been highlighted as a key process in the success of invasive individuals (AR Kanarek ,CT Webb, 2010). Alternatively invasive individuals may have been preadapted such that individuals from the native area already contained traits that turned out to be beneficial in the new environment (KM Dlugosch ,IM Parker, 2007). In a number of species shifts were demonstrated in major traits and allocation patterns upon introduction in the new area (O Bossdorf ,H Auge ,L Lafuma *et al.*, 2005; J Joshi ,K Vrieling, 2005; EA Leger ,KJ Rice, 2003; E Siemann ,WE Rogers, 2001). To distinguish between the two possibilities mentioned above, it is necessary to compare traits of the exact source population with those from the invasive individuals (O Bossdorf ,H Auge ,L Lafuma *et al.*, 2005). This requires detailed information about the origin of the invasive populations which is mostly lacking. Preadaptation becomes less likely if multiple introductions from different areas have taken place, because there is little chance that multiple introductions from different source populations all contain "preadapted"indivduals. So the number of introductions, even as determination of (the) source population(s), can give insight if invasive species evolved after introduction or if they were already preadapted before introduction.

Whatever the mechanism, rapid evolution or preadaptation, the success of an introduced species depends on the amount of genetic variation introduced in the new area (NC Ellstrand ,KA Schierenbeck, 2000). A number of studies observed that genetic variation of invasive populations is decreased compared to the native populations (L Amsellem ,JL Noyer ,T Le Bourgeois *et al.*, 2000; A Grapputo ,S Boman ,L Lindstrom *et al.*, 2005; BC Husband ,SCH Barrett, 1991). Only a small part of the genetic variation of the native population is introduced and as a consequence not all alleles are present in the invasive area. Thus founder effects reduce the amount of genetic diversity in the invaded area compared to the native area (L Amsellem ,JL Noyer ,T Le Bourgeois *et al.*, 2000; S Lachmuth ,W Durka ,FM Schurr, 2010). A further decrease of genetic variation is brought about by initial small population sizes leading to inbreeding and genetic drift (H Meimberg ,JI Hammond ,CM Jorgensen *et al.*, 2006). If invasive individuals went through a bottleneck and the species was introduced once the level of genetic variation in the invasive area will be low. Than, it is unlikely this species becomes abundant due to reduction in genetic variation for most traits. Alternatively when invasive populations are founded multiple times and admixture of different source populations takes place in the invasive area genetic variation can be maintained or even increased and new combinations of traits may become invasive (BJ Genton ,JA Shykoff ,T Giraud, 2005; S Lavergne ,J Molofsky, 2007). It has been hypothesized that admixture may lead to problematic invaders capable of fast evolutionary response to selection pressure because of the increased genetic variation. (RA Hufbauer ,R Sforza, 2008; PJ Prentis ,JRU Wilson ,EE Dormontt *et al.*, 2008). In addition admixture can alleviate the inbreeding load (KFJ Verhoeven, M Macel, LM Wolfe et al, 2010).

Jacobaea vulgaris or tansy ragwort is a plant species that is native within Europe and western Asia and introduced circa 150 years ago into Australia, New Zealand and North America. Within the invasive areas this plant species received a pest status because of its toxicity to livestock and vigorous growth. Joshi and Vrieling (2005) showed that compared with native populations *J. vulgaris* from invasive areas grew bigger, produced more seeds and were better defended against generalist herbivores but less well defended against native specialist herbivores, which were absent in the areas where it was introduced (LJ Doorduin ,K van den Hof ,K Vrieling *et al.*, 2010; LJ Doorduin ,K Vrieling, 2011; J Joshi ,K Vrieling, 2005). The altered traits in the invasive area compared to the native area suggest that a fast evolution took place. However preadaption can not be excluded as traits of the invasive individuals stil show an overlap with traits in the native area. An AFLP study on 15 native- and 16 invasive populations of *J. vulgaris* showed that the amount of genetic variation did not differ between native and invasive populations (LJ Doorduin ,K van den Hof ,K Vrieling *et al.*, 2010). Furthermore the high levels of genetic variation in all studied invasive populations suggest that multiple introductions occurred followed by admixture (AV Suarez ,ND Tsutsui, 2008). However the source population(s) could not be clearly pinpointed due to limited sample sizes and the low resolution of AFLP markers (LJ Doorduin ,K van den Hof ,K Vrieling *et al.*, 2010). In this study we use a larger set of individuals to determine if and which multiple source populations founded the invasive populations. Furthermore we will use single nucleotide polymorphisms (SNPs) and microsatellites of the chloroplast genome. These markers have a higher resolution rate compared to nuclear markers used in the AFLP study because of maternal transmission only and the absence of recombination. To identify the source population(s) we need to know the genetic structure of individuals originating from the native area to pinpoint possible source populations which were not included in the set that was genotyped. Therefore we investigate with a Mantel test if genetic and geographical distances are correlated. We expect to find a higher degree of relatedness between individuals that are geographically closeby and less relatedness between individuals that are geographically widespread.

We address the following questions: 1) is ragwort introduced more than once and if so can preadaptation be excluded? 2) What is/are the source population(s) of the invasive ragwort populations? 3) are genetic distance and geographical distance positively correlated in the native area? 4) Does the genetic diversity between native populations differ? 5) Are ragwort populations in the native and the invasive areas genetically differentiated? 6) Is the genetic variation in invasive ragwort populations reduced compared to native populations?

Methods

Species description

Jacobaea vulgaris formerly known as *Senecio jacobaea*, is a monocarpic perennial plant species and belongs to the family of the Asteraceae. It is native to Europe and western Asia, ranging from Norway through Turkey, and from Great Britain to Siberia. In the 1850s this species was first reported from the east coast of Canada (Nova Scotia) (P Harris ,ATS Wilkinson ,ME Neary *et al.*, 1971) and shortly thereafter in New Zealand (1875) (GM Thomson, 1922) and Australia (L Schmidl, 1972). In 1900 *J. vulgaris* also invaded the west coast of North America (HM Gilkey, 1957) and in 1913 it was recorded on the

west coast of Canada (P Harris ,ATS Wilkinson ,ME Neary *et al.*, 1971). Control is difficult because the lifecycle can vary from annual to short-lived perennial and seeds remain viable in the soil for several years (E van der Meijden ,RE van der Waals-Kooi, 1979).

J. vulgaris contains about 37 different hepatoxic PA's and it causes four million dollar losses annually to cattle poisoning in Australia alone (PD Roberts ,AS Pullin, 2007).

In Great Britain *J. vulgaris*. is attacked by 30 specialist and more than 40 generalist herbivores (JL Harper ,WA Wood, 1957) while in the invasive area mainly generalist herbivores are reported (KE Frick, 1972). In several invasive populations biological control agents has been introduced with mixed results (M Julien, 1987).

Chloroplast markers

To identify the source population often polymorphic markers from neutrally evolving areas of the genome are used because these will not be affected by natural selection (RA Marrs ,R Sforza ,RA Hufbauer, 2008). The chloroplast genome is transmitted as a single locus through the maternal line and does not recombine. Dispersal of chloroplasts genomes in the population is limited because they are only dispersed through the seeds. As a consequence the chloroplast genome has an effective population size of approximately one- fourth of the nuclear genome (RL Small ,RC Cronn ,JF Wendel, 2004). So chloroplast markers are more affected by genetic drift but genetic patterns will fade away more slowly compared to nuclear markers (DE McCauley ,JE Stevens ,PA Peroni *et al.*, 1996) due to the lack of recombination. The low mutation rate of single nucleotide polymorphisms of the chloroplast genome (1.0 x 10^{-9} – 3.0 x10^{-9}) (KH Wolfe ,WH Li ,PM Sharp, 1987) and chloroplast microsatellites (3.2 x 10^{-5}- 7.9 x 10^{-5}) (J Provan ,N Soranzo ,NJ Wilson *et al.*, 1999) contributes furthermore to the conservation of genetic patterns. These characteristics make chloroplast markers good markers for finding (a) source population(s) in population studies if an appropriate number of polymorphic markers can be found. In other studies chloroplast microsatellites have already proven to be valuable for their polymorphisms (M Jakobsson ,T Sall ,C Lind-Hallden *et al.*, 2007; J Provan ,W Powell ,PM Hollingsworth, 2001; BA Richardson ,J Brunsfeld ,NB Klopfenstein, 2002).

Sampling

From each population, seeds of individuals growing at least 2 m apart were collected. From each maternal plant one seed was germinated and grown in a climate-room. Fresh leaf samples (5 leaf punches of 1 cm. diameter each) were collected from each individual and stored at -80 ºC. DNA was extracted using the CTAB protocol (JJ Doyle, JL Doyle, 1987).

In total 177 individuals were used for the analysis (see Table 1), 90 native individuals (11 populations) and 87 invasive individuals (29 populations). Since we expected to find less variation within populations of the invasive range, we chose to sample more populations but fewer individuals per population in the invasive range. However we were also interested in the genetic variation within invasive populations and therefore from every invasive area (New Zealand, Australia and North America) one population was sampled more intensively.

Detection of polymorphic loci on the chloroplast genome

In a previous study, the total chloroplast genomes of 12 native and 5 invasive individuals of *J. vulgaris* were sequenced using next generation sequencing techniques (LJ Doorduin ,B Gravendeel ,Y Lammers *et al.*, 2011). Comparison of the 17 chloroplast genomes with each other yielded 32 Single Nucleotide Polymorphisms (SNPs). In this study, eight SNPs were used to screen polymorphisms in 177 individuals. Two different methods were used; six SNPs were screened with high resolution melting and two SNPs, of which scoring by high resolution was difficult, showed good results with restriction mapping (Table 2).

In addition a total of 33 potential microsatellite regions with more than nine mononucleotide repeats were identified. Primers were developed to amplify the ten largest repeat regions (between 10 and 18 repeats). Nine of these amplified regions showed variation in repeat length between individuals (Table 3).

Table 1: Origin of sampled populations of *J. vulgaris*.
Pop. nr. = number of the population, AUS= Australia, CAN= Canada, NZ = New Zealand, USA = United States of America, N= number of individuals.

pop. nr.	country	location	latitude	longitude	N
	Invasive				
1	AUSba	Barramunga (Victoria)	E 143.41°	S 38.33°	3
2	AUSbe	Beech forest (Victoria)	E 143.33°	S 38.38°	2
3	AUSc	Cape Schank (Victoria)	E 144.54°	S 38.28°	3
4	AUSd	Dairy Plains (North Tasmania)	E 146.31°	S 41.38°	3
5	AUSf	Franklin (South Tasmania)	E 147.00°	S 43.05°	3
6	AUSt	Turton's Creek (Victoria)	E 146.14°	S 38.33°	12
7	AUSw	Wild Dog Road (Victoria)	E 144.15°	S 37.26°	2
8	CANcd	Cardigan	W 62.37°	N 46.14°	3
9	CANd	Dundas	W 62.31°	N 46.19°	3
10	CANg	Green Cables	W 63.24°	N 46.29°	3
11	CANc	Carvell	W 63.07°	N 46.15°	2
12	CANb	Beludere	W 63.07°	N 46.15°	4
13	CANe	Ellen's Creek	W 63.09°	N 46.15°	3
14	CANm	Marco Polo (Prince Edward Island)	W 63.20°	N 46.29°	2
15	NZms	Maruia (South Island)	E 172.13°	S 42.11°	1
16	NZmn	Mangatoki (North Island)	E 174.13 °	S 39.25°	1
17	NZcs	Craigieburn (South Island)	E 171.51°	S 43.06°	1
18	NZfs	Fox Glacier (South Island)	E 170.01°	S 43.28°	1
19	NZss	Southland (South Island)	E 167.55°	S 45.28°	2
20	NZhs	Haast (South Island)	E 169.02°	S 43.53°	7
21	USAh	Humboldt County (California)	W 123.52°	N 40.45°	10
22	USAs	Suprise Hill-Sylvia Lake (Montana)	W 115.21°	N 48.21°	2
23	USAin	Indian Creek (Oregon)	W 124.25 °	N 42.26°	2
24	USAi	Island Lake (Montana)	W 114.58°	N 48.14°	2
25	USAl	Larch Slope (Oregon)	W 121.50°	N 45.29°	1
26	USAn	No Bear (Oregon)	W 114.53°	N 48.14°	2
27	USAsi	Silvertown (Oregon)	W 122.47°	N 45.29°	2
28	USAsh	Surprise Hill (Montana)	W 114.59 °	N 48.15°	2
29	USAw	West Crest (Oregon)	W 121.50°	N 45.29°	3
	Native				
30	ENG	New Castle upon Tyne	W 01.36°	N 54.58°	10
31	FRA	Sainte Marguerite	E 00.50°	N 49.27°	10
32	GER	Halle	E 11.56°	N 51.28°	7
33	HUN	Lénárddaróc	E 20.22°	N 48.08°	7
34	IRE	Caherdaniel	W 10.06°	N 51.45°	6
35	NLv	Veluwe	E 05.24°	N 51.49°	8
36	NLw	Wageningen	E 05.39°	N 51.58°	8
37	NOR	Sør Trøndelag	E 63.00°	N 10.23°	7
38	POL	Warsaw	E 21.01°	N 52.13°	9
39	SPA	Porte de San Glorio	W 4.45°	N 43.04°	8
40	SWE	Uppsala	E 59.51°	N 17.38°	10

Table 2: Primers and probes used for SNP analysis in *J.vulgaris*
Primer = name of the primer, between brackets the used restriction enzyme, SNPpos = SNP position (bp) on cp genome *J. vulgaris* HQ234669, T = annealing temperature (°C), HRM = high resolution melting, HRMP = high resolution melting with probe, REST= restriction.

Primer	SNP pos	Primer sequence (5′> 3′)	T	Identification method
SNP 1F	4032	GACTTCGGTTTGCTCCCTTT	55	HRM
SNP 1R		CTTCTTTCACTTTTTCAATT		
SNP 2F	7837	AACCTTCGATTCAAACATTG	55	HRM
SNP 2R		CTCGCGGCTATCCGATAATT		
SNP 3F	11353	ATCAACTTCCTTTCATCTCC	55	HRM
SNP 3R		TTTATTTGTCAAGTCTACCT		
SNP 4F	18287	TTGTGGCTGCGGTGGCGACT	55	HRM
SNP 4R		GCAGCAATAACGCGTTGATC		
SNP 5F	60245	CAATCCAATTAATCAAGATA	55	HRM
SNP 5R		ATGAATATGACCCTCGTTGT		
SNP 6F	5555	TCATTTGTACTCATAACTCAAGTTCAA	60	HRMP
SNP 6R		CACGGATCCGAATCAAGAAT		
SNP6 probe		GATAGATATTTTTTTATTGAGTGGTCTTTAACCCC		
SNP 7F	118779	TGTCTTTACCACGAACAACTTTCCTTG	60	HRMP
SNP 7R		TTGTCCTATTTCCTTTATGTGGAAGA		
SNP7 probe		GTTTTACCAATATTTGCGGGTTCCTTAATTTTC		
SNP 1F (Dra I)	4032	GCCTTCCCGTATTGGGTACT	55	REST
SNP 1R	4032	GAAGCGAGACATTCGTCCAT		
SNP 3F (Sty I)	11353	CCATCTGATAGTAGGTTGCCAAA	55	REST
SNP 3R	11353	TTTATTTCCTAAGGGTGGTTGG		
SNP 8F (Xcm I)	39829	ACATGCCCAGTTGAGATGTG	55	REST
SNP 8R		GGCTAAGTGATACTGCACACCA		
SNP 9F (Bsr BI)	69567	CGAATTCAAAGTGCCATGCT	55	REST
SNP 9R		AAAAAGAATTGAAATCTACACATTGA		
SNP 10F (Bsa HI)	70234	TTTCTTGTTCTTAAACGAGCCTCT	55	REST
SNP 10R		CGTTTTCTCCCCAATCGAG		
SNP 11F (Ava I)	97496	CCAATTCCTTCCCGATACCT	55	REST
SNP 11R		GACTCACTAAGCCGGGATCA		

Table 3: Primers used for amplifying microsatellite regions in *J. vulgaris*.
Primer = primer name, Ms pos= start position of repeat (bp) in cp genome of *J. vulgaris* HQ234669, T = annealing temperature (°C), Label = used fluorescent label, Length of product = length of amplified PCR product in bp. Forward primers all contain the M13 primer sequence at the 5′end.

Primer	Ms pos	Primer sequence (5′> 3′)	T	Label	Length of product
MS 1F	6705	CACGACGTTGTAAAACGACCGAAT TGT CAATGATG	50	Fam	133-137
MS 1R		TGTGAAAAATAATGAGCATCCCTA			
MS 2F	41459	CACGACGTTGTAAAACGACCAATCACGCGAGCAGACATTAGCTATTA	50	Tamra	242-246
MS 2R		TTGGGAAGAACCAACCAAAG			
MS 3F	79774	CACGACGTTGTAAAACGACCGAGCCCCACTGTTATCTGCT	50	Fam	239-247
MS 3R		AATAGCAGCGTCCAAAATGC			
MS 4F	24848	CACGACGTTGTAAAACGACCGGGCCACCATTGACTAGTTT	48	Hex	259-264
MS 4R		CAAAGGGAATTTTAGGAAAAAGA			
MS 5F	109743	CACGACGTTGTAAAACGACCTGGTCCTCCTCGAAAAACAC	48	Tamra	255-257
MS 5R		CCTTTTCCGTTTGAGTTTCA			
MS 6F	54013	CACGACGTTGTAAAACGACCTGGAATTCGAACCTGAACTCT	48	Hex	238-243
MS 6R		TCGAAATACCTAAAAATCACTCAAA			
MS 7F	27760	CACGACGTTGTAAAACGACCATTGGGGACGATGAAACAAA	52	Fam	185-189
MS 7R		GCTCAATACGTTCGCGGATA			
MS 8F	12459	CACGACGTTGTAAAACGACCTCCACCTATCTCATAGATTCCAGTC	52	Hex	208-212
MS 8R		TGTGGACATTGCGTCTATCC			
MS 9F	76775	CACGACGTTGTAAAACGACCAGATCCTGGGAGGCAATTCT	46	Tamra	215-217
MS 9R		TCCCGGAATTTGATTGATTT			

Single Nucleotide Polymorphisms (SNPs)

High resolution melting

For SNP genotyping, two high resolution melting protocols were used. The first protocol was used to identify nucleotide changes from A/T to G/C. When strands from the DNA duplex are separated, fluorescence rapidly drops. Different alleles result in different melting temperatures with lower temperatures for A/T genotypes compared to C/G genotypes (GH Reed ,JO Kent ,CT Wittwer, 2007) . For remaining cases where only the base pair orientation changed (A to T and G to C) a probe was added that allows fine discrimination of variants under the probe (LM Zhou ,AN Myers ,JG van der Steen *et al.*, 2004). A single base change will cause the probe to melt at a lower temperature than if the probe is completely complementary. Primers were developed in such a way that the SNP was located in the centre of the PCR product (total PCR product of 100-150 bp). Probes were developed including one of the SNP variant in its sequence. For primer and probe information see Table 2.

Four SNPs were identified with the first protocol using a PCR mixture of 25 µL containing 15 µL mineral oil, 6.45 µL water, 1.0 µL 10x PCR buffer with 20 mM $MgCl_2$, 0.3 uL 10 µM forward and reverse primer (Table 2), 0.8 µL 2.5 mM dNTPs mixture, 0.1 µL 10 µM low calibrator oligo's, 1 µL LC- Green Plus, 0.1 µL 5U/µL *Taq* polymerase (Roche, Woerden, the Netherlands) and 2.0 µL genomic DNA (circa 10 ng/ µL). Reactions were carried out in a 96 well plate covered with thermowell aluminium sealing tape to avoid evaporation. The PCR cycling conditions were as follows: 10 minutes at 95ºC for initial denaturation; following by 40 cycles of 20 seconds at 95 ºC, 30 seconds at 55 or 60 ºC and 40 seconds at 72 ºC; followed by a final extension for 5 minutes at 72ºC and a final denaturation step of 1 minute at 95 ºC. For the two SNPs that only had a change in base pair orientation, the same protocol was used but without adding oligo's (LM Zhou ,AN Myers ,JG van der Steen *et al.*, 2004) . Furthermore primers were added in different amounts, 0.05 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM) with the probe added in the same amount as the reverse primer. To end up with 25 µL, 3.6 µL H2O was added in stead of 6.45 µL in the former protocol. The PCR cycling conditions were as follows: 10min at 95ºC for initial denaturation; following by 55 cycles of 20 seconds at 95 ºC, 30 seconds at 55 ºC and 40 seconds at 72 ºC; followed by a final extension for 5 minutes at 72ºC and a final denaturation step of 1 minute at 95 ºC. Melting analyses were performed on the LightScanner (Idaho Technology) at the Leiden Genome Technology Center. Melting curves were generated by monitoring the fluorescence of the saturating dye LC- Green Plus. Missing data of SNP 1 and SNP 3 were completed by restriction (see below for details).

Restriction mapping

At four SNP positions primers were developed to amplify products of around 200 basepairs with the SNP position located at 2/3 of the amplicon. As mentioned two of the six SNPs, with position 4032 and 11353, were already identified with high resolution melting, but missing data were obtained by restriction. For two SNPs all individuals were screened with restriction mapping. Individuals of which the cp genome was already sequenced (LJ Doorduin ,B Gravendeel ,Y Lammers *et al.*, 2011) and which represented different nucleotides were used as a control. Primers and restriction enzymes that were used are indicated in Table 2. Amplification was carried out in a 25 µL PCR reaction containing 16.0 µL water, 2.5 µL 10x PCR buffer, 1.0 uL 25mM MgCl2, 1.0 µL 10 µM forward and reverse primer, 2.0 µL 2.5 mM dNTPs mixture, 0.5 µL 5U/µL *Taq* polymerase and 1.0 µL genomic DNA (about 1 ng/ µL). The PCR cycling conditions were as follows: 5 min at 95ºC for initial denaturation; following by 40

cycles of 30 seconds at 95ºC, 30 seconds at 55ºC and 30 seconds at 72ºC; followed by a final extension for 5 minutes at 72ºC. PCR products were checked on a 1% agarose gel and the following mix was added to 2.5 µL of the visualized PCR products;16.0 µL water, 1 µL 10x recommend buffer for enzyme (New England Biolabs) and 1.0 µL of the restriction enzyme (see Table 2). After spinning the mix was incubated in a PCR machine for 16 hours at 37ºC. After incubation 7.5 µL restriction mix was loaded on a 1.5% agarose gel next to a control (uncut PCR product). With the help of the negative and positive control all samples on the gel were analysed. For samples that did not have a PCR product, the whole protocol was carried out again but this time with approximately 10 ng of DNA per sample. For three invasive individuals we were unable to amplify one locus resulting in three missing data points for the SNP data.

Microsatellites

Microsatellites were amplified using the M13-tailed PCR protocol to label the PCR products with fluorescent dyes (I Boutin-Ganache ,M Raposo ,M Raymond *et al.*, 2001). A PCR mixture with a final volume of 10 µL containing 4.9 µL water, 1.0 µL 10x PCR buffer, 0.4 µL 10 µM M13- tailed forward primer, 0.4 µL 10µM reverse primer, 0.4 µL 10µM M13-labelled primer (CACGACGTTGTAAAACGAC), 0.8 µL 2.5 mM dNTPs mixture, 0.1 µL 5U/µL *Taq* polymerase (QIAGEN, Venlo, the Netherlands) and 2.0 µL genomic DNA (about 0.1 ng/ µL). M13 primers were labelled with Fam, Tamra and Hex fluorescent dyes. The PCR cycling conditions were as follows: 5 minutes at 94ºC for initial denaturation; following by 40 cycles of 1 minute at 94ºC, 1 minute at the annealing temperature of each primer pair and 1 minute at 72ºC; followed by a final extension for 10 minutes at 72ºC. Primer information can be found in Table 3. All nine amplified microsatellites were pooled because they differed in length and fluorescent label, and run on a MegaBACE sequencer (GE Health Care, Eindhoven, the Netherlands) with ROX 400 as internal standard. The allele size of each microsatellite locus was scored with Fragment Profiler, version 1.2 (Amersham Biosciences, 2003). Samples with weak signals or unclear peaks were amplified and run again to reduce errors. One invasive individual still had an unclear peak for one locus and therefore this data point was considered as a missing value. In total 0.13% of the SNP and microsatellite data are missing (4 out of 3179 data points).

Data analysis

Combining of SNP and microsatellite data

Single Nucleotide Polymorphisms were scored as nucleotides whereas for microsatellites the number of repeat nucleotides was scored. To analyze both SNPs and microsatellites, data were combined by converting fragment sizes into a sequence. Sequences for microsatellites were formed by taking the longest repeat and replacing bases by gaps for smaller repeats. For SNPs the alternative base for that particular SNP was inserted. All microsatellites and SNPs were concatenated to one sequence per individual and used for TCS (M Clement ,D Posada ,KA Crandall, 2000) and Arlequin (L Excoffier ,G Laval ,S Schneider, 2005).

In contrast Fstat and Geneclass were run with fragment sizes, because these programs only taken into account allele difference rather than allele length differences and because sequences could not be analysed. For SNPs allele length 101 was given to one variety whereas 102 was given to the alternative allele.

Genetic diversity

First the distribution of alleles was determined for SNP and microsatellite data separately, by counting the number of individuals sharing alleles. The Fisher exact test calculated if there were significant differences between the native and invasive area in the distribution of alleles per locus.

After combining the datasets, the total number of alleles per locus of the native and invasive area was also determined and significance was tested with an independent sample T- test.

Genetic diversity over all loci (Hs) for native and invasive areas was calculated with the program Fstat (J Goudet, 1995). Hs was calculated following Nei's F- statistic (1987) with 1000 permutations.

The number of private haplotypes and the number of non-private haplotypes were counted for both native and invasive areas and were tested with a Chi-square test to test for differences between the native and invasive area.

Genetic differentiation between populations, regions and areas.

Genetic differentiation among populations, among regions in the invasive area and among the native and invasive area was analyzed with an analysis of molecular variance (AMOVA) using Arlequin 3.5. Significance of variance was tested by comparing obtained values to a null distribution generated by 1000 permutations. The correlation between genetic distance (Fst) and geographical distance within the native area was tested with a Mantel test (N Mantel, 1967).

Within population genetic distance of native populations

To compare the within population genetic distances, in each population the number of basepair changes between a pair of individuals was calculated for all possible combinations within a population. The average within population genetic distance is the average of all possible combinations within a population. Differences between populations in average within population genetic distance were tested with an ANOVA with population as a random factor.

Haplotype networks

We used the program TCS 1.21 to construct haplotype networks of native and invasive individuals alone and of all individuals together. Pairwise differences were calculated and the default 95% connectivity limit was used as the maximum of mutational connections between pairs (M Clement ,D Posada ,KA Crandall, 2000). The number of independent haplotype networks gives an indication about the diversity within the native and invasive area. Furthermore this program shows shared haplotypes between native and invasive individuals which can indicate potential source populations.

Assignment analysis

Rannala and Mountain (1997) (B Rannala ,JL Mountain, 1997) developed a Bayesian method to calculate for each invasive individual the likelihood that it is related to a native individual. In Geneclass 2.0 (S Piry ,A Alapetite ,JM Cornuet *et al.*, 2004) this method was used and likelihood scores were calculated. Because missing data strongly affect the outcome of likelihood scores, only individuals without missing data were used. In total 4 invasive individuals with one missing locus were not included in the analysis. Total likelihood scores were ordered from high to low. Populations containing native individuals with highest likelihood scores were most probably involved in introduction of invasive individuals. Furthermore for invasive individuals that had low likelihood scores for every comparison with a native individual, the source population was most likely not sampled.

Results

Genetic diversity

All of the eight SNP loci were polymorphic in the native area and six were polymorphic in the invasive area. Furthermore the distribution of the alleles over native and invasive individuals differed significantly for four out of eight loci (Fisher-exact test P< 0.05). The microsatellite loci of native individuals were highly polymorphic for every locus. For invasive individuals eight microsatellite loci were polymorphic whereas one locus was monomorphic.

We recorded 46 alleles for nine microsatellite loci in the 40 native and invasive populations. For the invasive area the number of alleles per microsatellite locus ranged from one to six. In the native area the number of alleles ranged from three to six. In the native area forty-one microsatellite alleles in 11 populations with 90 native individuals were found, compared to 30 alleles in 29 populations with 87 individuals in the invasive area. Sixteen private microsatellite alleles were present in the native area compared to five in the invasive area. The distribution of alleles over native and invasive individuals differed for seven out of nine microsatellite loci (Fisher-exact test P<0.05).

For the combined dataset of SNPs and microsatellites the total number of alleles per locus averaged over all loci did not differ between the native and invasive area (independent sample T- test, $F_{1,32,}$= 2.432 p= 0,158).

Genetic diversity (Hs) within populations was significantly higher in native populations (Hs =0.184) compared to invasive populations (Hs = 0.109) (Fstat, P < 0.05). Moreover the native area consisted of significantly more haplotypes than the invasive area, 63 versus 26 respectively (χ^2= 9.17 df=1, P< 0.01) despite equal sample size but a larger number of populations in the invasive area. The number of private haplotypes was also significantly higher in the native area, 46 versus 19 respectively (χ^2= 8.97 df=1, P< 0.01).

Within population genetic distance of native populations

The average within population genetic distance varied between 1.6 and 7.6 basepair changes with an average of 4.5 basepair changes for all populations. Hungary, France, Sweden and The Netherlands-Wageningen showed significantly higher within population genetic distances than the other populations (Fig. 1) suggesting that these are composite populations.

Genetic differentiation between populations, regions and areas.

Native and invasive populations were significantly genetically differentiated (Table 4). Of the total genetic variation, 23% was explained by native or invasive origin and 32% of genetic variation was due to differences among populations.

Within the native area only half of the total genetic variation was attributed to among population differences and the other half to variation within populations (Table 4).

Within the invasive area, the four regions (New Zealand, Australia, Canada east coast and North America west coast) accounted for only 14 % of the genetic variation, despite the large geographical distances. The among population genetic variation was 19% showing that the genetic differentiation in the invasive area is much smaller than the genetic differentiation between populations in the native area (Table 4). Variation among populations in the native area was not dependent on geographical distance (Mantel-test, Native area: r= -0.0918 n= 90, NS).

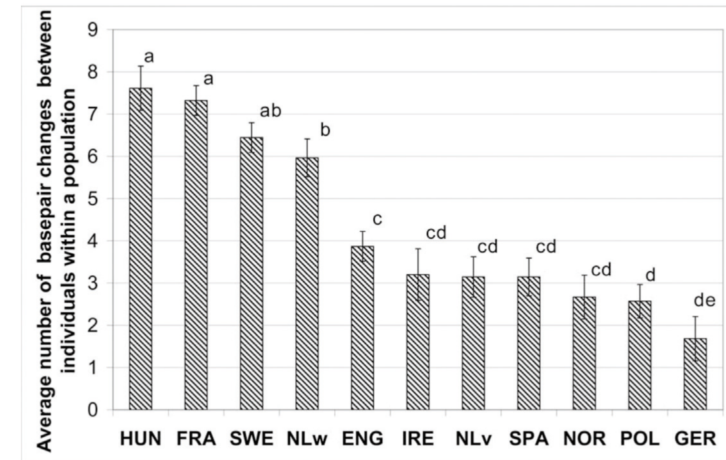

Fig. 1: Genetic distances within 11 native *J. vulgaris* populations. Different letters indicate significant differences. Bars indicate standard errors. Anova ($F_{10,646}$ = 22,98, p<0.001) For abbreviations of the populations see Table 1.

Haplotype network

Native area

For the native area we found 19 independent haplotype networks (Fig. 2). Thirteen of these networks consisted of single haplotypes and two networks consisted of two haplotypes from the same population (Fig. 2d). Four networks were found that consisted of 19,18,7 and 2 haplotypes, respectively (Figs. 2a,2b,2c,2d). The majority of the other native individuals were distributed over three large networks. One network, further on called the "North West European network" (Fig. 2a) consisted of all individuals from Ireland (6) and most individuals from Sweden (8), Norway (6) and The Netherlands-Veluwe (7), and one individual of The Netherlands-Wageningen. The second large network, further on called "South East European network" (Fig. 2b) consisted of all individuals from Germany (7) and most individuals from Poland (7), France (5), The Netherlands-Wageningen (6) and some individuals from Hungary (2) and England (1). The third network, further on called "England network" (Fig. 2c) consisted of individuals from England (7) Hungary (4) and The Netherlands-Wageningen (1). Most of the haplotypes in the native area are unique and only three haplotypes are shared by individuals from different native populations which is indicative of the large genetic variation found in the native area. The four networks were plotted on the geographic map showing the relatedness of these networks with particular geographic areas (Fig. 3).

Invasive area

In the invasive area five independent networks were detected by TCS. Three networks consisted of only one individual Carvell (CANc), Wild dog road (AUSw) and Humboldt County (USAh). One network consisted of two individuals namely Barramunga (AUSba) and Silvertown (USAs). The other network consisted of all other invasive individuals (Fig. 4).

The 19 independent networks of the native area and the five independent networks of the invasive area show that genetic diversity is higher in the native area compared to the invasive area. In contrast to the native area nine haplotypes are shared between individuals from different invasive populations. Surprisingly, shared haplotypes often contain individuals from the different invasive regions.

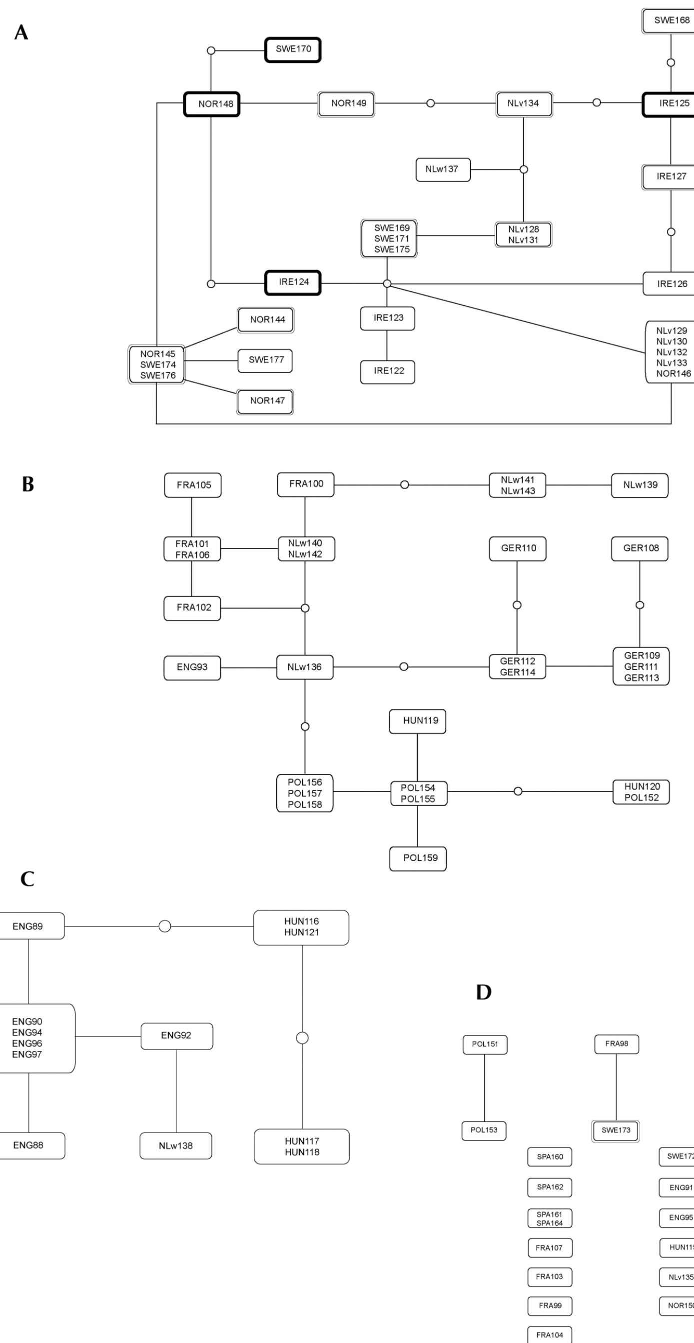

Fig. 2: Haplotype networks of *J. vulgaris* based on chloroplast microsatellites and SNPs in the native area. Abbreviations are the same as in Table 1. Numbers indicate individuals. A bold line around a haplotype indicates that an identical haplotype is also present in the invasive network. A double line indicates a haplotype that is assigned to an invasive haplotype with a high likelihood score above the cut-of point (Fig. 5). a) North-west European network, b) The south European network, c) The England network and d) Independent networks consisting of a single or two haplotypes.

Four haplotypes from the native area (2 from Ireland, 1 from Sweden, 1 from Norway), all belonging to the North West European Network, were shared by invasive individuals in Australia, New Zealand, the east coast of Canada and the west coast of North America (Figure 2a and 4) . All shared haplotypes between native and invasive areas consisted of only one native individual shared with several individuals from different populations and regions, the exception being the Irish haplotype that is shared only with four Canadian populations. The Irish, Swedish and Norwegian populations sharing the haplotypes with invasive populations can therefore be considered source populations.

Furthermore the shared haplotypes in the invasive area with individuals from different invasive populations that are geographically far apart suggesting either multiple introductions or geneflow between regions.

Assignment analysis

Likelihood scores of how well a native individual matches an invasive individual were ranked. The first 40 likelihood scores are the pairs that share an identical haplotype between the native and invasive area (Fig. 5). That the log likelihood scores differ for these individuals with exactly the same haplotype is caused by differences in frequency of alleles. The graph shows a sudden drop after the 164th pair (representing 2.2 % of all invasive individuals). All the individuals in these pairs have near identical haplotypes between native and invasive individuals. All native individuals with these near identical sharing haplotypes belong to the North-Western European haplotype network reaffirming that possible source populations are from this particular native area (Figs. 2 and 4).

Invasive individuals with likelihood scores beyond the 164th pair probably originate from other source populations than the native individual they formed a pair with. These source populations are not represented in this study.

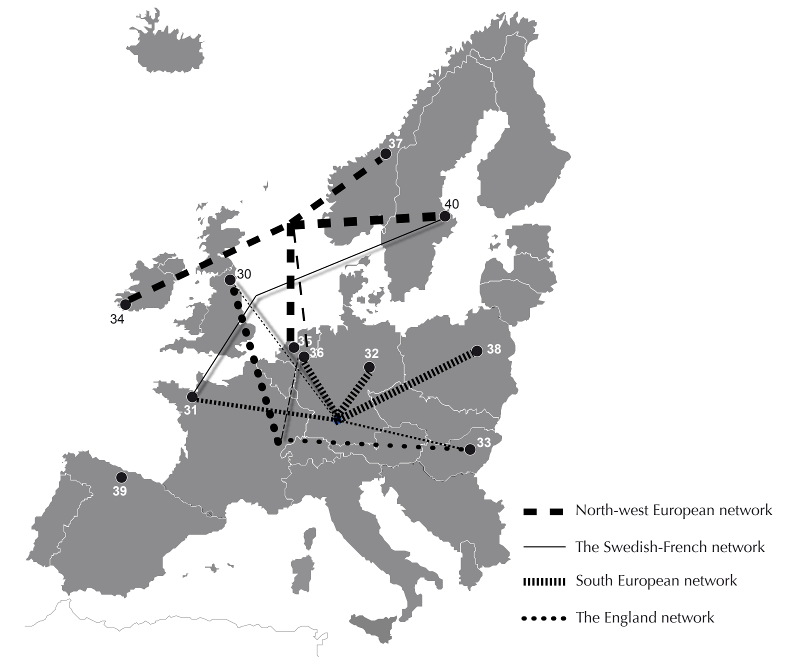

Fig. 3: Overview of four haplotype networks representing the native area in Europe. The thickness of the lines is proportional with the number of haplotypes in the network. Population number refers to Table 1.

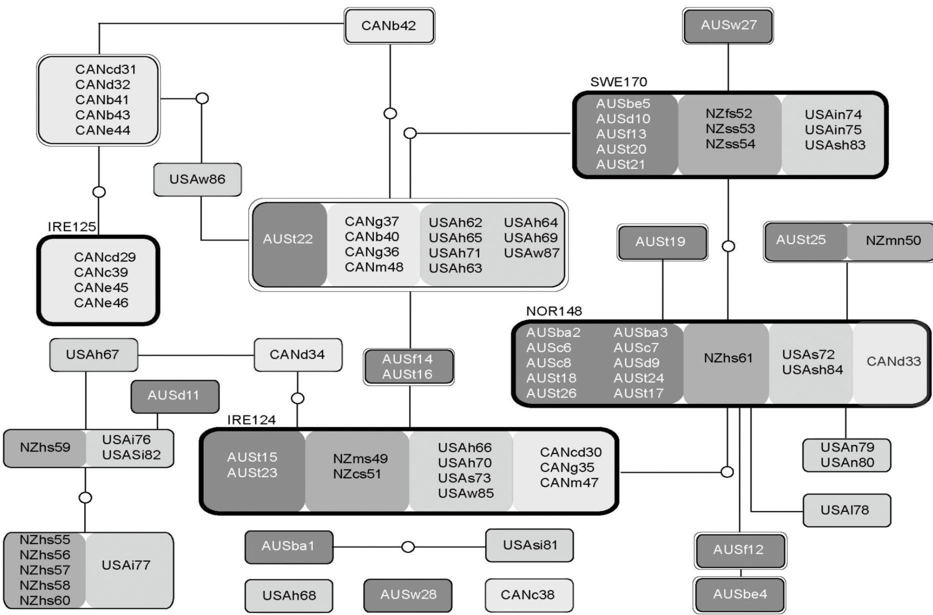

Fig. 4: Haplotype networks of *J. vulgaris* based on chloroplast microsatellites and SNPs in the invasive area. Abbreviations are the same as in Table 1. Numbers indicate individuals. A bold line around a haplotype indicates that an identical haplotype is also present in the native network. A particular native haplotype is indicated above the box. A double line indicates a haplotype that is assigned to a native haplotype with a high likelihood score above the cut-of point (Fig. 5) Grades of shading indicate the four invasive regions from dark to light: Australia, New Zealand, North America west coast, North America east coast.

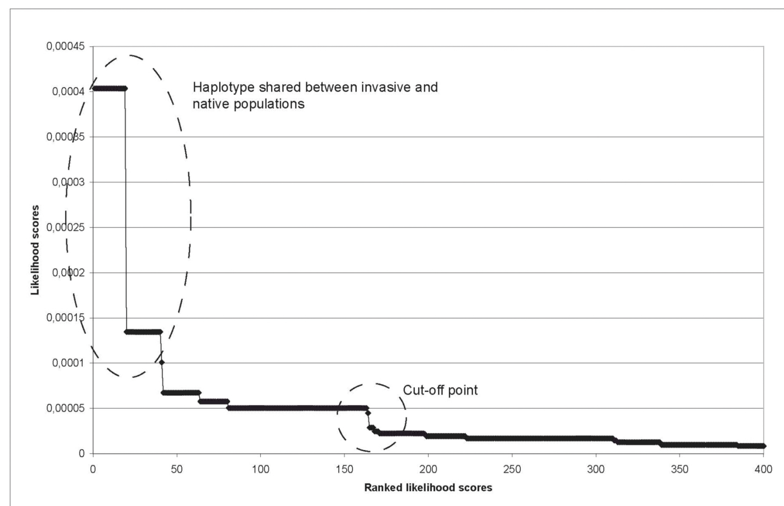

Fig. 5: The first 400 ranked likelihood scores of the assignment analysis between all possible pairs of invasive and native populations.
The likelihood scores in the circle on the left are likelihood scores for pairs with identical haplotypes in the native and invasive area. The likelihood scores for these pairs differ as the magnitude of the likelihood score depends on the allele frequencies. Individuals above the cut-off point have the highest chance of being the source population of (an) invasive individual(s). In total for 7470 pairs the likelihood scores were calculated, in the graph only the 400 highest likelihood scores are depicted.

Discussion

In this study we came a long way in establishing the source populations of invasive *J. vulgaris*. Our data strongly suggest that at least four but probably more introductions took place of *J. vulgaris*. The four native individuals from Ireland, Norway and Sweden sharing the same haplotype of invasive individuals are likely to be among the source population of the invasive individuals. All these populations belong to North-Western haplotype network. The assignment analysis showed that all native individuals with "near identical" haplotypes to invasive individuals were also only found in the North_Western European haplotype network. Collectively these results suggest that individuals from multiple source populations from North-Western Europe invaded Australia, New Zealand and North America. In the AFLP study of Doorduin et al 2010, populations from North-West Europe were the most likely source populations too. (LJ Doorduin ,K van den Hof ,K Vrieling *et al.*, 2010). A population from Ireland was assigned in both studies, Norway and Sweden were only assigned in this study and England only in LJ Doorduin ,K van den Hof ,K Vrieling *et al*, (2010). An explanation for this apparent discrepancy is that these samples, although originating from the same country, were sampled on different locations. This holds also true for Sweden, with high likelihood scores and an identical haplotype with invasive individuals in this study opposite to low assignment scores in the study of Doorduin et al (2010). In the invasive area nineteen private alleles were detected. Furthermore nine haplotypes were detected in the invasive area that were separated 2 or more mutations from the closest native individual. This indicates that we did not sample all native sources populations of the invasive individuals from this study. The latter shows that more source population are involved that have not yet been detected in the native area.

We found strong evidence that Irish populations were involved in the introduction of *J. vulgaris* to invasive areas. The Canadian individuals were sampled on the east coast of Canada. The introduction of *J. vulgaris* on the East coast of Canada was first recorded in 1850. Between 1845-1850 the potato famine took place in Ireland and as a response millions of Irishmen fled to Canada and also to North America (HPH Nusteling, 2009). This movement may have been responsible for the introduction of *J. vulgaris* from Ireland into the east coast of Canada and North America.

Subsequent introductions to one invasive region followed by admixture and spread to the other regions or independent introductions from the same source areas to all four regions, or both are likely scenarios because several haplotypes are shared between the invasive regions.

Although genetic diversity is lower in the invasive area, there are still many different haplotypes present and both the AFLP and this study indicate that populations and even different regions in the invasive area share their genetic diversity more than populations in the native area. In the common garden study of Joshi and Vrieling (2005) (J Joshi ,K Vrieling, 2005) individuals from the different invasive regions all showed the same changes in life history traits like growth, defence and reproduction. Because it is unlikely that all the introduced populations contained the same pre-adaptive traits we suggest that changes in life-history traits, herbivory and chemical defence of invasive individuals are caused by evolution rather than preadaptation.

We did not find a significant correlation between geographical and genetic distance suggesting that also native populations recently might have been admixed. Ragwort is a species from disturbed areas and often has ephemeral populations that exist in a metapopulation structure (E van der Meijden ,RE

van der Waals-Kooi, 1979). Within the native area most variation was detected in populations from Hungary, France, Sweden and The Netherlands-Wageningen. The significantly larger within population genetic distances in these four populations compared to the other populations suggests that these have been founded, or received immigrants, from different populations (Fig. 1). Although we did not find a correlation between genetic and geographical distance for all native populations, the two biggest independent networks showed, despite a small overlap, a significant distinction between populations from North- West Europe and South- East Europe (Fig 3).

Despite the fact that in the invasive area a similar number of individuals was analysed in more populations compared to the native area, more independent networks were found in the native area. This shows once more the larger genetic diversity of native populations compared to invasive populations.

The genetic variance among populations in the native area was 50% compared to 14% in the invasive area (Table 4). Genetic variance between populations was much higher in this study compared to the study of Doorduin et al 2010 using nuclear markers. This can be explained by the continuous recombination of nuclear markers that allow all possible combinations and therefore lead to a reduced differentiation between populations. These recombinations do not occur in chloroplast markers. The reduced genetic variation found between the invasive populations and even between different regions can be explained by admixture upon arrival in the invasive area. Both the AFLP data and the current data on microsatellite markers are in line with the admixture hypothesis in the invasive area of Verhoeven et al. (2010).

Table 4: Analysis of Molecular Variances (AMOVA's) for native and invasive populations combined, native population and invasive population. In the "invasive population only" analysis, the invasive area is split up in the four regions: Australia, New Zealand, west coast and east coast of America. n= number of populations ** p < 0.01 * p < 0.05.

Source of Variation	d.f.	Sum of Squares	Percentage of Variance
All populations combined (n=40)			
Native-vs.-invasive	1	93.65	23.18**
Among populations within native/invasive areas	38	280.83	31.91**
Within populations	137	249.50	44.91**
Total	176	623.98	
Native populations only (n=11)			
Among populations	10	185.18	49.54**
Within populations	79	162.35	50.46**
Total	89	347.52	
Invasive populations only (n=29)			
Among regions	3	30.00	13.84**
Among populations within regions	25	65.65	18.63**
Within populations	58	81.11	67.53**
Total	86	168.93	

In conclusion these results show that several populations from North-West Europe are the most likely source populations. The presence of alleles in the invasive area that were not found in the native area suggests that some source populations went undetected. Collectively the data suggest that multiple introductions have occurred. Furthermore the data show a reduced genetic variation in the invasive area. At last results show that upon arrival there has been a strong admixture before the invasive population spread over the different regions.

Acknowledgements

We are thankful to B. Stolk and N. Schidlo for helping with the SNP and microsatellite analyses and U. Schaffner, K. Wolff, J. van Alphen, H. Auge, A. Balogh, M. Bartelheimer, L. Joosten, N. Sletvold-Hommelvik and P. Olejnicrak for collecting seeds.

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Summary

The introduction of species from one area into another is a natural process that has always been a part of evolutionary history. However, the deliberate and undeliberate transport of species by humans, starting circa 10.000 years ago during the Neolithic Revolution, added considerably to the frequency of new introductions. As an early example, around 4.000 B.C. domesticated pigs were introduced in Europe from Asia and crossed with wild pigs. The increase of human migrations and trade in the 19th century was accompanied by the spread of domesticated species like cereals, rice and cattle and the accidental spread of natural species as transport contaminants, such as weeds and species like the brown rat and the zebra mussel. With these activities, species were even able to spread from one continent to the other, crossing almost insurmountable biogeographical barriers and maintained themselves in these new environments in many cases.

Invasive plant species are defined as species that manage to cope with the new environment, disperse to other local communities and become extraordinarily prominent in their new range. Species can receive a pest status if they have a negative impact on human health, are a pest in agricultural crops, lead to a loss of native biodiversity due to competition or predation, or cause habitat degradation and disruption. Besides the impact on the environment, invasions can have an economic impact in two ways. Firstly invasive species may negatively affect crop and forestry production and grazing capacity. Secondly there are the costs of combatting invasions like control and quarantine measures. For the United States the annual cost of all invasive species (plants, animals and microorganisms) is estimated to exceed 138 billion dollar per year.

Out of the thousands of species that are introduced into new area's only a few percent become invasive. It is still relatively poorly understood why some species become invasive and others do not. In this thesis I will focus on the mechanisms that contribute to the invasiveness of the plant species *Jacobaea vulgaris* or common ragwort. This species belongs to the family of Asteraceae and is native in Europe and Asia where this species does not have a pest status. About 130 years ago, it has been introduced to New Zealand, Australia, North America and Canada where it developed into a pest species. This species leads to problems because it can reach high densities and therefore can decreases native biodiversity locally. Besides this, *J. vulgaris* causes problems because it produces defence compounds, pyrrolizidine alkaloids (PAs), which are poisonous to cattle. After consumption of *Jacobaea vulgaris*, the cumulative storage of PAs in the liver leads to a sudden death in apparently healthy cattle. Furthermore PAs can enter the human food chain through milk and honey.

The PAs produced by *J. vulgaris* are defence compounds against herbivores. However, PAs are not equally effective against all types of herbivores. Generalist herbivores, attacking plant species from several plant families, are deterred by PAs in host plants. In contrast, specialist herbivores, attacking

only one or several plant species of one family, are often adapted to PAs and even can be attracted by these compounds. In the invasive area specialist herbivores of *J. vulgaris* like the cinnabar moth (*Tyria jacobaeae*) and the fleabeetle (*Longitarsus jacobaeae*) were initially absent.

A plant introduced in a new area has the direct benefit of leaving behind its specialist herbivores. The EICA (Evolution of Increased Competitive Ability) hypothesis predicts that, under reduced enemy pressure due to the absence of specialist herbivores, selection may shift the resource allocation of invasive plant species from defence to growth. This allocation to growth also results in a higher reproduction, giving the invasive plants a competitive advantage over local plants. This increases the chance of becoming a pest species in the introduced area.

The EICA hypothesis does not take into account the presence of generalist herbivores in the invasive area which can threaten introduced plants. The Shifting Defence Hypothesis (SDH) predicts that invasive plants will adapt their amount of quantitative and qualitative defence compounds to the presence of generalist herbivores and the absence of specialist herbivores.

Quantitative defences act against specialist as well as generalist herbivores. These defence compounds are digestibility reducers (e.g. tough leaves, thorns) and occur in high concentrations which make them expensive to produce. Qualitative defences act against generalist herbivores. These defence compounds are toxins (e.g. phenolics, alkaloids) and occur in relatively low quantities, which make them a cheaper defence compared to quantitative defences. Specialist herbivores are often adapted to these defences and can even use these chemicals as a cue to locate their host plant, as a feeding or oviposition stimulant and may sequestrate them for their own defence. So, qualitative defence compounds produced by plants are no longer repellent but often attractive to specialist herbivores. As a consequence, in the native area there is a risk of attracting specialist herbivores when high amounts of such compounds are produced. To be protected against specialist as well as generalist herbivores, selection in the native area will lead to a balance between quantitative and qualitative defences. For invasive areas, where specialist herbivores are absent, the SDH predicts that levels of expensive digestibility reducers are decreased at the expense of cheap toxins, through natural selection. The energy surplus can than can be diverted to growth and reproduction.

In a previous study on the invasiveness of *J. vulgaris* native and invasive individuals were grown in the same environment. Invasive plants produced significantly more PAs and were better protected against generalists, but less defended against specialists. These outcomes are fully explained by the SDH and it suggests that fast evolution has taken place after introduction.

The potency of an introduced species to adapt depends on the genetic variation introduced in the new area. With high levels of genetic variation, selection can take place without the necessity of new mutations. Such selective processes can lead to genetic differences between individuals in native and invasive areas. These genetic differences can also occur when multiple native populations are introduced into the invasive area and admixture takes place. In this case invasive populations can have higher genetic variation compared to the native populations. An alternative explanation for differences between native and invasive individuals is that by coincidence introduced individuals already contained the traits that were beneficial to maintain themselves in the new area, this is called preadaptation. To establish whether the invasive success of *J. vulgaris* is caused by evolution or preadaptation, it is

necessary to trace the source population(s) in the native area. Evolution has taken place if the introduced and source population differ significantly in the ecological traits of interest. If multiple introductions have taken place that lead to invasiveness, there is very little chance that all introduced individuals from different populations already obtaines the preadapted traits before introduction. Therefore the assumption is that preadaptation only can take place with one or few introductions.

My thesis is divided into two parts. In the first part (chapter 2 and 3) I will focus on the mechanisms related to herbivore pressure that can have contributed to the invasiveness of *J. vulgaris*. The second part (chapter 4,5 and 6) is based on tracing the (native) source population(s) of introduced *J. vulgaris* individuals. Detecting the source population is important to investigate if trait differences between native and invasive individuals are driven by evolution or preadaptation. In my thesis the following research questions are posed.

1. Has invasion led to a reduction in costly quantitative defence products and if so, what are the consequences for other fitness related traits?
2. Is the shifting defence a general phenomenon in invasive plant species?
3. What are the source population(s) of invasive individuals of *J. vulgaris*?
4. Is the genetic diversity of *J. vulgaris* lower in invasive populations compared to native populations and did admixture occur?
5. Are trait differences between native and invasive individuals of *J. vulgaris* driven by evolution or preadaptation?

In chapter 2 research was carried out on differences in anatomical, physiological- and growth parameters between native and invasive plants of *J. vulgaris*. Due to a decreased herbivore pressure in the invasive area, I hypothesized that selection would lead to a lower production of quantitative defence compounds like thicker cell walls and tougher leaves that are more difficult to digest. Cell walls do contain a substantially amount of nitrogen. The surplus of nitrogen, due to the reduced cell wall thickness can be used for photosynthesis, resulting in more competitive individuals. Results of chapter 2 showed that total photosynthesis is equal or higher in invasive *J. vulgaris* individuals compared to individuals from the native area. However, when photosynthesis was measured per surface unit, no difference was found between native and invasive individuals. Furthermore no differences were found between native and invasive individuals of *J. vulgaris* concerning traits related to quantitative defence, like the amount of cell wall material and leaf dry weight per area, thickness of cell walls and leaf toughness. However a difference in allocation of native individuals of *J. vulgaris* to cope with herbivore pressure of specialists was found. The root-shoot ratio was higher for native *J. vulgaris* plants. A bigger investment in root mass is detrimental to photosynthetic capacity and results in a smaller plant. So, investment in root mass is costly. However, a bigger investment in roots is positively correlated with the capacity of shoot regrowth after defoliation. Native individuals of *J. vulgaris* are often completely defoliated by the larvae of the cinnabar moth (*T. jacobaeae)*. In the native area *J. vulgaris* individuals will be selected for investment in roots, because these plants can regrow fast after defoliation. In the invasive area where *Tyria jacobaeae* is absent, selection favours plants with less investment in roots, yielding a faster growth.

In chapter three I investigated two predictions of the SDH based on a literature study. I have used a biogeographical approach, using only results of studies that compared native and invasive individuals under the same circumstances. My research question was if invasive plants indeed contained a higher level of qualitative defence compounds (toxins) and reduced levels of quantitative defence compounds (digestibility reducers). As expected invasive plants produced a higher concentration of toxins compared to native plants. However, in contrast to our expectation, no difference was found in the amount of quantitative defence compounds between native and invasive individuals. Our results do not completely support the SDH.

In chapter 4 I have used nuclear AFLP ("Amplified Fragment Length Polymorphisms") markers to trace the source population of *J. vulgaris* and to unravel the route of introduction. Neutral markers like AFLPs are useful for this research because no selection takes place on these markers. In total 38 native individuals spread over 15 populations and 44 invasive individuals spread over 16 invasive populations were analysed. Only ten percent of the total genetic variation in AFLP markers was explained by the difference between individuals coming from the native and invasive area. Within the native area populations of *J. vulgaris* differed significantly from each other in genetic variation, in contrast with invasive populations. Despite the big geographical distance, populations from the different invasive regions (Australia, New Zealand and North America) did not differ from each other in the amount of genetic variation. Besides, no decrease was found in the number of polymorphic AFLP markers although the allele frequencies did differ of individuals from the invasive area compared with individuals from the native area. This suggests that there have been multiple source populations. Moreover the lack of differentiation between invasive regions suggests that either introductions may have occurred from the native sources in all invasive regions or subsequent introductions took place from one into another invasive region and the same mix of genotypes was subsequently introduced into all invasive regions. With an assignment test, populations from Ireland, The Netherlands and the United Kingdom most resembled the invasive populations and were the most likely source populations of invasive *J. vulgaris* individuals of all populations tested.

To get more insight about the route of introduction and the source population(s) of *J. vulgaris* there was a need to develop markers with higher resolving power than AFLP markers. The chloroplast genome behaves as one locus, does not recombine and is only passed on through the maternal line (seeds). Because of the limited seed dispersal and the absence of recombination, it is easier to trace the source of populations. In chapter 5 I describe how I made use of a next generation DNA sequencing technique to sequence the DNA of seventeen chloroplast genomes. Twelve chloroplast genomes derived from native individuals and five genomes derived from invasive individuals. By comparing these genomes with a length of circa 150.000 basepairs, I found 32 SNPS ("Single Nucleotide Polymorphisms") and over 34 microsatellite locations. To find as much polymorphic markers as possible, selected individuals were geographically wide spread. These markers can be used to trace the source population(s) of *J. vulgaris*.

Eight SNPs and 9 microsatellite markers were selected to genotype native and invasive individuals. In chapter 6 in total 90 native and 87 invasive individuals were genotyped, spread over respectively 11 and 29 populations. The genetic variation was significantly higher in the native area. This outcome was also expressed in the number of allele combinations that was found, the so called haplotypes. In the native area 63 haplotypes were found compared with 26 in the invasive area. In agreement with the AFLP study low genetic variation was found between individuals coming from the invasive regions Australia, New Zealand, Canada and North America. Four haplotypes from Europe were identical to the invasive haplotypes, these were two individuals originated from Ireland, one individual from Norway and the fourth individual was coming from Sweden. Possibly these populations contained individuals that were introduced into the invasive area. This finding is partly in agreement with the AFLP study where the most likely source populations also originated from Northwest- Europe. In the invasive regions individuals with identical haplotypes did often occur in multiple or even all invasive regions. This result in combination with the low genetic variation between regions suggests once more multiple source populations originated from Europe and introduced into the new areas.

Conclusions

Native and invasive individuals of *Jacobaea vulgaris* differ from each other in a number of traits related to defence and growth. Despite the more vigorous growth of invasive individuals, defence related products were not lower for invasive individuals compared to native individuals as predicted by the EICA hypothesis. Furthermore a shift to a bigger investment in quantitative defence products of native individuals compared to invasive individuals, as predicted by the SDH, was also not found. Two separate studies with different genetic markers and partly also with different individuals both reveal that the most likely scenario of invasive *J. vulgaris* individuals is that they originated from multiple source populations. The chance of preadaptation is very little because it is very unlikely that all these different source populations contained individuals that were already adapted to the new environment. An alternative explanation for the differences in defence and growth between native and invasive individuals is that after introduction fast evolution has taken place. The introduction of different source populations and the admixture of individuals from different populations have likely contributed to the fast evolution of *J. vulgaris*. Admixture increased the genetic variation and has also lead to recombination of native individuals that were isolated from each other in the native area. The occurrence of new genetic combinations increased the potency of natural selection in the invasive areas.

The invasive character of *Jacobaea vulgaris* is especially expressed by the lower root- shoot ratio. With this change the competition with local species is increased. Bigger shoots leads to a higher photosynthetic capacity and more growth. Besides, within *J. vulgaris* plant size is positively correlated with the amount of seeds produced. As a result of this, spread and abundance of *J. vulgaris* easily increases.

De introductie van soorten naar een nieuw gebied is een natuurlijk proces en heeft altijd deel uitgemaakt van de evolutionaire geschiedenis. Echter, het bewuste en onbewuste transport door de mens van plant- en diersoorten hebben de frequentie van nieuwe introducties aanzienlijk verhoogd. Ter illustratie, rond 4000 voor Christus werden gedomesticeerde varkens al geïntroduceerd van Azië in Europa. De toename van migraties en handel door mensen in de 19de eeuw leidde tot de verdere verspreiding van gedomesticeerde soorten zoals graan, rijst en vee en daarnaast ook tot de onbewuste verspreiding van natuurlijke soorten zoals de bruine rat en de zebramossel. Door middel van menselijke activiteiten werden soorten in nieuwe continenten geïntroduceerd, waarbij biogeografische barrières werden overbrugd en veel soorten zich konden handhaven in de nieuwe geïntroduceerde gebieden.

Invasieve planten worden gedefinieerd als soorten die kunnen overleven in het geintroduceerde gebied, zich verspreiden en uiteindelijk in grote aantallen voor komen. Soorten kunnen een pest status krijgen wanneer zij een negatief effect hebben op de gezondheid van mensen, de groei van landbouw gewassen, op inheemse biodiversiteit of wanneer zij habitat degradatie en verstoring veroorzaken. Naast het ecologische effect, kunnen invasieve soorten op twee manieren een negatief economische effect hebben. Ten eerste kan een invasieve soort een negatief effect hebben op de productie van gewassen en bosbouw en op de begrazingscapaciteit van vee. Ten tweede zijn er de directe kosten om invasies te voorkomen en te beheersen zoals controle en quarantaine maatregelen. Voor de U.S.A. worden de jaarlijkse kosten voor de bestrijding van invasieve soorten (planten, dieren en micro organismen) geschat op 138 miljard dollar.

In dit proefschrift rapporteer ik over mijn studie naar het invasieve karakter van het Jakobskruiskruid (*Jacobaea vulgaris*). Deze soort behoort tot de familie van de samengesteldbloemigen en is inheems in Europa en Azië waar deze soort niet tot grote problemen leidt. Ongeveer 130 jaar geleden is het Jakobskruiskruid geïntroduceerd in Australië, Nieuw Zeeland en Noord Amerika. Daar veroorzaakt deze plantensoort problemen omdat zij hoge dichtheden kan bereiken en (plaatselijk) de biodiversiteit verlaagt. Daarnaast zorgt het Jakobskruiskruid voor problemen doordat het afweerstoffen, pyrrolizidine alkaloiden (PAs) produceert die erg giftig zijn voor vee. Na consumptie van Jakobskruiskruid treedt ernstige lever schade op leidend tot de dood. PAs kunnen via melk en honing ook in de menselijke voedselketen terecht komen.

De PAs die het Jakobskruiskruid produceert dienen als afweerstoffen tegen herbivoren. Echter deze afweerstoffen heeft niet bij elke type herbivoor dezelfde werking.

Generalistische herbivoren, herbivoren die van meer dan 1 plantensoort van verschillende plantenfamilies eten, worden afgeweerd wanneer zij met PAs in aanraking komen. Specialistische

herbivoren, herbivoren die heel specifiek een of een aantal soorten binnen een plantenfamilie eten, zijn daarentegen aangepast aan de afweerstoffen en worden er zelfs vaak door aangetrokken. In het invasieve gebied zijn specialistische herbivoren van het Jakobskruiskruid zoals de larven van de Jakobsvlinder (*Tyria jacobaeae*) en de aardvlo (*Longitarsus jacobaeae*) afwezig.

Een plant die geïntroduceerd wordt in een nieuw gebied heeft het directe voordeel dat zijn specialistische herbivoren meestal niet worden mee geïntroduceerd. De EICA ("Evolution of Increased Competitive Ability") hypothese stelt dat bouwstoffen, in plaats van aan afweer, in invasieve gebieden waar geen specialistische herbivoren zijn, kunnen worden gebruikt voor groei en reproductie. De reallocatie van bouwstoffen naar groei kan leiden tot verhoogde concurrentiekracht in het invasieve gebied wat de kans vergroot dat een soort zich ontwikkelt tot een plaag.

De EICA hypothese gaat voorbij aan het feit dat ook in invasieve gebieden al generalistische herbivoren aanwezig zijn die geïntroduceerde planten kunnen belagen. De "Shifting Defence Hypothesis"(SDH) stelt dat invasieve planten hun kwalitatieve en kwantitatieve afweer gaan aanpassen aan de aanwezigheid van generalistische herbivoren en de afwezigheid van specialistische herbivoren. Kwantitatieve afweer is werkzaam tegen zowel specialistische als generalistische herbivoren. Deze afweer is gebaseerd op hoge concentraties van vaak verteringsremmende stoffen. Kwalitatieve afweer is werkzaam tegen generalistische herbivoren. Deze afweer bestaat vaak uit toxines, zoals pyrrolizidine alkaloiden, die bij lage concentraties werkzaam zijn. Specialistische herbivoren hebben zich vaak aangepast aan de kwalitatieve afweer en gebruiken deze zelfs om hun waardplant te herkennen of als stimulans om te eten en/of eieren te leggen. Bovendien kunnen de toxische stoffen worden opgenomen door de specialistische herbivoren en zo dienen als verdediging tegen de predatoren van de specialistische herbivoor. Deze kwalitatieve afweer is door de aanpassing van de specialistische herbivoor verworden van afweerstof tot een lokstof. In het inheemse gebied is er dus het risico dat bij hoge toxine productie specialistische herbivoren worden aangetrokken. Om toch beschermd te zijn tegen aanvallen van zowel generalistische als specialistische herbivoren zal er in het inheemse gebied selectie plaatsvinden op individuen met een goede balans tussen kwantitatieve en kwalitatieve afweer. Kwantitatieve afweer is kostbaarder voor een plant om te produceren dan kwalitatieve afweer omdat eerstgenoemde in grotere concentratie aanwezig is in de plant. De SDH voorpelt daarom dat in een invasief gebied waar specialisten afwezig zijn, selectie plaatsvindt op plantenindividuen met hoge gehaltes aan kwalitatieve afweer omdat deze vorm van afweer minder kostbaar is. De plant is goed beschermd tegen generalistische herbivoren en omdat de specialistische herbivoren afwezig zijn, kan de plant gedijen met een laag gehalte aan kwantitatieve afweer. De kostbare kwantitatieve afweer wordt dus ingeruild voor een minder kostbare kwalitatieve afweer. De bespaarde kosten kunnen worden aangewend voor een snellere groei.

In een eerdere studie waarbij inheemse en invasieve planten van het Jakobskruiskruid in dezelfde omgeving werden geplaatst, bevatten invasieve planten siginificant meer PAs en waren vatbaarder voor specialisten en minder vatbaar voor generalisten in vergelijking met de inheemse planten. Deze uitkomst komt overeen met de SDH en suggereert dat er snelle evolutie is opgetreden na introductie.

Of een soort in staat is zich aan te passen aan een nieuw gebied is grotendeels afhankelijk van de hoeveelheid genetische variatie van de geïntroduceerde individuen die een populatie vormen. Bij een grote geïntroduceerde genetische variatie kan er selectie plaatsvinden zonder dat nieuwe mutaties noodzakelijk zijn. Een dergelijke selectie leidt tot genetische verschillen tussen individuen in inheemse gebieden en invasieve gebieden. Deze genetische verschillen kunnen ook ontstaan wanneer meerdere inheemse populaties geïntroduceerd worden in het invasieve gebied waardoor vermenging van populaties kan plaatsvinden. Door vermenging kan de genetische variatie van invasieve populaties groter zijn dan van inheemse populaties. Een alternatieve verklaring voor dergelijke verschillen is, dat door toeval, geïntroduceerde individuen al voor introductie de goede eigenschappen bezaten die nodig waren om zich te handhaven in het nieuwe gebied. Dit noemen we preadaptatie.

Om te achterhalen of het invasieve succes van het Jakobskruiskruid te danken is aan evolutie of preadaptatie is het noodzakelijk om de bronpopulatie(s) in de inheemse gebieden te achterhalen. Wanneer eigenschappen van individuen uit de bronpopulatie afwijken van de eigenschappen van de geïntroduceerde individuen, heeft er evolutie plaatsgevonden. Wanneer er meerdere introducties zijn geweest uit meerdere bronpopulaties, is de kans erg klein dat al deze geïntroduceerde individuen precies die eigenschappen bezaten om zich te handhaven in het nieuwe gebied. Daarom is de aanname dat preadaptatie alleen kan plaatsvinden wanneer er een of weinig succesvolle introducties geweest zijn.

Mijn proefschrift is opgedeeld in twee delen. In het eerste deel (hoofdstuk 2 en 3) ligt de focus vooral op mechanismen gerelateerd aan herbivoren druk die het invasieve karakter van het Jakobskruiskruid mogelijk kunnen verklaren. Het tweede gedeelte (hoofdstuk 4,5 en 6) is gericht op het traceren van (inheemse) bronpopulatie(s) van de Jakobskruiskruid planten in de invasieve gebieden. Het traceren van de bronpopulatie is van belang omdat hiermee onderzocht kan worden in hoeverre het Jakobskruiskruid na introductie zich heeft aangepast. In mijn proefschrift zijn de volgende onderzoeksvragen behandeld:

1. Heeft invasie van het Jakobskruiskruid geleid tot een afname in kostbare kwantitatieve afweer producten en indien ja, heeft dit consequenties voor andere gerelateerde kenmerken?
2. Is de SDH een algemeen fenomeen in invasieve plantensoorten?
3. Wat is/ zijn de bronpopulatie(s) van invasieve individuen van het Jakobskruiskruid?
4. Is de genetische diversiteit en differentiatie snelheid van *J. vulgaris* lager in invasieve populaties in vergelijking tot inheemse populaties en heeft vermenging van populaties plaatsgevonden?
5. Zijn verschillen in kenmerken tussen inheemse en invasieve individuen van het Jakobskruiskuid gedreven door pre-adaptatie of evolutie?

In hoofdstuk 2 is onderzocht of er een verschil is in anatomische, fysiologische- en groeiparameters tussen inheemse en invasieve planten van het Jakobskruiskruid. Aangezien er een verminderde herbivorendruk aanwezig is in het invasieve gebied, is de hypothese dat er minder selectie plaatsvindt op planten met een verhoogde kwantitatieve afweer zoals dikkere celwanden en stuggere bladeren die moeilijker te verteren zijn. Door de afname in kwantitatieve afweer blijft er meer stikstof over in de plant die gebruikt kan worden om de fotosynthese te bevorderen en om zo de concurrentiekracht te verhogen. Uit hoofdstuk 2 is gebleken dat de fotosynthese inderdaad gelijk of hoger is voor invasieve Jakobskruiskruid planten vergeleken met Jakobskruiskruid planten uit de inheemse gebieden. Per

oppervlakte eenheid werd echter geen verschil gevonden tussen inheemse en invasieve planten. Ook werden geen verschillen gevonden tussen inheemse en invasieve Jakobskruiskruid planten in eigenschappen gerelateerd aan kwantitatieve afweer, zoals de hoeveelheid celwand materiaal en droge stof in een blad, de dikte van de celwanden en de sterkte van het blad. Wel werden er andere aanwijzingen gevonden voor een veranderde allocatie van inheemse Jakobskruiskruid planten om met specialistische herbivorendruk om te gaan. De wortel-spruit verhouding was groter voor inheemse Jakobskruiskruid planten. Een grotere investering in wortel massa gaat ten koste van het fotosynthetisch vermogen en resulteert in een lagere groei. Een investering in wortel massa is dus kostbaar. Echter een grotere investering in de wortel is ook positief gecorreleerd met de capaciteit tot hergroei van de spruit na vraat. Inheemse Jakobskruiskruid planten worden regelmatig volledig kaalgevreten door de rupsen van de Jacobsvlinder. Voor inheemse Jakobskruiskruid planten zal er door de herbivorie druk van deze specialistische herbivoren selectie plaatsvinden op planten die relatief meer investeren in wortels, die na vraat snel een nieuwe spruit kunnen vormen. In het invasieve gebied is de Jakobsvlinder niet aanwezig. Door de verminderde herbivorie druk vindt hier, in tegenstelling tot in het inheemse gebied, geen selectie plaats op Jakobskruiskruid planten met een sterke hergroei capaciteit.

In hoofdstuk 3 heb ik aan de hand van een literatuur onderzoek twee voorspellingen van de SDH hypothese getoetst. Hierbij heb ik gebruik gemaakt van een biogeografische benadering, waarbij resultaten van inheemse en invasieve individuen van een zelfde soort zijn vergeleken met experimenten onder dezelfde omstandigheden. Hierbij heb ik getoetst of invasieve planten inderdaad hogere gehaltes aan kwalitatieve afweer (toxines) hadden en inheemse planten hogere gehaltes aan kwantitatieve afweer (verteringsremmers). Invasieve planten produceerden zoals verwacht hogere concentraties toxines in vergelijking met inheemse planten. Er was echter geen verschil in de hoeveelheid kwantitatieve afweer tussen inheemse en invasieve individuen.

In hoofdstuk 4 heb ik gebruik gemaakt van nucleaire AFLP ("Amplified Fragment Length Polymorphisms") merkers om de bronpopulatie van het Jakobskruiskruid en de route van introductie te achterhalen. Neutrale merkers zoals AFLP merkers zijn erg geschikt voor dit onderzoek omdat er geen selectie plaatsvindt op deze merkers. In totaal zijn 38 inheemse individuen verspreid over 15 populaties en 44 invasieve individuen verspreid over16 invasieve populaties geanalyseerd. Slechts 10 procent van alle genetische variatie in AFLP merkers werd verklaard door het verschil tussen individuen uit het inheemse en invasieve gebied. Binnen het inheemse gebied verschilden Jakobskruiskruid populaties significant van elkaar in genetische variatie, in tegenstelling tot de invasieve populaties. Ondanks de grote geografische afstand verschilden zelfs populaties uit de verschillende invasieve regio's (Australië, Nieuw Zeeland en Noord Amerika) niet van elkaar in de hoeveelheid genetische variatie. Daarnaast was er geen afname in het aantal polymorfe AFLP merkers, hoewel de allelfrequenties wel verschilden van individuen uit het invasieve gebied ten opzichte van individuen uit het inheemse gebied. Dit suggereert dat er meerdere bronpopulaties geweest zijn. Daarnaast duidt de grote genetische gelijkheid tussen de invasieve regio's erop dat in deze regio's introducties geweest zijn vanuit dezelfde bronpopulaties of op opeenvolgende introducties van de ene regio naar de andere. Door middel van een mathematische toewijzingsmethode werden de populaties uit Ierland, Nederland en het Verenigd Koninkrijk aangewezen als meest aannemelijke bronpopulaties.
Om meer duidelijkheid te krijgen over het introductie proces en de bronpopulatie(s) van het Jakobskruiskruid was er de behoefte om merkers te ontwikkelen die een hoger oplossend vermogen hebben dan AFLP merkers. Het chloroplast genoom gedraagt zich als 1 locus. Er vindt geen recombinatie plaats en wordt alleen via de moederlijn (zaden) doorgegeven. Door de geringe verspreiding en het ontbreken van recombinatie zijn lijnen makkelijker te herleiden tot bepaalde populaties. In hoofdstuk 5 beschrijf ik hoe er met behulp van nieuwe DNA sequencing technieken het DNA van 17 chloroplast genomen is gesequenced. Twaalf chloroplastengenomen waren afkomstig van inheemse individuen en 5 waren afkomstig van invasieve individuen. Door deze chloroplast genomen met een lengte van circa 150.000 baseparen met elkaar te vergelijken, kon ik 32 SNPs ("Single Nucleotide Polymorphisms") en 34 microsatelliet locaties vinden. Om zoveel mogelijk polymorfe merkers te vinden, waren geselecteerde individuen geografisch zo wijd mogelijk verspreid. Deze merkers kunnen worden gebruikt om de bronpopulaties op te sporen.

Met de chloroplast microsatellieten en SNPs die gevonden werden met behulp van DNA sequencing zijn 8 SNP en 9 microsatelliet merkers geselecteerd om inheemse en invasieve individuen te genotyperen. In hoofdstuk zes zijn er in totaal 90 inheemse en 87 invasieve individuen gegenotypeerd, verspreid over respectievelijk 11 en 29 populaties. De genetische variatie was significant hoger in het inheemse gebied. Dit kwam ook tot uiting in het aantal gevonden allelcombinaties, ook wel haplotypes genoemd. In het inheemse gebied werden 63 haplotypes gevonden tegenover 26 in het invasieve gebied. In overeenstemming met de AFLP studie werd erg weinig genetische variatie gevonden tussen individuen afkomstig uit de invasieve regio's Australia, Nieuw Zeeland, Canada en Noord Amerika. Vier haplotypes uit Europa waren identiek aan invasieve haplotypes. Dit waren twee individuen uit Ierland, een individu uit Noorwegen en het vierde individu was afkomstig uit Zweden. Mogelijk zijn deze populaties dus betrokken geweest bij de introductie van het Jakobskruiskruid in de invasieve gebieden. Dit komt deels overeen met de AFLP studie waarin de meest aannemelijke bronpopulaties ook afkomstig waren uit Noordwest- Europa. In het invasieve gebied waren individuen met gelijke haplotypes vaak afkomstig uit meerdere of alle regio's. Dit in combinatie met de lage genetische variatie tussen regio's duidt wederom op meerdere bronpopulaties vanuit Europa.

Conclusies

Inheemse en invasieve individuen van het Jakobskruiskruid verschillen van elkaar in een aantal eigenschappen gerelateerd aan afweer en groei. Ondanks de sterkere groei van invasieve individuen waren waardes van eigenschappen gerelateerd aan afweer niet lager voor invasieve individuen in vergelijking met inheemse individuen. Deze uitkomst is niet in overeenstemming met de EICA hypothese. De SDH voorspelt dat inheemse individuen meer investeren in kwantitatieve afweer in vergelijking tot invasieve individuen. Er werd echter geen verschil gevonden in relatie tot kwantitatieve afweer. Twee afzonderlijke studies met verschillende merkers en deels ook met verschillende individuen laten zien dat het aannemelijk is dat invasieve individuen van het Jakobskruiskruid afkomstig zijn uit meerdere inheemse bronpopulaties. De kans op pre-adaptatie is klein aangezien het vrijwel uitgesloten is dat al deze afzonderlijke populaties individuen met genotypen leveren, die al aangepast zijn aan het nieuwe gebied. Een alternatieve verklaring voor de aangetoonde verschillen tussen inheemse en invasieve individuen is dat na introductie snelle evolutie is opgetreden. De introductie uit verschillende bronpopulaties en de kruisbestuiving van individuen uit de verschillende populaties heeft waarschijnlijk snelle evolutie bevorderd omdat het heeft geleid tot een grote genetische variatie en recombinatie

van individuen die in de inheemse gebieden geen genetische uitwisseling met elkaar hadden. Het ontstaan van nieuwe genetische combinaties is een goede uitgangspositie voor natuurlijke selectie in de invasieve gebieden.

Het invasieve karakter van het Jakobkruiskruid typeert zich vooral door de productie van een kleinere wortel- spruit verhouding waardoor de kracht om te concurreren met inheemse soorten wordt verhoogd. De veranderde wortel spruit verhouding ten bate van een grotere spruit leidt tot meer fotosynthetische capaciteit en een krachtigere groei. Bovendien is voor het monocarpe Jakobskruiskruid de grootte van de plant positief gecorreleerd met de zaadproductie. Door de productie van meer bovengrondse biomassa wordt dus uiteindelijk meer zaad geproduceerd. Vanzelfsprekend komt dit ook de dichtheid en de verspreiding van het Jakobskruiskruid ten goede.

Upon the completion of this thesis I would like to thank Eddy van der Meijden, my supervisor during the first years, for his open mind and enthusiasm. Furthermore I would like to thank the technical team of the section plantecology and phytochemistry. Thanks to Cilke Hermans, Karin van der Veen and Henk Nell for their practical help in raising plants and organising experiments. For the support in the molecular lab and the runs on the Megabace many thanks to Natasha Schidlo, Rene Glas and Marcel Eurlings.

For the opportunities to present my findings and the discussions we had, I would also like to thank all of my other colleagues of the section plantecology and phytochemistry. Also many thanks to the trainees Sonja Lodder, Michiel Hooykaas, Jelle Zandveld, Andries Temme, Remco de Zwijger, Achid Farooq and Bas Stolk for their effort in gathering and discussing data. I am also very grateful to Rolf Vossen, Yavuz Ariyurek and Sophie Greve of the team of the Leiden Genome Technology Center for their time and knowledge in genomic sequencing and SNP analyses. Finally I would like to thank all the people who provided seeds of tansy ragwort to me from all over the world.

Leonie Doorduin werd geboren op 3 december 1983 in Rozenburg (Zuid- Holland). Na het behalen van haar V.W.O. diploma aan de Angelus Merula te Spijkenisse in 2001, begon ze september van dat jaar aan haar studie Biologie aan de Universiteit van Leiden. Na haar propedeuse in 2002 te hebben gehaald, volgden er nog twee jaar waarin gekozen werd voor studieblokken gerelateerd aan ecologie en evolutiebiologie. Haar eerste stage liep ze in het derde jaar bij evolutiebiologie en zij bestudeerde de invloed van hormonen op de eileg van een Afrikaanse vlindersoort. Leonie werd begeleid door Jeroen Pijpe en Jeanette Bot. Haar tweede stage volbracht zij in het vierde jaar bij de groep van dierenecologie waar zij onder leiding van Tom van Dooren en Femmie Kraaijeveld onderzoek deed naar de onderlinge verwantschap van killies (zoetwatervissen afkomstig uit Zuid- Amerika). In het laatste studiejaar heeft Leonie samen met Anne de Vries vijf maanden stage gelopen in Kameroen en de onderlinge relaties tussen gazelles en nijlpaarden onderzocht. Zij werd vanuit Kameroen begeleid door Ralph Buij en in Nederland door Hans de Iongh en Paul Loth beide werkzaam bij het Centrum voor Milieukunde (CML). In oktober 2006 heeft Leonie haar diploma in ontvangst genomen waarna zij in januari 2007 is begonnen als promovendus bij de groep plantecologie aan het in dit proefschrift beschreven onderzoek. Gedurende haar promotie heeft ze meerdere stagiairs begeleid afkomstig van het MLO en van de bachelor en master opleiding Biologie. Daarnaast heeft zij gedurende drie jaar practica begeleid tijdens de bachelor cursus moleculaire technieken. Per maart 2011 is Leonie werkzaam als pre- breeder Cucurbitaceae bij het zaadveredelingsbedrijf “Rijk Zwaan” in de Lier.

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