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Natural hybridization between *Senecio jacobaea* and *Senecio aquaticus* : ecological outcomes and evolutionary consequences

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THESIS LEIDEN UNIVERSITY

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**Natural hybridization between
Senecio jacobaea and *Senecio aquaticus*:
Ecological outcomes and evolutionary consequences**

PROEFSCHRIFT

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General introduction

Heather Kirk

In his evolutionary doctrine 'The Origin of Species' (1859), Charles Darwin devoted a chapter to the implications of hybridization for the species concept. Darwin asks:

'do ... complex and singular rules [mediating interspecific crossing and hybrid fertility] indicate that species have been endowed with sterility simply to prevent their becoming confounded in nature? I think not. For why should the sterility be so extremely different in degree, when various species are crossed, all of which we must suppose it would be equally important to keep from blending together? Why should the degree of sterility be innately variable in the individuals of the same species? Why should some species cross with facility, and yet produce very sterile hybrids; and other species cross with extreme difficulty, and yet produce fairly fertile hybrids? Why should there often be so great a difference in the result of a reciprocal cross between the same two species? Why, it may even be asked, has the production of hybrids been permitted?'

Today, 150 years later, Darwin's insightful questions are still under investigation by evolutionary biologists. While Darwin used observation of variability in hybrid success to support the theory of evolution versus special creation, modern biologists are focusing on the potential evolutionary contributions of hybridization to speciation and phenotypic diversity. Since Darwin's time, two polarized views on the biological importance of inter-specific hybridization have emerged.

As discussed by Darwin, many researchers, historical to modern, have viewed inter-specific hybridization as an evolutionary dead end (Mayr, 1942; Wilson, 1965; Barton & Hewitt, 1985). Hybridization has frequently been approached as a thorn in the side of taxonomists, since hybrids lead to a blurring of species delineations, and a sort of evolutionary noise in studies of divergent evolution. Also, classical speciation models viewed hybridization as a process that either reinforces reproductive isolation, or causes hybridizing populations to merge (Dobzhansky, 1940; Stebbins, 1950; Grant, 1963). Thus much effort has been invested to minimize, and even ignore the consequences of hybridization in taxonomic and evolutionary studies. A good example is the frequent application of only cytoplasmic markers (which are generally non-recombinant) for phylogenetic reconstruction. Hybridization events in these cases are not detected, and for convenience are assumed to be absent.

On the other hand, the possibility that hybridization could have evolutionary outcomes was recognized as early as the mid-19th century (Herbert, 1847; Mendel,

1866; Naudin, 1863). Over the past 150 years, a number of evolutionary biologists have dabbled in studies of experimental and natural hybridization (Anderson, 1949; Stebbins, 1950; Lewontin & Birch, 1966). Yet, natural hybridization as a major evolutionary theme only really became established in the early 1990s (Harrison, 1990; Arnold, 1992; Whitham *et al.*, 1994). In recent years, hybridization in plants and animals has been credited with an astounding number of evolutionary processes, including the evolution of invasiveness (Ellstrand & Schierenbeck, 2000; Petit, 2004; Blair *et al.*, 2008), the generation of genetic (Rieseberg *et al.*, 1999) and phenotypic diversity (Orians, 2000; Rieseberg *et al.*, 2003a), introgression (Rieseberg *et al.*, 2000; Kim *et al.*, 2008), speciation (Rieseberg, 1997; Rieseberg *et al.*, 2003a), and adaptive radiations (Seehausen, 2004).

Despite claims that hybridization has wide-ranging evolutionary consequences, the question of whether hybridization is of universal adaptive importance remains to be answered. The taxonomic distribution of hybridization events is widespread; frequent reports of hybridization arise from birds (Grant & Grant, 1996) and plants (Ellstrand *et al.*, 1996), with less frequent but appreciable reports from fungi and bacteria, and invertebrates (marine invertebrates, Knowlton *et al.*, 1997; crickets, Harrison, 1986; grasshoppers, Marchant *et al.*, 1988). In plants, which are the focus of this thesis, it has been estimated that up to 50-70% of modern species have arisen from a hybridization event (Stace, 1975). Moreover, a recent review of five extensive floras (Ellstrand *et al.*, 1996) places the average number of extant interspecific plant hybrids (i.e., the number of hybrids with unique pairs of parental species) at 11% of plant species. This estimate represents a minimum estimate of actual hybridization events, since many natural hybrids likely remain unobserved and unreported. Furthermore, hybridization may be an increasingly common and therefore important phenomenon, as widespread worldwide human transplantation of plant species, both intentional and unintentional, may provide many new opportunities for previously geographically isolated species or population to hybridize, or for hybrid individuals to break free from parental populations (i.e., Abbott *et al.*, 2003).

Yet, is hybridization a process that frequently contributes to adaptive evolution? The overall evolutionary contribution of hybridization is currently difficult to estimate, since only a few groups of interspecific hybrids have been investigated in detail. In plants, model genera among which hybridization is common and frequently studied include irises (Arnold, 1990), sunflowers (Rieseberg *et al.*, 2003a), poplars (Martinsen *et al.*, 2001), *Eucalyptus* (Whitham *et al.*, 1999) and *Senecio* (Abbott & Lowe, 2004; Kim *et al.*, 2008). In animals, hybridization studies have focused on fish species including lake whitefish (Rogers *et al.*, 2001), cichlid fishes (Salzburger *et al.*, 2002; Smith *et al.*, 2003), and bird species including Darwin's finches (Grant & Grant, 1996). In most of these systems, it has been shown that hybridization has major ecological and/or evolutionary outcomes. Nevertheless, it is not yet clear whether hybridization is an evolutionary force among all species, or a process that contributes to adaptive evolution within a limited number of taxa. If hybridization frequently changes the evolutionary trajectory of organisms, then Darwin's (1859) divergent view of evolution may provide an inaccurate picture of life. Recent para-

digm shifts regarding the evolutionary importance of hybridization, combined with technological advances that facilitate detailed genetic studies of evolutionary processes, will facilitate deeper insights into the evolutionary role of hybridization over the coming years.

APPROACHES TO HYBRIDIZATION RESEARCH

Research into hybridization and its consequences has been initiated from a number of perspectives, from functional, to ecological to evolutionary. On a functional scale, researchers have investigated the immediate consequences of hybridization, in mechanistic terms, and also in relation to the immediate fitness consequences for early generation (F_1) hybrids. Functional research has been primarily initiated and utilized by the agricultural industry, which now uses superior (mostly intra-specific) hybrid strains for many economically important crops. Ecologists have been interested primarily in the question of how hybrid swarms are maintained in nature. Are hybrid populations maintained by a balance between continuous inter-specific crossing followed by negative selection (Barton & Hewitt, 1985)? Or are hybrids generally found in zones where they are more fit than parental species (Moore, 1977; Emms & Arnold, 1997)? Finally evolutionary biologists and systematists ask ultimate questions pertaining to the historical significance of hybridization: what significance has hybridization had for the evolution of both new traits and of new species?

A distinction is usually made between polyploid and homoploid hybridization, the first referring to a multiplication of the genome, and the second involving hybridization between taxa with equal chromosome numbers. Mechanisms of speciation via polyploidy are fairly easily understood, since polyploidy often occurs simultaneously with reproductive isolation. The ecology and evolutionary role of homoploid hybridization is less well understood, and we focus on homoploid hybridization here.

Although I focus mostly on ecological and evolutionary research in this thesis, I summarize current knowledge of hybridization derived from all three scales of research below.

THE FUNCTIONAL PERSPECTIVE: WHAT HAPPENS WHEN PLANTS HYBRIDIZE?

The immediate consequences of hybridization events have been well studied, both in agricultural and natural systems. Premating and postmating (prezygotic and postzygotic) factors can influence the initial success of interspecific crosses. When initial crosses are successful, the genomes from two different species are merged (F_1 hybridization), without recombination. Many F_1 systems express heterosis, or an increased fitness of hybrids over parents. Heterosis may have a number of causes, including a masking of deleterious alleles from each parental species (dominance), overdominance, or positive epistatic interactions between genes from different species. Additionally, it has been shown that for single traits, hybrids may inherit beneficial alleles at separate loci from both parents, yielding additive effects from

different loci that are greater in hybrids than in either parent (Rieseberg *et al.*, 1999).

Subsequent intercrossing and backcrossing to parental species can lead to genetic recombination, and changes in allele frequencies (Fritz *et al.*, 1999; Hochwender *et al.*, 2000; Rieseberg *et al.*, 2000). Such genetic recombination can have both positive and negative effects on hybrid fitness. Recombination can lead to the breakup of epistatic gene complexes (complexes of genes that work together, but are physically disparately located in the genome), which results in a decrease in fitness, a process known as hybrid breakdown. In contrast, recombination can also lead to the fixation of new combinations of beneficial alleles, and the expression of high fitness traits in hybrid individuals. Random recombination in a large number of hybrid offspring can create a heterogeneous pool of hybrid genotypes, such that hybrid individuals vary greatly with respect to each other and to parental individuals in terms of fitness (Whitham *et al.*, 1994; Emms & Arnold, 1997).

THE ECOLOGICAL PERSPECTIVE: ESTABLISHMENT, PERSISTENCE, AND SPREAD OF HYBRIDS IN NATURE

Once viable hybrids have been formed in nature, hybrid populations can persist, expand, or become locally extinct. Stable or expanding hybrid swarms have formed the basis of hybridization studies, since ephemeral populations are difficult to observe (i.e., Ellstrand *et al.*, 1996), and are likely have few ultimate consequences.

A number of models have been developed to explain the persistence of natural hybrid zones. Such models can generally be classified as environmentally dependent or environmentally independent. Environmentally independent models are epitomized by the tension zone model proposed by Barton & Hewitt (1985), which relies on intrinsic fitness inferiority of hybrids irrespective of environment. Barton & Hewitt (1985) propose that maintenance of most hybrid swarms results from an equilibrium between continuous dispersal of hybrids into hybrid zones, and subsequent negative selection against such hybrids.

However, modern researchers agree that environment plays a role in the maintenance of most hybrid populations (Arnold & Hodges, 1995a,b; Arnold, 1997; Fritz, 1999). Environmentally dependant models (Moore, 1977; Howard, 1986; Harrison, 1986, 1990) of hybrid zone stability usually involve 'genotype-by-environment' interactions (Emms & Arnold, 1997), which imply that variable hybrid genotypes interact with environmental gradients to produce zones where hybrids have superior fitness in relation to parental species.

Many studies confirm that hybrids often vary in fitness in relation to parental species across environmental gradients (Emms & Arnold, 1997; Campbell & Waser, 2001), and that hybrids can in fact be relatively more fit than parental species in certain environments (Arnold & Hodges, 1995a,b). Moreover, studies with sunflowers (Rieseberg *et al.*, 2003a) have shown that hybrids may be most fit in entirely novel environments, which cannot be colonized by either parental species.

HYBRIDIZATION AND EVOLUTION

On a longer time scale, evolutionary biologists have focussed on the contribution of hybridization to the creative processes in evolution. Hybridization is now known to play a number of roles in the evolution of new species and traits. These roles include i) the generation of novel traits, ii) introgression of traits between species, and iii) speciation.

Generation of novel traits

The generation of novel traits can be classified quantitatively, or qualitatively. The expression of quantitatively extreme phenotypes by hybrids in relation to parental species is referred to as transgressive segregation. The generation of entirely novel characters has received most attention by researchers investigating the expression of secondary metabolites in plants (e.g., Orians, 2000).

Transgressive segregation

Transgressive segregation is specific to segregating hybrid populations. Natural and artificial hybrids are frequently reported to possess phenotypes that are extreme relative to parental phenotypes. In a recent review of 171 studies examining traits in hybrids versus parental species, Rieseberg *et al.* (1999) found that 91% of all studies reported at least one transgressive trait. Overall, 44% of 1229 traits observed were transgressive in hybrids. Transgressive segregation occurs most often in plants versus animal, inbred species versus outbred species, and intra-specific crosses versus inter-specific crosses (Rieseberg *et al.*, 1999).

As with heterosis, hypotheses that account for transgressive segregation include overdominance of alleles at heterozygous loci, and the masking of deleterious alleles from either parental species. Yet, genetic studies have shown that complementary action of additive alleles from both species (Table 1; Rieseberg *et al.*, 2003b) may explain transgressive segregation in most cases.

Unexpectedly, Rieseberg *et al.* (2003b) found that, in most QTL studies, species that express traits with high values possess antagonistic, alleles that decrease trait values at some loci. For example, marker-based QTL studies from inter-specific crosses between two tomato species, *Lycopersicon esculentum* and *L. pennellii*,

Table 1 An illustration of the mechanism by which the complementary action of genes with additive effects can contribute to transgressive segregation in hybrids (modified from Rieseberg *et al.*, 2003b).

QTLs	Phenotype		Transgressive F2	Transgressive F2
	Species A (AA genotype)	Species B (BB genotype)		
1	+1	-1	+1 (AA)	-1 (BB)
2	+1	-1	+1 (AA)	-1 (BB)
3	+1	-1	+1 (AA)	-1 (BB)
4	-1	+1	+1 (BB)	-1 (AA)
5	-1	+1	+1 (BB)	-1 (AA)
Total	+1	-1	+5	-5

showed that many loci had antagonistic effects on the species that possessed the highest trait values (i.e., height, stem diameter, number of internodes, etc.; DiVicente & Tanksley, 1993). For instance, although, *L. esculentum* exhibited a significantly larger stem diameter than *L. pennellii*, more than 50% of the QTLs detected in the study had a decreasing effect on stem diameter of *L. esculentum* individuals. Segregation in hybrids can thus facilitate the purging of alleles that have negative effects on high fitness traits, such that some hybrids can have higher (or lower) trait values than both parental species. Overall, a majority of traits (63.6% of 572 traits from plants and animals) are controlled by multiple quantitative genes (QTLs) that are influenced by antagonistic loci (Rieseberg *et al.*, 2003b).

Transgressive segregation may be extremely important for adaptive evolution in hybrid populations. Hybrids, which often encompass greater genetic variation than parental species, may respond more quickly to selection pressure than the latter, and may therefore have higher rates of evolution (i.e., Hercus & Hoffman, 1999b). High adaptive potential in hybrids may be particularly important in rapidly changing environments, or for drastic ecological divergence (Lexer *et al.*, 2003a).

Qualitative novelty

The generation of entirely novel traits may result from unique interactions between genes combined from both parental species (i.e., epistatic interactions between separate loci). At the metabolic level, novel compounds may be generated via at least three mechanisms (Orians 2000). Obstruction of a biosynthetic pathway in hybrids may lead to the accumulation of intermediaries that do not normally accumulate in parental individuals. Furthermore, pathway elaboration may allow novel compounds to be produced through the combination of unique parental components in hybrid individuals (i.e., the basic skeleton from one parent with the side chains or enzymes of another parents). Lastly, the location of metabolite production or modification may be shifted from one organ to another.

In turn, qualitative changes in plant chemistry may have myriad consequences for hybrid ecology, affecting interactions with parasites (Fritz *et al.*, 1999), or even primary growth habits (i.e., tolerance to damage; Hochwender *et al.*, 2000). Overall, mechanistic studies of the consequences of epistatic gene interactions in hybrids are lacking in the literature.

Introgression of traits between species

During or after divergence, species may evolve derived, high fitness genes. Subsequent hybridization between divergent taxa may allow the transfer of such high fitness traits from one species to another, functioning as a sort of adaptive shortcut. It has been shown that small fragments of the genome are more likely to introgress than large fragments (Martinsen *et al.*, 2001), since high fitness genes in large fragments are more likely to be linked to deleterious alleles.

Studies of introgression in poplar (Martinsen *et al.*, 2001) and sunflowers (see Rieseberg *et al.*, 2000, and references therein) have demonstrated that different areas of the genome introgress at different rates in natural hybridizing populations. These results provide evidence for positive, negative, and neutral selection on dif-

ferent areas of the genome in hybrid lines. Also, in both poplar (Martinsen *et al.*, 2001) and sunflower (Rieseberg *et al.*, 1990, 1991), chloroplast introgression rates are higher than rates of nuclear introgression, perhaps due to neutral or low levels of negative selection on cytoplasmic genes compared to nuclear genes. Results from these systems provide evidence for a strong genetic ‘filter’ in at least some hybrid zones. Such a filter should prevent the introgression of deleterious alleles, and could preclude genetic assimilation of one species into the other.

Speciation

Homoploid hybrid speciation presents a dilemma for evolutionary biologists, because hybrids generally occur in sympatry with parents, and are not reproductively isolated (Rieseberg, 1997). Hybrid founder events, cases in which hybrids can establish in a geographically isolated location, are thought to be the most likely avenue for homoploid hybrid speciation (Charlesworth, 1995). Rapid chromosome rearrangements (Grant, 1981) may also be a mechanism to establish speedy reproductive isolation between hybrids and parental species.

However, genotype sorting according to local environments may also provide opportunities for divergence of hybrid lines from parental species (ecological speciation; McCarthy *et al.*, 1995; Rieseberg *et al.*, 2003a; Gross & Rieseberg, 2005), as long as some form of reproductive isolation prevents continuous genotype mixing (Rieseberg, 1997). Ecological speciation may occur when extreme or novel ecological tolerance levels in hybrids allow them to perform better in some environments than parental species, or to colonize habitats that are outside the range of parental tolerance (Rieseberg *et al.*, 2003a). A number of flowering plant species, from genera including *Helianthus*, *Iris*, *Peaonia*, *Pinus*, and *Stephanomeria* (see Rieseberg, 1997, and references therein) are known to be derived from homoploid hybrids. While homoploid hybrid speciation clearly occurs in some instances, it is currently difficult to estimate the frequency of this mode of speciation among plants and animals.

SENECIO AND HYBRIDIZATION

The genus *Senecio*¹⁾ is ubiquitous taxa containing more than 1500 species, members of which occur across the planet. Not only species diversity, but also phenotypic diversity is extremely high within the genus. *Senecio* species can be found in highly variable habitats, from alpine habitats, to aquatic environments, to extremely salty or dry locales. Moreover, life history strategies are extremely variable, even between closely related species, such that growth forms include annual and perennial herbs, woody trees, succulents, and vines. It is known that hybridization occurs frequently between a number of species within the genus (Lowe & Abbott, 2004; Kim *et al.*, 2008), and hybridization may partially contribute to species and/or phenotypic diversity within *Senecio*.

¹⁾*Senecio jacobaea* and some related species are now placed in the *Senecio* Section *Jacobaea*, based upon the DNA sequences of their plastid genomes. *Senecio jacobaea* is now called *Jacobaea vulgaris*. I have not followed this change since it would have led to the use of different names for the same plant species in this thesis.

In this thesis, I develop a model hybrid system between *Senecio jacobaea* and *Senecio aquaticus*. These species were selected because there are potentially interesting functional, ecological, and evolutionary aspects to hybrid systems between them.

Firstly, *S. jacobaea* and *S. aquaticus* are both considered to be noxious weeds in agriculture systems where they frequently occur. *Senecio jacobaea* is highly invasive in regions where introduced (Bain, 1991), including Australia, North America, and New Zealand, and is becoming more pervasive within its native range. Reports of livestock poisoning by *S. aquaticus* are increasing in frequency, and either inter-specific or inter-population hybridization may contribute to the invasiveness of these species (i.e., Ellstrand & Schierenbeck, 2000).

Additionally, preliminary field observations have indicated that *S. aquaticus* grows in water rich marshland areas, while *S. jacobaea* grows in dry sandy sites. Putative hybrids appeared to be found on intermediate sites. Large differences in both abiotic and biotic conditions in the local environments of these species are interesting to study ecologically. How do hybrids cope with environmental extremes faces by either parental species? What factors determine the distribution of the parental population compared to parental individuals?

Furthermore, *S. jacobaea*, and to a lesser extent *S. aquaticus*, have been studied extensively because they are widely known to produce secondary defense compounds (pyrrolizidine alkaloids), which are used by plants for anti-herbivore, and potentially anti-microbial defense. Pyrrolizidine alkaloids (PAs) are highly diversified within the *Senecio* genus, and the composition of PA bouquets is highly species specific. Up to ten PAs can be found within a single *S. jacobaea* plant. It has been hypothesized that hybridization can lead to the generation of chemical diversity (Orians, 2000), which may partially explain the diversity of PAs in *Senecio*.

RESEARCH QUESTIONS

In this thesis, I investigate whether hybrids between *S. jacobaea* and *S. aquaticus* are, in some environments, more fit than parental species, in order to deduce whether hybridization may contribute to hybrid swarm stability, or to invasiveness or speciation in *Senecio*. I also search for evidence of novelty in chemical traits, in order to determine whether hybridization between *Senecio* species may provide an avenue for the generation of the staggering chemical diversity found in this genus.

- (1) Do *S. jacobaea* and *S. aquaticus* hybridize in natural populations?
- (2) Are hybrids vigorous, intermediate, or unfit in relation to parental species?
- (3) Is there any evidence that hybridization can lead to evolutionary innovation in *Senecio* hybrids?
 - Does hybridization result in production of novel PAs, or novel combination of PAs?
 - Does hybridization result in novelty or unique combinations of other primary and secondary metabolites?

OUTLINE OF THESIS

This thesis is divided into two general themes. Chapters 2-4 explore the ecology of a natural population of hybrids between *S. aquaticus* and *S. jacobaea*. In Chapter 2, I first show using molecular and chemical techniques that hybrids of *S. jacobaea* and *S. aquaticus* occur in natural populations. Chapter 3 details a number of growth studies, in which the vegetative growth of *S. jacobaea*, *S. aquaticus*, artificial hybrids, and natural hybrids was quantified over a range of water and nutrient treatments. Results presented in Chapter 4 describe the reproductive potential of F_1 hybrids compared to parental species, and this chapter investigates whether directional crossing success can influence the dynamics of a natural hybrid population.

The second theme of the thesis is the potential role of hybridization in the evolution of chemical diversity in *Senecio*. Chapter 5 uses a metabolomics approach to study the expression of common primary and secondary metabolites in parental species versus hybrids. The aim of the experiments presented in Chapter 6 was to test whether novel PAs, novel combinations of PAs, or greater concentrations of PAs are produced in natural and artificial hybrids, across a range of environmental conditions. Chapter 7 summarizes the findings presented in this thesis, and outlines future research that will take place as a result of the work outlined here.

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2

Natural hybridization between *Senecio jacobaea* and *Senecio aquaticus*: molecular and chemical evidence

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Hybridization is known to be involved in a number of evolutionary processes, including species formation, and the generation of novel defense characteristics in plants. The genus *Senecio* of the Asteraceae family is highly speciose and has historically demonstrated significant levels of inter-specific hybridization. The evolution of novel chemical defense characteristics may have contributed to the success of *Senecio* hybrids. Chemical defense against pathogens and herbivores has been studied extensively in the model species *Senecio jacobaea*, which is thought to hybridize in nature with *Senecio aquaticus*. Here, we use AFLPs and pyrrolizidine alkaloid (PA) composition to confirm that natural hybridization occurs between *S. jacobaea* and closely related species *S. aquaticus*, and we use AFLPs to estimate ancestry of hybrids. We also demonstrate that even highly backcrossed hybrids can possess a unique mixture of defense chemicals specific to each of the parental species. This hybrid system may therefore prove to be useful in further studies of the role of hybridization in the evolution of plant defense and resistance.

Key words: *Senecio jacobaea*, *Senecio aquaticus*, hybridization, chemical defense, pyrrolizidine alkaloids, amplified fragment length polymorphisms (AFLPs)

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INTRODUCTION

Early theories regarded hybridization events as counter-productive to speciation, creating gene flow between otherwise divergent clades. However, recent estimates indicate that plant hybridization events are at the root of thirty to eighty percent of modern angiosperm species (Rieseberg & Ellstrand, 1993 and references therein).

Increased awareness of the role of hybridization in plant evolution has led to a rapid expansion in the use of hybrid systems in ecological and evolutionary research. Researchers are now using hybrid systems to explore the evolution of various plant traits including susceptibility to herbivory (Fritz *et al.*, 1994; Fritz, 1999), plant defenses (Fritz *et al.*, 1994; Orians, 2000) and tolerance to damage (Hochwender *et al.*, 2000), and broader studies of species formation (see Barton & Hewitt, 1985; Arnold, 1997).

However, hybridization does not occur frequently in all families (Ellstrand *et al.*, 1996), and may therefore contribute variably to evolutionary processes among taxa. Within the *Senecio* genus (Senecioneae) of the Asteraceae family, reports of inter-specific hybridization are common; confirmed natural hybridizations are known to occur between *Senecio vulgaris* and *S. squalidus* (Lowe & Abbott, 2000), *S. vulgaris* and *S. vernalis* (Comes, 1994), *S. germanicus*, *S. hercynicus*, and *S. ovatus* (Hodalova & Marhold, 1996; Hodalova, 2002), and *S. keniodendron* and *S. keniensis* (Beck *et al.*, 1992). Also a number of modern *Senecio* species, including *S. cambrensis* (Harris & Ingram, 1992), and *S. squalidus* (Abbott *et al.*, 2000) have arisen from hybrid origins. Hybridization has therefore played a potentially large role in *Senecio* species evolution.

Additive and novel chemical defense characters are among the factors that are postulated to play a role in superior hybrid fitness and persistence (Fritz, 1999; Fritz *et al.*, 1999; Hochwender *et al.*, 2000; Orians, 2000). *Senecio* species are well known for production of pyrrolizidine alkaloids (PAs), defense compounds that are known for their toxic and repellant effects on vertebrate (Cheeke, 1988) and invertebrate (Macel, 2003) herbivores. In particular, *Senecio jacobaea* L. has been used as a model organism in studies of PA effects on fungal pathogens (Hol & van Veen, 2002) as well as generalist and specialist herbivores (Macel, 2003), and is therefore an ideal contender as a model for studies of hybridization in relation to plant defense evolution.

A candidate hybrid system includes *S. jacobaea* and *Senecio aquaticus* Hill. *S. aquaticus* is closely related to, but not a sister species of *S. jacobaea* (Pelser *et al.*, 2003). Putative hybrids between *S. jacobaea* and *S. aquaticus*, identified based on morphology, have been described from multiple locations in Western and Central Europe (Chater & Walters, 1976). Here, we investigate a putative *S. jacobaea* × *S. aquaticus* hybrid population from the Zwanenwater reserve (The Netherlands).

Putative hybrids from the Zwanenwater were initially identified in 1979 based on highly variable and usually intermediate flower and leaf lobe morphology compared with parental species (Ruud van der Meijden, personal communication). *S. aquaticus* possesses well developed ligulate flowers and large terminal leaf lobes, while *S. jacobaea* always has divided terminal leaf lobes (Chater & Walters, 1976) and in the Zwanenwater reserve, displays undeveloped ligules. Because morphological variation can be quite high within *Senecio* species (Benoit *et al.*, 1975), and because *S. jacobaea* is known to exhibit considerable variation in ligule morphology (Andersson, 2001), we chose to verify hybrid status using molecular and chemical techniques.

Both molecular and chemical character expression are far more predictable in hybrids than morphological characters, which can vary greatly in relation to parental characters (Rieseberg & Ellestrand, 1993). The amplified fragment length polymorphism (AFLP) technique developed by Vos *et al.* (1995) is reliable, informative at species and population levels, and requires no knowledge of nucleotide sequences. In addition, AFLPs have been successfully used to identify hybrids and/or estimate degree of backcrossing between a number of plant and animal species (see Teo *et al.*, 2002; Pooler & Riedel, 2002).

Among potential chemical markers, secondary metabolites have been shown to be reliable for identifying hybrids between many pairs of species (see review by Rieseberg & Ellestrand, 1993). Within the *Senecio* genus, PAs are highly diversified, can be species-specific (Hartmann & Witte, 1995), and are at least partially genetically regulated (Vrieling *et al.*, 1993). We therefore expect to find reliable PA markers from both *S. aquaticus* and *S. jacobaea* which we can use to identify hybrid individuals.

Here, we aim to confirm the natural existence of *S. jacobaea* × *S. aquaticus* hybrids using molecular and chemical markers. We also examine secondary metabolite patterns in parental species and natural hybrids to determine whether hybridization may lead to novel combinations of defense characteristics in natural *Senecio* hybrids.

MATERIALS AND METHODS

Plant material

Putative hybrids were collected from a zone (10 m broad) at the border of a small lake within the Zwanenwater nature reserve. *S. jacobaea* individuals were collected from sand dunes at least three hundred meters distant from the putative hybrid zone, and *S. aquaticus* individuals were collected from an agricultural pasture separated from the dune/lake area by a road and approximately 500 m distant from the putative hybrid swarm. *Senecio aquaticus* was not collected from the immediate hybrid-zone locality because *S. aquaticus*-like individuals occur rarely along the lake fringe, but in insufficient quantities for collection and analysis.

For the first of two AFLP analyses, we identified and collected rosette plants of *S. jacobaea* ($n = 22$), *S. aquaticus* ($n = 18$), and putative hybrids ($n = 20$), based on leaf lobe morphology, as no plants were flowering at this time. Reference *S. jacobaea* plants ($n = 6$) were collected in the Meijendel dune reserve approximately 50 km south of the Zwanenwater reserve.

Seeds of *S. jacobaea* (n , number of parental plants = 13), *S. aquaticus* ($n = 13$) and putative hybrids ($n = 15$) used in a second set of AFLP analyses were collected from plants in the field and were identified based on leaf lobe and flower morphology. Reference *S. jacobaea* ($n = 8$) and *S. aquaticus* ($n = 10$) individuals used in the second AFLP analyses and PA analysis were selected from a seed collection at Leiden University, The Netherlands; *S. jacobaea* individuals originated from The Netherlands (Chaam, $n = 1$), France (Chérenge, $n = 1$; second population, $n = 2$),

Switzerland (L'Himelette, $n = 2$), and Germany ($n = 2$). *S. aquaticus* reference individuals originated in Denmark ($n = 2$), Switzerland ($n = 2$ from each of two populations), Germany (Darmstadt, $n = 2$), and Italy ($n = 2$). Reference individuals were used to confirm the validity of diagnostic molecular markers and PAs identified from Zwanenwater individuals.

Seeds were germinated in Petri dishes on moist filter paper (light 16h, temperature 20 °C, relative humidity 100%). Approximately 1 week after germination, seedlings were planted in potting soil and were grown in a climate room for 6-10 weeks (light 16h, temperature 20/15 °C, relative humidity 70%).

Molecular analyses

We conducted two rounds of AFLP analysis to confirm that characterization of the hybrid population was consistent regardless of selected AFLP primers and plant individuals.

DNA isolation

In all cases, single young leaves were removed from test plants and stored at -80 °C prior to DNA extraction. During the first round of molecular analysis (AFLP analysis 1), DNA was extracted using a modified version (Vrieling *et al.*, 1999) of the procedure described by Dellaporta *et al.* (1983). DNA was isolated during the second round of molecular analysis (AFLP analysis 2) using a Nucleon extraction and purification kit for plant tissue (Amersham International, England) according to the manufacturer's instructions.

AFLP analyses

AFLP analyses generally adhered to the protocol described by Vos *et al.* (1995). In short, genomic DNA was digested and ligated to adapters (Gibco, BRL) in one step, using MseI and EcoRI restriction enzymes (New England Biolabs). The reaction was conducted using ligation buffer provided by the supplier (New England Biolabs). Restriction-ligation was carried out overnight at 37 °C, after which the ligase was heat inactivated. Restriction-ligation products were diluted 10-fold for use in polymerase chain reaction (PCR). Diluted restriction ligation products were preamplified using 1 selective nucleotide with each AFLP primer (A for EcoRI primer, and C for MseI primer). A second round of selective amplification was conducted, using three selective nucleotides with each primer. All PCR reactions were carried out using AFLP core mix (Applied Biosystems). In total, one primer combination was used in the first analysis (Mse-CAG/Eco-ACA), and two primer combinations were used in the second analysis (Mse-CTG/Eco-ACA and Mse CTG/EcoAGG). Eco primers were fluorescently labelled (Fam and Joe labels, Applied Biosystems). Selective amplification products were separated on 5% polyacrylamide gel using an ABI Prism 377 automatic sequencer.

PA analysis

All plants used in AFLP analysis 2 were analysed for PA composition. In addition, we analysed reference *S. aquaticus* and *S. jacobaea* (see *Plant Material* above). Dried

leaves and roots from each plant were separately milled to a fine powder. Milled samples were stored in a freezer at -80°C until use. Fifteen mg of plant material was extracted according to a modified version (de Boer, 1999) of the acid-base extraction method (Hartmann & Zimmer, 1986). Extracts were dissolved in methanol containing heliotrine (Latoxan, France) as an internal standard and analysed using gas chromatography (GC). Conditions (injector 250°C , temperature program 0-22-5-250, split mode 1-30, carrier gas N_2 0.9 ml min^{-1} , pressure 56 kPa; detector NPD) were controlled by a Hewlett Packard gas chromatographer (model 6890). GC traces were compared with known references to identify sample composition.

Data analysis

AFLP analyses

Initial analysis of data was carried out in Genescan (Applied Biosystems), after which data was extracted to Genographer 1.4.0. for scoring of bands. Fragments ranging from 100-500 bases with a fluorescent intensity >50 were scored as present. We scored bands as dominant markers, giving bands present a value of 1, and bands absent a value of 0.

For qualitative identification of hybrid individuals, we defined diagnostic markers as those that are present in one species and not present in the other species. Diagnostic markers are thus a subset of all polymorphic markers identified in the study, as some polymorphic markers are present in both parental species, but in differing frequencies. Uniform (always present in a species) and variable (present sometimes in a species) diagnostic markers were identified in *S. aquaticus* and *S. jacobaea* reference individuals, and cross-checked in Zwanenwater parental individuals (referred to from here forth as Zw). Only those markers that were always present in both references and Zw parents were considered to be uniform. Individual putative hybrids were considered to be confirmed hybrids if they possessed at least one diagnostic marker from each parental species, or if they possessed at least one diagnostic marker from one parental species, and were missing at least one uniform diagnostic marker from the same parental species.

To quantify ancestry of putative hybrids, we analysed all polymorphic AFLP markers from all Zw individuals according to an admixture model in the program Structure 2.1 (Pritchard *et al.*, 2000), which uses a Bayesian model-based clustering method to infer individual proportions of ancestry deriving from multiple populations. While the admixture model has not been explicitly tested for analysis of dominant markers, the authors assert that the use of many markers, as is the case in this study, should assure unbiased results. For data entry, we considered absent markers to be homozygous (aa), and present markers to be either hetero- or homozygous (Aa or AA). Absent markers were thus assigned values of 0 for both alleles. Present markers were assigned a value of 1 for one allele, and the second allele was considered to be missing data. We assumed that all individuals were derived from two separate populations, representing *S. jacobaea* (Zw) and *S. aquaticus* (Zw). We used a burn-in period of 50,000 iterations, at which time summary statistics were approximately stationary. Results presented are based on runs of 100,000 iterations, which yielded consistent outcomes over several independent runs.

To test whether our two separate analyses generally yielded the same results, we conducted a two-way ANOVA on estimates of inferred ancestry (proportion derived from *S. jacobaea* cluster), defining sampling location (*S. jacobaea*, *S. aquaticus*, and hybrid) and analysis as random factors. Because we found no effect of analysis ($df = 1$, $F = 3.846$, $P = 0.189$), and no interaction between sampling location and analysis ($df = 2,95$; $F = 1.281$; $P = 0.283$), we combined all data from both analyses by assigning missing value scores to markers that were not utilized in each analysis. We then re-analysed the combined data set as described above, and the results presented here represent those yielded by the combined analysis.

PA analysis

Species-specific PAs were identified in *S. aquaticus* (Zw) and *S. jacobaea* (Zw) individuals. Species-specificity of such diagnostic PAs was confirmed by comparing such PAs to reference *S. aquaticus* and *S. jacobaea* individuals, and by cross-checking with literature regarding known PA composition for both parental species (Hartmann & Witte, 1995; Christov *et al.*, 2002; Macel *et al.*, 2002). Individual putative hybrids were considered to be confirmed hybrids if they possessed at least one diagnostic PA from each parental species. No informative, strictly species-specific PA markers were identified during the analysis, so the absence of such PAs was not used in hybrid characterization.

RESULTS

AFLP analyses

Diagnostic Markers

The first AFLP analysis yielded a total of 11 diagnostic bands for *S. jacobaea* (of which four were uniform) and nine diagnostic bands for *S. aquaticus* (of which seven were uniform). Of 20 putative hybrids analysed during the first analysis, we confirmed that 15 were hybrids based on diagnostic AFLP bands (Fig. 1A).

The second AFLP analysis yielded a total of 26 diagnostic bands for *S. jacobaea* (of which five were uniform) and seven diagnostic bands for *S. aquaticus* (of which two were uniform). Of 15 putative hybrids analysed during the second analysis, we confirmed that eight were hybrids based on diagnostic AFLP bands (Fig. 1B).

Figure 1 Presence and absence of *S. aquaticus* and *S. jacobaea* diagnostic PA and AFLP markers in putative hybrids used in two rounds (identified as A and B) of AFLP analysis. Uniform markers indicate markers that are always present in one parental species and never present in the other, while variable markers indicate markers that are sometimes present in one parental species and never present in the other. Ancestry, expressed as proportion of individuals derived from *S. jacobaea*, is indicated for each individual (columns). Rows represents markers. Note that PA composition was not measured for analysis 1. Hybrid based on AFLP bands (*), PAs (**), or AFLP bands and PAs (***)

A

Hybrid individual	H10a	H7a	H71a	H3a	H12a	H5a*	H6a*	H9a*	H1a*	H15a*	H51a*	H71a*	H31a*	H8a*	H41a*	H13a*	H61a*	H81a*	H31a*	H2a*
Inferred proportion from <i>S. jacobaea</i> ancestry	.997	.998	.998	.995	.998	.997	.998	.962	.958	.942	.679	.854	.854	.750	.980	.466	.995	.620	.646	.578
AFLP Markers: Primer combination and marker identity (# bases)																				
CAG/ACA 109																				
CAG/ACA 218																				
CAG/ACA 237																				
CAG/ACA 324																				
CAG/ACA 445																				
CAG/ACA 466																				
CAG/ACA 617																				
CAG/ACA 111																				
CAG/ACA 164																				
CAG/ACA 325																				
CAG/ACA 564																				
CAG/ACA 125																				
CAG/ACA 144																				
CAG/ACA 234																				
CAG/ACA 424																				
CAG/ACA 426																				
CAG/ACA 449																				
CAG/ACA 546																				
CAG/ACA 457																				
CAG/ACA 483																				

B

Hybrid Individual	H11b	H2b	H3b**	H4b	H5b	H6b	H7b**	H9b*	H10b***	H11b*	H14b*	H15b*	H12b***	H13b*	H8b***
Inferred proportion from <i>S. jacobaea</i> ancestry	.992	.996	.983	.998	.997	.997	.997	.271	.336	.193	.811	.979	.372	.454	.284
PA Markers: Retention time and PA location in plant [roots (r), shoots (s)]															
8.13 s															
10.20 r															
10.53 s															
13.18 r															
3.62 s															
AFLP Markers: Primer combination and marker identity (# bases)															
CTG/ACA 481															
CTG/ACA 470															
CTG/ACA 447															
CTG/ACA 437															
CTG/ACA 405															
CTG/ACA 370															
CTG/ACA 352															
CTG/ACA 262															
CTG/ACA 255															
CTG/ACA 241															
CTG/ACA 218															
CTG/ACA 209															
CTG/ACA 177															
CTG/ACA 146															
CTG/ACA 140															
CTG/AGG 334															
CTG/AGG 213															
CTG/AGG 200															
CTG/AGG 156															
CTG/AGG 126															
CTG/AGG 106															
CTG/AGG 381															
CTG/ACA 415															
CTG/ACA 253															
CTG/ACA 132															
CTG/ACA 124															
CTG/ACA 135															
CTG/ACA 250															
CTG/ACA 357															
CTG/ACA 355															
CTG/ACA 322															
CTG/AGG 307															
CTG/AGG 210															

	= <i>S. jacobaea</i> specific variable marker present
	= <i>S. jacobaea</i> specific uniform marker present
	= <i>S. aquaticus</i> specific uniform marker present
	= <i>S. aquaticus</i> specific variable marker present
	= marker absent

Bayesian Cluster Analysis

Forty-seven and 65 polymorphic markers were included from AFLP analyses 1 and 2, respectively, for use in cluster analysis. The clustering program estimates ancestry of each individual, expressed as proportion derived from each parental cluster (which we will refer to from here forth as *S. aquaticus* and *S. jacobaea* clusters), such that proportions derived from parental clusters adds to 100% for each individual.

Overall, *S. aquaticus* (Zw) individuals were derived almost completely from the *S. aquaticus* cluster (98.8%), and *S. aquaticus* (Zw) clustering never overlapped with that of *S. jacobaea* (Zw) or hybrid individuals (Fig. 2). Putative hybrids were mostly derived from the *S. jacobaea* cluster (79.8%), which indicates that the hybrid population is generally back-crossed to *S. jacobaea*. There is however evidence that *S. jacobaea* (Zw) and hybrid individuals were sometimes confused in the field based on morphology. While overall, *S. jacobaea* (Zw) individuals were derived mostly from the *S. jacobaea* cluster (95.7%), at least five of 35 *S. jacobaea* (Zw) individuals (Fig. 2) were partially derived from the *S. aquaticus* cluster (>10% from *S. aquaticus* cluster). Similarly 10 putative hybrids were not confirmed to be hybrids on the basis of diagnostic markers, and were derived almost completely from the *S. jacobaea* cluster (Fig. 1). Since the Bayesian clustering approach is robust to the presence of misclassified individuals (Pritchard *et al.*, 2000), we do not anticipate that such misclassification affects the accuracy of our results.

While results from Bayesian clustering generally agreed with classification based on diagnostic markers, we did note several incongruencies; a number of individuals (H5a, H6a, H4ia, H6ia) are missing uniform *S. jacobaea* specific markers and/or possess *S. aquaticus* specific markers but are almost entirely derived from *S. jacobaea* (>99%). Conversely, H9b is mostly derived from *S. aquaticus* according to Bayesian analysis but possesses a considerable number of *S. jacobaea*-specific markers and no *S. aquaticus*-specific markers. Such results may occur due to stochastic inheritance of the diagnostic markers identified in our study.

PA analysis

We considered 17 different PAs in our analysis (Table 1). We discarded 11 potential PAs from our analysis because they were very rare among sampled individuals and

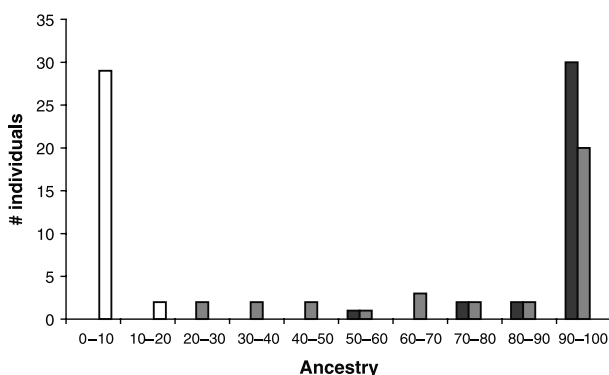


Figure 2 Histogram of ancestry of putative *S. jacobaea* (black bars), hybrids (grey bars) and *S. aquaticus* (white bars). Ancestry (x-axis) is expressed as percentage of genome derived from the *S. jacobaea* cluster.

Table 1 Pyrrolizidine alkaloids (PAs) (with GC retention times) included in PA analysis. Confirmation of identity using known standards was not possible for un-named PAs. Roots (R)/ shoots (Sh) indicates whether a given PA was found in roots, shoots, or both. (tr) indicates that a PA was found only in trace amounts; * used as species-specific markers

Retention time	PA	<i>S. jacobaea</i>	<i>S. aquaticus</i>	Hybrid
3.07		Sh	Sh	Sh
3.16		Sh/R	Sh/R	Sh/R
3.62*		Sh(tr)	Sh	Sh
3.77		Sh/R	Sh/R	Sh/R
6.11		R	R	R
7.42	Senecionine	Sh/R	Sh/R	Sh/R
7.65	Seneciophylline	Sh/R	Sh/R	Sh/R
8.13*	Spartiodine	Sh		Sh
8.20	Intergerrimine	Sh/R	Sh/R	Sh/R
9.10		Sh	Sh(tr)	Sh
9.58	Jacobine	Sh/R	Sh/R	Sh/R
10.02	Jacozine	Sh/R	Sh(tr)/R(tr)	Sh/R
10.20*	(otonecine type)	R		R
10.53*	Jacoline	Sh/R		Sh
11.13	Erucifoline	Sh/R	Sh/R	Sh/R
13.18*		R		R
13.38	Acetylerucifoline	Sh	Sh	Sh

therefore not informative, or because only trace amounts were detected by GC and data were therefore considered unreliable. For convenience, we refer to PAs using retention times (rt) where no reference samples were available.

We found 13 PAs in *S. aquaticus* (12 in shoots and nine in roots) and 17 PAs in *S. jacobaea* (14 in shoots and 12 in roots). Of the PAs included in our analysis, three were always present in both *S. aquaticus* and *S. jacobaea* in roots, in shoots, or in both roots and shoots (senecionine, seneciophylline, and jacozine). One PA (rt 3.62) was always present in *S. aquaticus* (reference and Zw individuals) and was rarely present in *S. jacobaea* (Zw). Several PAs were present sometimes in *S. jacobaea*, and never in *S. aquaticus*. These include spartiodine and jacoline in the shoots, and jacoline, rt 10.20, and rt 13.18 in the roots.

The PA rt 3.62 was particularly informative for identifying hybrids because it was always present in *S. aquaticus* in relatively high concentrations. rt 3.62 also appeared in four *S. jacobaea* (Zw) individuals but in very low concentrations (0.0152 ± 0.0047 mg PA/g plant dry weight) that never overlapped with concentrations occurring in *S. aquaticus* (0.2-0.9 mg PA/g plant dry weight). Of these *S. jacobaea* (Zw) individuals, one was shown to be partly derived from the *S. aquaticus* cluster (20.9%), and we speculate that the presence of rt 3.62 in *S. jacobaea*-like individuals may reflect introgression, since this PA was never found in reference *S. jacobaea* individuals. Nonetheless, PA rt 3.62 was considered to be a *S. aquaticus* specific marker only when present in hybrids in concentrations higher than the range in which this PA was found in *S. jacobaea* (Zw) individuals.

We confirmed hybrid ancestry if *S. aquaticus*-like concentrations of rt 3.62, and at least one *S. jacobaea* diagnostic PA were found within an individual (Fig. 2B).

Out of 15 putative hybrid individuals analysed for PAs, five were confirmed to be hybrids based on PA composition. Of these five, three were also confirmed to be hybrids based on AFLP markers. Expression of species-specific PAs was not predictable based on ancestry of hybrids, such that rt 3.62 was sometimes expressed in hybrids that were mostly derived from *S. jacobaea* (H3 and H7), and spartiodine and jacoline (*S. jacobaea* specific) were sometimes expressed in hybrids highly derived from *S. aquaticus*. In other cases, species-specific PAs were not expressed in hybrids highly derived from parental species in which such PAs are frequently found.

DISCUSSION

Reports of hybridization between *S. jacobaea* and *S. aquaticus* are common in literature. Two independent molecular analyses, in addition to chemical evidence, confirm that hybrids are present in a hybrid swarm in the Zwanenwater reserve in The Netherlands, and that hybrids are generally back-crossed to *S. jacobaea*.

Molecular analysis demonstrates that field identification based on morphology is not always reliable for identification of either parental or hybrid individuals. We can clearly conclude that hybridization has led to genomic introgression, and possibly introgression of one *S. aquaticus*-specific PA, to *S. jacobaea*-like individuals at least three hundred meters distant from the hybrid zone. It is less clear whether inability to confirm hybrid ancestry of some putative hybrids resulted from misidentification or insufficient sensitivity of our methods. We expect that highly back-crossed hybrids are difficult to distinguish from parental individuals because such individuals should cluster with pure *S. jacobaea* individuals using Bayesian methods, and diagnostic markers will occur infrequently in such back-crossed hybrids. Indeed, Boecklen & Howard (1997) suggest that upwards of 70 diagnostic markers are required to distinguish parental species from advanced back-crosses with reasonable confidence.

PA composition data was particularly useful for this study because PAs were complementary to molecular data for inter-specific hybrid identification, such that some putative hybrids could be confirmed on the basis of PA composition, but not using AFLP markers. However, we also observed that expression of PAs specific to parental species was not predictable in hybrids based on clustering results, and we thus stress that PA expression is not reliable for predicting degree of backcrossing, or as a sole technique for hybrid identification. That parental PAs are expressed inconsistently in hybrid individuals which have similar ancestry is not surprising; as Orían (2000) indicates, both qualitative and quantitative variation in expression of secondary chemicals can be quite high within and between hybrid classes.

Our finding that species-specific PAs from *S. aquaticus* and *S. jacobaea* occur within even highly backcrossed hybrid individuals is also significant from the perspective of hybrid ecology and the evolution of chemical defenses. Many authors postulate that enhanced defense characters in hybrids may play a role in hybrid persistence and hybrid fitness superiority in the environments in which they are found

(Fritz, 1999; Fritz *et al.*, 1999; Hochwender *et al.*, 2000, Orians, 2000 and references therein). Indeed, a recent review indicates that approximately 15% of hybrids tested for herbivore resistance in field, common garden, and laboratory tests demonstrate additive inheritance of resistance from parental species (Fritz *et al.*, 1999). Such increased resistance is generally thought to result directly from plant chemistry (Orians, 2000). However, there is relatively little evidence from natural systems that resistance superiority contributes to natural hybrid persistence (Fritz *et al.*, 1997).

That hybridization between *S. jacobaea* and *S. aquaticus* can lead to novel combinations of PAs even after extensive backcrossing warrants further study into both causes of the Zwanenwater hybrid swarm persistence and the evolutionary potential of *Senecio* hybrids for the development of new defense characteristics.

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3

Maternal effects and heterosis influence the fitness of plant hybrids

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We test two possible non-exclusive explanations for the maintenance of a hybrid swarm between *Senecio jacobaea* L. and *Senecio aquaticus* Hill: 1) Genotype-by-environment interactions involving water and nutrient clines are involved in hybrid fitness, and 2) heterosis in early hybrid generations may provide an initial hybrid advantage that contributes to hybrid persistence.

In three climate chamber studies, fitness and root growth were measured for parental species and natural and artificial F₁ hybrids, in order to determine whether hybrids occur in habitats where they are more fit than parental species.

Natural hybrids, which are generally back-crossed to *S. jacobaea*, always equaled *S. jacobaea* in growth characteristics. Maternal effects played a role in the fitness of F₁ hybrids, with offspring from *S. jacobaea* mothers exhibiting higher fitness than those from *S. aquaticus* mothers, and compared to parental species and natural hybrids.

Natural hybrids are not distributed in zones where they are most fit with respect to nutrient and water regimes. Superior fitness of early generation hybrids may contribute to hybrid swarm stability.

Key words: hybridization, genotype-by-environment interactions, hybrid fitness, heterosis, maternal effects, *Senecio jacobaea* L. (ragwort), *Senecio aquaticus* Hill

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INTRODUCTION

It is now widely accepted that hybridization can contribute to evolutionary processes (Arnold, 1997; Riesberg, 1997; Rieseberg & Carney, 1998). From introgression (Rieseberg *et al.*, 2000), to speciation (Rieseberg, 1997), to adaptive radiation

(Seehausen, 2004), hybridization has been credited with diversification of innumerable traits, as well as species. Yet, not all hybridization events have long term consequences; some natural hybrids, for example, may be ephemeral, or sterile, such that no future genetic contribution can occur via the hybrid lineage. The fate of hybrids in natural hybrid zones, and thus their evolutionary potential, is thought to be dependent on a number of factors, the most important of which may be the fitness of hybrids in relation to parental species: 'if hybrids were uniformly less fit than the parental species, the role of hybridization in adaptive evolution would be minimal...' (Rieseberg & Carney, 1998).

There are two classes of models explaining the stability of hybrid zones in nature. Environmentally independent models propose that hybrids demonstrate some fitness level in relation to parental species, which is fixed regardless of environment. Such models are epitomized by the tension zone model proposed by Barton & Hewitt (1985), which relies on intrinsic fitness inferiority of hybrids. Barton & Hewitt (1985) propose that maintenance of hybrid swarms results from an equilibrium between continuous dispersal of hybrids into hybrid zones, and subsequent negative selection against such hybrids.

Environmentally dependent models (Moore, 1977; Howard 1986; Harrison 1986, 1990) of hybrid zone stability involve 'genotype-by-environment' interactions, according to which variable hybrid genotypes interact with environmental gradients to produce zones where hybrid fitness differs in relation to parental fitness. Many empirical examples from plant literature confirm that at least in plants, hybrids indeed often vary in fitness in relation to parental species across environmental gradients (Arnold & Hodges, 1995; Campbell & Waser, 2001; Johnston *et al.*, 2001; Arnold, 1997). Furthermore, superior hybrid fitness in environments intermediate, or unique in comparison, to parental environments may contribute to the maintenance of hybrid populations (Fritz, 1999; Arnold & Hodges 1995a, b; Arnold, 1997). For example, in a study of F₁ reciprocal crosses between *Ipomopsis aggregata* and *I. tenuituba*, Campbell & Waser (2001) found an interaction between maternal species and clinal location of planting, such that hybrids from *I. tenuituba* mothers were more fit than other plant groups in the hybrid habitat. Similarly, Wang (1997) showed local adaptation of two subspecies of *Artemisia tridentata* and their hybrids to their respective environments. Other authors have pinpointed examples of single environmental factors which mediate hybrid genotype × environment interactions, such as light intensity (*Iris fulva* × *Iris hexagona*; Arnold, 1997).

One of the shortcomings of many hybridization studies is experimentation is limited only to early generation artificial hybrids, or to natural hybrids that have a complex crossing history. For instance, it is an agricultural paradigm that F₁ hybrids generally exhibit heterosis over parental species, and that such heterosis can be conserved in early generation back- and inter-crosses. Still, many researchers only use F₁, F₂, and BC generations for studies of hybrid fitness (e.g., Burke *et al.*, 1998; Campbell & Waser, 2001; Parris, 2001; Johnston *et al.*, 2001), which may result in misleading conclusions. Conversely, studies only using natural hybrids, which are often highly back- or inter-crossed, may fail to encompass the processes that occur

early in hybrid zone formation, or population dynamics affected by early (i.e., F₁, F₂, and BC) hybrid generations occurring in stable populations (Carney *et al.*, 2000).

Here, we select two *Senecio* (Senecioneae; Asteraceae) species of which hybridization reports are common and widely distributed, in order to test whether hybrid vigor and genotype-by-environment interactions may lead to hybrid superiority and persistence in nature. We include parental species, F₁ hybrids, and natural hybrids in an attempt to understand the processes leading to the maintenance of a natural hybrid swarm.

The *Senecio* genus is extremely diverse, containing at least fifteen hundred species distributed throughout the world. Hybridization is thought to be common within the genus (see Lowe & Abbott, 2000; Kirk *et al.*, 2004), and hybridization processes may have partially contributed to historical diversification. *Senecio jacobaea* and *S. aquaticus* are typically characterized as drought and flood resistant species respectively. While *S. aquaticus* and *S. jacobaea* are closely related (Pelser *et al.*, 2003), field observation and literature (Weeda & Deursen, 1994; Chater & Walters, 1976) indicate that these species occupy distinct ecological niches. Molecular analysis of our study populations confirms that natural hybrids are generally backcrossed to *S. jacobaea* (Kirk *et al.*, 2004).

We present the results of three climate room experiments which are intended to explore differences between *S. aquaticus* and *S. jacobaea* and hybrids with respect to growth across a variety of environmental conditions. We test the following hypotheses:

- (1) Genotype-by-environment interactions determine parental and hybrid fitness, such that each group is more fit than the others in endemic water and nutrient conditions.
- (2) Performance of F₁ hybrids differs depending on parental maternal species. If this is true, we expect that hybrids from *S. jacobaea* mothers are more fit, because we observe that natural hybrids are highly backcrossed to *S. jacobaea*.
- (3) Performance of F₁ hybrids is greater than that of natural hybrids. If this is true F₁ heterosis may prolong hybrid breakdown, or contribute to the success of natural hybrid swarms.
- (4) Root elongation ability is a key adaptation for adaptation of *S. jacobaea* to drought, and may contribute to the fitness of natural hybrids in drying environments.

MATERIALS AND METHODS

Study system

Senecio jacobaea and *S. aquaticus* are monocarpic, biennial to perennial, self-incompatible species native to Western Eurasia. *Senecio jacobaea* colonizes areas subject to high disturbance such as roadside ditches and sand dunes, and *S. aquaticus* is an established resident of flood-prone marsh areas. Little overlap seems to occur between the local distribution of these species (Weeda & Deursen, 1994).

Viable hybrids between *S. jacobaea* and *S. aquaticus* have been reported from a number of locations including the United Kingdom (Stace, 1975), Germany (Christian Düring, personal communication), and The Netherlands (Kirk *et al.*, 2004). In this investigation, we study natural *S. jacobaea* × *S. aquaticus* hybrids from the Zwanenwater reserve (The Netherlands). Composed mostly of sand dunes, the Zwanenwater reserve contains a small lake around which a hybrid population exists (Fig. 1). *Senecio jacobaea* are abundant in the dunes surrounding the lake, while *S. aquaticus* occurs infrequently at the lake fringe. Alleged hybrids, first observed at the Zwanenwater in 1979 (R van der Meijden, personal communication) can be found in a narrow zone spanning a bank at the edge the lake, which appears to be intermediate to parental sites with regards to soil organic content and humidity.

Seeds of *S. jacobaea*, *S. aquaticus*, and natural hybrids were collected from plants in the field during 2001 and 2002. Putative hybrids were identified in the field based on leaf lobe and flower morphology, and were later confirmed to be hybrids based on diagnostic amplified fragment length polymorphism (AFLP) markers (Kirk *et al.*, 2004).

F₁ hybrids were produced by collecting second year rosettes of parental plants, exhibiting the development of flowering stems, from the field. To minimize chances that introgressive genes were present in experimental parents, *Senecio aquaticus* individuals were collected from a marshy agricultural grassland approximately 500 meters from the hybrid zone (Fig. 1), and *S. jacobaea* individuals were collected from dunes located approximately 300 m from the hybrid zone. Plants from both species were placed in a greenhouse, allowed to flower, and were crossed in pairs of *S. jacobaea* × *S. aquaticus* by rubbing flowerheads together. Seeds were harvested from both parental plants.

Determination of soil water and organic content from field sites

Soil samples were obtained from soil surrounding the roots of hybrid and parental plants collected during August 2001. Ten soil samples were analyzed from each plant group (hybrids, *S. jacobaea*, and *S. aquaticus*). Soil samples were weighed,



Figure 1 Schematic diagram of field distributions of *Senecio jacobaea*, *S. aquaticus*, and natural hybrids within the Zwanenwater reserve, The Netherlands. *Senecio aquaticus* individuals used in this study were collected from the zone across the road from the lake.

dried in an oven, and weighed again to determine soil moisture content. Samples were then ignited at 500 °C for a period of 4 h to burn off organic matter, and were reweighed to determine organic content. Percent water and organic content were calculated based on weight.

Climate room experiments

In all cases, seeds were germinated in Petri dishes on moist filter paper (light 16h, temperature 20 °C, relative humidity 100%). Approximately 1 week after germination, equal sized seedling were selected and placed under experimental conditions (light 16h, temperature 20/15 °C, relative humidity 70%).

Experiment 1

Experiment 1 was designed to test whether fitness of natural hybrids is greater than parental species in intermediate nutrient and water conditions. Seeds from four different *S. aquaticus* maternal plants, four different *S. jacobaea* maternal plants, and seven different natural hybrid maternal plants were used in the experiment. We transplanted six equal sized seedlings from each maternal plant into separate 10 × 10 × 10 cm pots, three of which contained potting soil and three of which contained dune sand, so that one offspring from each maternal plant was subjected to each treatment. All plants were given normal water for one week to allow for establishment of seedlings. Following this week, dry, wet, and intermediate soil conditions were established. The six treatments thus included all combinations of soil types and water treatments. Dry treatments were given small amounts of water from the bottom of the pots three times during the course of the experiment in order to keep the plants alive while maintaining near drought conditions. Intermediate groups were kept in one cm of water, and wet group pots were submerged in water to a depth of ½ cm from the top of the pot.

Experimental conditions were maintained for 6 weeks until plants were harvested. Harvested plants were measured individually for total fresh and dry biomass.

Experiment 2

The purpose of Experiment 2 was to test whether root length and water table depth plays a role in the relative fitness of parental species and natural hybrids. We randomly selected seeds from four different *S. aquaticus* maternal plants, four different *S. jacobaea* maternal plants, and seven different natural hybrid maternal plants for experimental use. One equal sized seedling from each maternal plant was transplanted into each of four experimental column (15 cm diameter) lengths: 20, 45, 75, and 100 cm. Columns contained soil composed of 50% dune sand and 50% potting soil. All columns were given sufficient water at the beginning of the experiment to allow for seedling establishment. Therefore at the beginning of the experiment, soil throughout the total length of the column was moist. Experimental conditions were established 2 weeks after seedlings were transplanted to columns. Twenty cm columns were placed in bins in which the water table was varied from 1 cm above the soil surface to 3 cm below the soil surface. Plants in 45 cm columns were watered regularly from the top. Plants in 75 and 100 cm columns were given no fur-

ther water throughout the course of the experiment. Because evaporation occurred from the soil surface, plants in 75 and 100 cm columns experienced an increasing gradient from dry to moist with column depth during the course of the experiment. Plants were harvested 8 weeks after the establishment of experimental conditions, when growth curves began to level out. Roots were carefully washed, and root length was measured by extending roots to full length on a table. Harvested plants were measured individually for total fresh and dry biomass.

Experiment 3

Experiment 3 was designed to test whether artificial F_1 hybrids are more fit than natural hybrids, whether maternal effects play a role in the fitness of F_1 hybrids, and whether root length is plastic or fixed in *S. jacobaea* and hybrids. Small clonal plants (tissue culture) were used rather than seedlings in this experiment. We selected five *S. aquaticus* genotypes, five *S. jacobaea* genotypes, and five natural hybrid genotypes for experimental use. We also included genotypes from F_1 producing crosses, from both parental plants in the reciprocal cross, such that genotypes originated from five crosses, and ten parental plants (five *S. aquaticus* mothers, and five *S. jacobaea* mothers). F_1 genotypes were unrelated to parental genotypes used in this experiment. One equal sized clone from each genotype was transplanted into each of six experimental columns (1 m length, 15 cm diameter), yielding a total of 150 experimental plants. One plant from each genotype was thus subjected to each of six experimental treatments.

The experiment was established to test a combination of two nutrient and three water treatments. We used sieved dune sand to fill all columns. In half the columns, the dune sand was mixed with 'Osmocote' slow release fertilizer (N:P:K = 15:11:13 + 2 MgO) at a concentration of 1.3 g/l sand to provide a nutrient rich medium. After establishment of seedlings, columns were partially submerged in water of three different depths: 5, 50, and 100 cm.

All columns were given sufficient water at the beginning of the experiment to allow for seedling establishment. Therefore at the beginning of the experiment, soil throughout the total length of the column was moist. Experimental conditions were established 2 weeks after seedlings were transplanted to columns.

Eight weeks after establishing experimental conditions, we emptied two 'dry' columns in which seedlings had died, in order to observe the depth at which moisture was available to plants growing in dry treatments. We observed that moisture was available at a depth of 45 cm, and we therefore allowed the remaining 148 experimental plants to grow for two more weeks, after draining the 5 cm water from the 'dry' treatment tubes.

At harvesting, fresh and dry weight of above and below ground plant parts (shoots and roots) was measured. Roots were carefully washed, and root length was measured by extending roots to full length on a table.

Statistical analysis

All analyses were carried out using SPSS. Data were tested for normality, and data from Experiment 3 was square-root transformed to achieve normality. Dry mass was

used as an estimator of fitness, as dry mass is highly correlated with seed production in monocarpic plants (see Klinkhamer *et al.*, 1987).

Data were analyzed using mixed model two-way ANOVAs to determine whether dependent variables (dry weight and root length) differed according to plant group (random factor), treatment (fixed factor), or an interaction between plant group and treatment. Plant group was treated as a random factor because the groups tested here represent only a few of a large number of classes (i.e., there are a range of hybrid classes, and we only sampled two of them here; see Green & Tukey, 1960, for a more detailed explanation). In Experiments 1 and 3, treatment was broken up according to soil type and water regime for multi-factorial analysis. In Experiment 3, we first tested for differences between F_1 hybrids from differing maternal species, and based on the results, defined offspring from different parental species as separate groups for analysis of the total data.

In cases where species was a significant factor, we further analyzed data by separating data into species and using one-way ANOVAs followed by Tukey multiple comparisons to identify differences between treatments per species.

RESULTS

Determination of soil water and organic content from field sites

Water content of soil collected from the root zone of all three plant groups differed significantly, while organic content of *S. aquaticus* soil differed from that of the other two plant groups (Fig. 2). Hybrids are found on sites intermediate to parental species sites in soil water content, and similar to *S. jacobaea* sites in soil organic content. *Senecio aquaticus* is found on moist organic soil, while *S. jacobaea* occupies dry sites with little organic content.

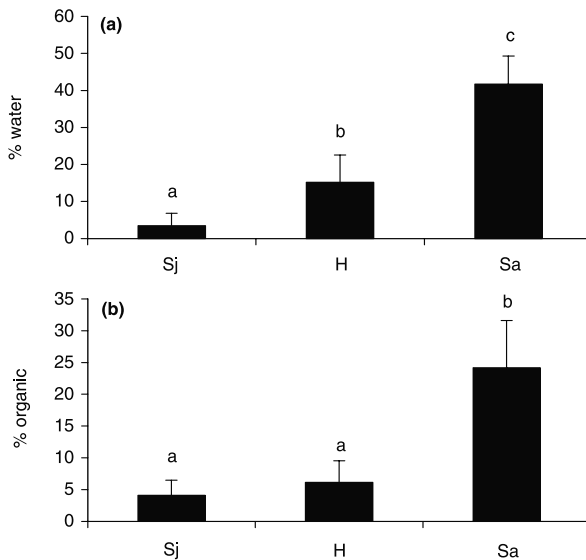


Figure 2 Water (a) and organic (b) content, expressed as mass percentage, of soil collected around the base of *S. aquaticus* (Sa), *S. jacobaea* (Sj), and natural hybrids (H) plants in the Zwanenwater reserve. Letters indicate significant differences between groups (Tukey test, $P < 0.05$; $N = 10$ soil samples per group).

Experiments

Overall, all plant groups attained maximal dry mass when grown in organic soil with sufficient but not excess water (medium organic/nutrient treatment) (Figs. 3, 5), except F_1 hybrids in Experiment 3, which performed best in wet, nutrient rich soil (Fig. 5). Except under extreme drought (Experiment 2; Fig. 4), *S. aquaticus* outperformed or equaled *S. jacobaea*, and we never observed a significant difference between natural hybrids and *S. jacobaea* under any treatment.

Experiment 1

ANOVA demonstrates that plant dry weight was affected by an interaction between treatment, soil type, and plant group (Table 1). Except in sand, *S. aquaticus* performed best in all treatments. In sand, *S. aquaticus* increased in dry weight with increasing soil moisture, while in potting soil, dry weight was highest in the medium water treatment. Conversely, *S. jacobaea* and hybrids were always negatively affected by flooding in comparison with the intermediate water treatments (Fig. 3), though in sand the negative effect is not significant. Although a significant three-way interaction occurs, natural hybrids never outperform either parental species in any of the treatments.

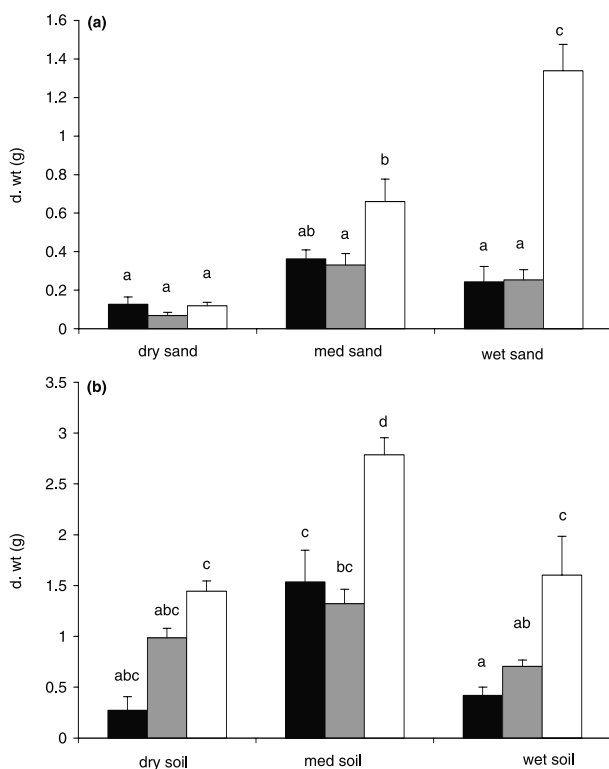


Figure 3 Dry weight of *S. aquaticus* (white), *S. jacobaea* (black), and natural hybrids (grey) after 8 weeks of growth in 10 cm pots, subject to various treatments (soil and water conditions). Letters above bars indicate significant differences between plant groups within treatments (Tukey test, $P < 0.05$). Dry, medium (med) and wet refer to moisture regime, while sand and organic refer to soil type. Vertical bars indicate standard error.

Table 1 ANOVA results Experiment 1: The effects of soil medium (S), water treatment (W), and plant group (P) on total dry weight.

Dependent variable	Factor	df	MS	F	P
Total dry weight	S	1	17.876	42.3613	0.022
	W	2	2.067	3.130	0.151
	P	2	4.174	5.047	0.108
	S*W	2	2.409	8.891	0.033
	S*P	2	0.429	1.562	0.315
	W*P	4	0.674	2.448	0.204
	S*W*P	4	0.275	3.238	0.017
	Error	70	8.497*10 ⁻²		

Experiment 2

Plant group and column length had an interactive effect on both total dry weight and root length (Table 2). Again, we noted no significant differences in dry weight or root characteristics between *S. jacobaea* and hybrids. *Senecio aquaticus* attained higher dry weight than *S. jacobaea* and hybrids when grown in conditions subject to frequent flooding, but performed worse in all other treatments (Fig. 4a). *Senecio jacobaea* and hybrids decreased in dry weight as column length increased, performing best when water availability was high (20 and 45 columns), and significantly worse when water availability was low (75 and 100 cm columns). Plant group × environmental interactions never led to circumstances in which natural hybrids performed better than parents.

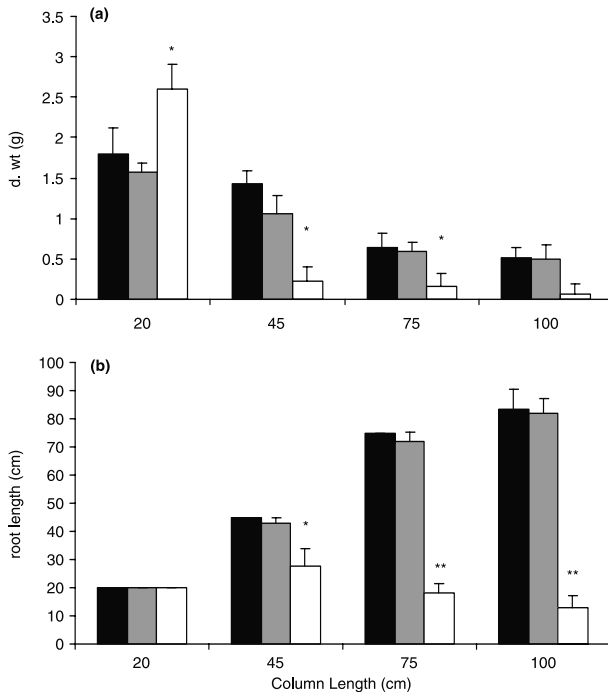


Figure 4 Root length (a), and total dry weight (b) of *S. aquaticus* (white), *S. jacobaea* (black) and hybrids (grey) after 8 weeks of growth in columns ranging in length from 20 to 100 cm in length. Vertical bars represent standard error [note that groups lacking standard error (B) grew roots that always reached the maximum pot depth]. Means marked (*) indicate significant differences between *S. aquaticus* and other plant groups (Tukey test, * $P < 0.05$, ** $P < 0.001$).

Table 2 ANOVA results Experiment 2: The effects of column length (C) and plant group (P) on total dry weight and root length.

Dependent variable	Factor	df	MS	F	P
Total dry weight	C	3	7.774	7.423	0.019
	P	2	0.461	0.431	0.669
	C*P	6	1.071	8.418	0.000
	Error	46	0.127		
Root length	C	3	4219.786	3.184	0.105
	P	2	6584.079	4.816	0.057
	C*P	6	1371.844	21.396	0.000
	Error	46	64.117		

Table 3 ANOVA results testing for maternal effects in F_1 hybrids: Interactions between nutrient (N) and water (W) treatments and maternal species (M) on total dry weight.

Dependent variable	Factor	df	MS	F	P
Total dry weight	N	1	53.915	2341.319	0.013
	W	2	2.752	133.435	0.007
	M	1	0.454	10.580	0.062
	N*W	2	2.303	3408.164	0.000
	N*M	1	2.303×10^{-2}	20.575	0.005
	W*M	2	2.062×10^{-2}	30.521	0.032
	N*W*M	2	6.757×10^{-4}	0.002	0.998
	Error	45	0.385		

Table 4 ANOVA results Experiment 3: Interactions between nutrient (N) and water (W) treatments and plant group (P) on total dry weight and root length.

Dependent variable	Factor	df	MS	F	P
Total dry weight	N	1	12.253	58.113	0.002
	W	2	0.427	6.052	0.025
	P	4	0.449	1.636	0.275
	N*W	2	0.559	74.636	0.000
	N*P	4	0.211	28.352	0.000
	W*P	8	7.058×10^{-2}	9.532	0.002
	N*W*P	8	7.405×10^{-3}	0.183	0.993
	Error	116	4.044×10^{-2}		
Root length	N	1	10263.822	27.257	0.006
	W	2	19845.626	158.195	0.000
	P	4	417.955	1.325	0.452
	N*W	2	2485.050	13.301	0.003
	N*P	4	376.833	2.017	0.185
	W*P	8	125.182	0.670	0.708
	N*W*P	8	186.737	0.839	0.570
	Error	116	222.510		

Root length was affected by a significant interaction between plant species (random factor) and column length (fixed factor) (Table 2). Root length of *S. aquaticus* remained constant across column lengths, while root length of *S. jacobaea* and hybrids increased with increasing column length. As a result, root length of *S.*

aquaticus was lower than that of both hybrids and *S. jacobaea* at column lengths of 45, 75, and 100 cm.

Experiment 3

Four plants died during the experiment, and we excluded them from our analysis.

Maternal effects interacted significantly with nutrient and water treatments to determine the fitness of F_1 hybrids (Table 3), such that offspring from *S. jacobaea* mothers perform generally better than those from *S. aquaticus* mothers (Fig. 5).

We found significant interactions between both plant group and nutrient treatment, and plant group and water treatment (Table 4). Natural hybrids performed equally well to *S. jacobaea* in all conditions (Fig. 5a), supporting results presented from Experiments 1 and 2. Artificial hybrids from *S. jacobaea* mothers exhibited hybrid vigor, always performing better than either parent (though differences were not always significant). F_1 offspring from *S. aquaticus* mothers performed better than parental species only in nutrient rich treatments.

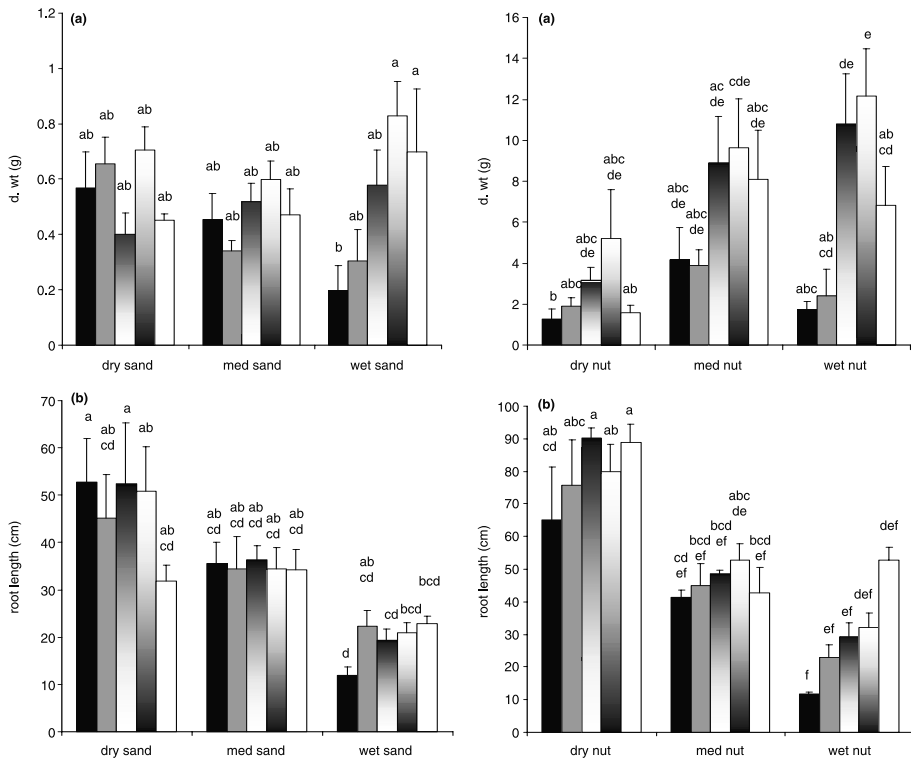


Figure 5 Dry weight (a) and root length (b) of *S. aquaticus* (white), *S. jacobaea* (black), natural hybrids (grey), F_1 hybrids (*S. aquaticus* mother; white at base) and F_1 hybrids (*S. jacobaea* mother; black at base) planted in columns (1 m) after 8 weeks of growth in sand and nutrient treatments, and according to varying water conditions. Letters above bars indicate significant differences between plant groups (Tukey test, $P < 0.05$). Dry, medium (med) and wet refer to moisture regime. Vertical bars indicate standard error.

While species was not a significant factor in the overall ANOVA (Table 4), there was some evidence for species-by-environment interactions in root length (Fig. 5b). In sand, *S. jacobaea* and both hybrid classes exhibited more plasticity in root length than did *S. aquaticus* (Fig. 5b); as in Experiment 2, root length of *S. aquaticus* did not differ across differing treatments, while root length was significantly different between dry and wet treatment for *S. jacobaea* and hybrids (Fig. 5b). However, in the nutrient treatment, *S. aquaticus* was able to extend its roots equally well to *S. jacobaea*, suggesting that given sufficient nutrients, and a weak drying gradient (as compared to Experiment 2), *S. aquaticus* can indeed extend roots to lengths reaching at least one meter. Both natural and artificial hybrids have significantly longer roots in dry conditions than in wet, in both sand and nutrient treatments, and thus exhibit plastic root elongation responses to drying which are similar to *S. jacobaea*.

DISCUSSION

While laboratory and field studies confirm that natural hybrids often vary in fitness in relation to parental species across environmental gradients (see Arnold, 1997; Rieseberg & Carney, 1998; Campbell & Waser, 2001), and that natural hybrids can in fact be relatively more fit than parental species in certain environments (Arnold & Hodges, 1995), we did not find that this is the case here. Contrary to the expectations of environmentally dependent models of hybrid zone stability, we did not find that hybrids, either artificial or natural, vary in fitness with relation to parental species, or that natural hybrids are distributed in zones where fitness is greatest.

In contrast to environmentally dependent models, Barton & Hewitt (1985) propose that maintenance of hybrid swarms results from an equilibrium between continuous dispersal of hybrids into hybrid zones, and subsequent negative selection against such hybrids. While passive processes such as dispersal and neutral selection could certainly play a role in the persistence of natural *S. jacobaea* × *S. aquaticus* hybrids in the Zwanenwater, we did not find that natural hybrids are less fit than parental species, as expected in a tension zone. *Senecio aquaticus* is relatively rare in the immediate area of the hybrid zone, while *S. jacobaea* is fairly abundant. Repeated hybridization events followed by back-crossing to relatively abundant *S. jacobaea* could be an explanation for the observed distribution, and genetic composition (high levels of back-crossing; Kirk *et al.*, 2004) in the hybrid population.

Our data, however, are not completely compatible with environmentally independent models such as that of Barton & Hewitt (1985), which assume that most hybrids have a fixed fitness level relative to parents. We have found that different hybrid generations differ in fitness, with early generations performing much better than natural hybrids, which have presumably undergone many generations of inter-crossing and back-crossing (Kirk *et al.*, 2004). F₁ hybrids possess a combination of adaptations, including flooding and drought resistance, which are normally found exclusively in either one or the other parent. Such adaptations, combined with superior fitness of F₁ hybrids (from *S. jacobaea* mothers) in all environments, may allow early generation hybrids to expand their range to the habitats of both parents.

Unfortunately for the hybrids, which cannot reproduce clonally, back-crossing and inter-crossing must occur, reducing the fitness of subsequent generations, such that heterotic F_1 s cannot dominate the population. Our findings do not completely agree with any current models that explain hybrid zone stability, and we propose that heterosis followed by a decrease in hybrid fitness could contribute to the stability of hybrid swarms.

That maternal effects have a significant role in the fitness of hybrids may also have notable consequences for hybrid swarm dynamics. Not much is known about the evolutionary genetics of, and mechanisms behind maternal effects, though it is thought that such effects can be evolutionarily adaptive and subject to natural selection (ie. Theide, 1998; Fox *et al.*, 1999). In the case described here, experimental plants were tissue cultured and selected for size equality at the beginning of the experiment, which rules out possible maternal effects on seed size, germination, and early growth, factors which are often influenced by maternal environment (Platenkamp and Shaw, 1993). Thus, maternal benefits to offspring might have been conferred by non-nuclear inheritance of genetic material from cytoplasm and organelles of maternal plants (Roach & Wulff, 1987). Alternatively, fitness of offspring might be epigenetically regulated (eg. Jaenisch & Bird, 2003) by the mother plant, such that mother plants control nuclear gene expression in the offspring through cues passed via the cytoplasm.

Maternal effects on the fitness of hybrids might also partially explain back-crossing of natural hybrids in the Zwanenwater reserve to *S. jacobaea* (Kirk *et al.*, 2004). If *S. jacobaea* mothers produce offspring which are more fit than those produced by *S. aquaticus* mothers, back-crosses to *S. jacobaea* might have a competitive advantage, and/or greater reproductive success than other hybrid classes. At least one other study (Campbell & Waser, 2001) showed that fitness of F_1 hybrids can be mediated by parental species. To test whether natural *Senecio* hybrids indeed originate mostly from *S. jacobaea* mothers, it would be interesting to examine the origin of cytoplasmic genes in natural hybrids.

Distribution of parental species *S. jacobaea* and *S. aquaticus* is well accounted for by adaptation of root responses to local water and nutrient regimes in the respective environments of these species. Differences between root elongation ability of *S. jacobaea* and *S. aquaticus* in response to low water tables are striking. When soil moisture is available at greater depths and nutrients are lacking, *S. jacobaea* uses an avoidance strategy in order to reach such water sources that are unavailable to *S. aquaticus*, and therefore maintains a significantly higher dry weight than *S. aquaticus* when water is scarce. In all other growth situations we have shown that *S. aquaticus* is equally or more fit than *S. jacobaea*. As expected, *S. aquaticus* performs best (both relative to other species and to itself) in conditions similar those in which this species grows in the field (medium to wet soil with high organic content).

Unlike *S. aquaticus*, *S. jacobaea* and natural hybrids suffer reductions in dry weight when subjected to soil saturation. Furthermore, leaf yellowing of *S. jacobaea* and natural hybrids becomes apparent after several weeks of growth in wet soils (data not shown). It is therefore clear that *S. jacobaea* and natural hybrids are sus-

ceptible to anoxia related stress, which may explain why these groups are excluded from flood susceptible environments.

To our knowledge, this is the first instance in which superiority of F_1 hybrids over natural hybrids has been considered as a potential contributing factor for hybrid swarm stability. It is often assumed that hybrid swarms that exhibit low fitness or hybrid breakdown reinforce reproductive isolation between parental species (Barton & Hewitt, 1985), and thus have little evolutionary potential (Rieseberg & Carney, 1998). If the expansion and/or persistence of hybrid populations can be facilitated by the success of early generation hybrids, negative selection against less fit later generation hybrids may be delayed. The prevalence of back-crossing in such populations, as is the case here (Kirk *et al.*, 2004), might provide the opportunity for introgression to occur even if the average fitness of natural hybrids is low. A previous study (Kirk *et al.*, 2004) has shown evidence for introgression of chemical expression specific to *S. aquaticus* into *S. jacobaea* in the Zwanenwater area, which may suggest that hybrids don't have to be superior to make an evolutionary contribution.

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4

Reproductive fitness of hybrids between *Senecio jacobaea* and *Senecio aquaticus* (Asteraceae)

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Natural hybridization is increasingly recognized as an important process for the ecology and evolution of natural plant populations and species. There is a great need to initiate more studies based on natural populations in order to elucidate the possible role of hybrids in nature. The reproductive success of early generation hybrids can make or break hybrid lineages and may determine the genetic structure of hybrid swarms or the potential for gene flow through future generations, but studies of hybrid reproductive success are lacking. Here we measured components of male and female reproductive success in *Senecio jacobaea* and *S. aquaticus* (Asteraceae) species and F_1 hybrids between these species under laboratory conditions, and we measured reproductive output from crosses producing F_1 , F_2 , and backcross (BC) generation hybrids. F_1 hybrids were readily produced, and on average, the success of crosses producing subsequent generations (F_2 , BC) decreased (though remained substantial), but the success of crosses was highly dependent on the genotypes involved. Also, F_1 hybrids were bigger, produced more flowers, and therefore produced more pollen than parental plants. Finally, crosses between parents were asymmetrical, such that *S. aquaticus* produced more and larger F_1 seeds than did *S. jacobaea*.

Key words: Asteraceae, fertility, hybridization (hybridisation), male fitness, Netherlands, reproductive success, *Senecio aquaticus*, *Senecio jacobaea*

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INTRODUCTION

Already in 1859, Darwin recognized the potentially important role of hybridization in his *Origin of Species*, and since Darwin's time, many authors have touched upon the possible ecological and evolutionary consequences of gene flow between species (Stebbins, 1950; Lewis, 1966; Grant, 1981). Especially in the last decade, an increase in research into natural hybridization has extended our knowledge about the evolutionary significance of this process (Arnold, 1997; Rieseberg & Carney, 1998; Rieseberg *et al.*, 1999).

While many researchers have in the past considered that hybridization can reduce biological diversity by homogenizing divergent genomes (Mayr, 1942; Wilson, 1965), we now know that hybridization can increase biological diversity within populations, and also potentially within and between species. For instance, at the population level, Whitham *et al.* (1999) showed that hybrid zones in plants support a higher diversity of phytophagous insects than pure parental populations. With regards to diversity within and between species, hybridization may generate novel traits (Rieseberg *et al.*, 1999; Orians, 2000) or novel combinations of traits, which can be introgressed to parental species (Anderson, 1949; Arnold, 1997; Abbot *et al.*, 2003), or can allow hybrid lineages to adapt to new environments (e.g., sun-flowers; Rieseberg *et al.*, 2003). Not only can hybridization facilitate diversification at the trait level, but it can also result in the evolution of new species (Rieseberg *et al.*, 2003), perhaps to the extent of contributing to adaptive radiations (Seehausen, 2004).

Although there is mounting evidence for the contribution of hybridization to the evolutionary history of plants in particular (Ellstrand *et al.*, 1996), the ecological fates of individual natural plant hybrid populations are varied, with some highly ephemeral populations (Barton & Hewitt, 1985) and some stable, or growing hybrid zones (see Arnold, 1997). The success of natural hybrids is dependent on a wide variety of factors, including the compatibility of parental genomes (e.g., Dobzhansky, 1933; Muller, 1942), vegetative performance according to abiotic conditions (e.g., Campbell & Waser, 2001; Kirk *et al.*, 2005b), and interactions with pollinators and herbivores (Campbell *et al.*, 1997; Fritz *et al.*, 1999).

Many recent papers examining the success of natural hybrids have focused on vegetative survival in natural environments (Burke *et al.*, 1998; Campbell & Waser, 2001; Johnston *et al.*, 2001; Kirk *et al.*, 2005b). Especially early hybrids are often found to be superior to parents in terms of growth, often surviving, growing, and competing better than parental species in all or a limited number of habitats, and such hybrids are frequently expected to have a high ecological or evolutionary impact. Yet, the evolutionary impact of such hybrids can be restricted if they are sterile or if gene flow from hybrids is restricted by genomic instability or genetic incompatibilities. Even hybrids that are superior to parents in vegetative phases may fail to reproduce, which can effectively enforce isolation of parental populations. Alternatively, hybrids could breed most successfully with one or other of the parental species, or with each other, which may result in unidirectional introgression or even in sympatric speciation. Measurements of reproductive fitness of hybrids

have been underrepresented in hybrid literature, likely because studies involving reproduction involve a longer time scale, especially for biennial and perennial plants.

Here, we measure the success of crosses that produce early generation hybrids (F_1 , F_2 , and BC) between *Senecio jacobaea* L. and *S. aquaticus* Hill (Senecioneae; Asteraceae). *Senecio* is a genus containing more than 1500 species with a world-wide distribution. Many *Senecio* species are known to hybridize in nature (e.g., Lowe & Abbott, 2000; Abbott & Lowe, 2004; Kirk *et al.*, 2004), and hybridization may make a large contribution to the evolutionary history of the genus. Phylogenetic study of *Senecio* sect. *Jacobaea*, which contains *S. jacobaea*, *S. aquaticus*, and at least 23 of their closest relatives, has provided some evidence that hybridization may contribute to the evolutionary history of these species (Pelser *et al.*, 2003).

Senecio jacobaea and *S. aquaticus* have been widely reported to produce hybrid swarms throughout Europe (Chater & Walters, 1976), with a confirmed population in the Zwanenwater nature reserve in The Netherlands (Kirk *et al.*, 2004). The Zwanenwater hybrid population has been present for at least 25 yr (R van der Meijden, National Herbarium of the Netherlands, personal communication), and there is some evidence that hybridization there facilitates introgression of chemical traits from *S. aquaticus* into *S. jacobaea* individuals that are located at some distance from the hybrid zone (Kirk *et al.*, 2004). Under laboratory conditions, hybridization between these species may lead to the generation of altered pyrrolizidine alkaloids (H Kirk, unpublished data) and unique expression of primary and secondary metabolites in hybrid individuals (Kirk *et al.*, 2005a). Furthermore, studies of the Zwanenwater population have shown that F_1 hybrids are superior in vegetative fitness to parental species across a variety of abiotic growth conditions (Kirk *et al.*, 2005b). Combined, these results indicate that natural hybridization between *S. jacobaea* and *S. aquaticus* plays a potentially important ecological and/or evolutionary role.

The aims addressed by this study were (1) to test whether early generation hybrids can be easily formed in nature, (2) to make initial estimates of both male and female reproductive success of F_1 hybrids and parental species, and (3) to evaluate whether there is asymmetry in the success of crosses.

MATERIALS AND METHODS

Crossings

Six *S. jacobaea* and six *S. aquaticus* plants were collected from the Zwanenwater Nature Reserve (The Netherlands) during May 2003 (see Kirk *et al.*, 2005b, for a detailed description of the layout of the hybrid population and of collection zones). When plants were beginning to develop flowering stems, they were transferred to a greenhouse, matched in pairs between species, and crossed pairwise twice per week by rubbing flower heads together. Seeds, which represented F_1 offspring, were harvested from both parents.

F₁ seeds and seeds from unrelated parental individuals were placed in tissue culture to produce clonal plantlets. Of nine F₁ genotypes included in the experiment, there were three sets of two full-sibs. Thus in total, six F₁ genotypes were completely unrelated to one another.

In total, eight *S. jacobaea* genotypes, six *S. aquaticus* genotypes, and nine F₁ hybrid genotypes were used in the experiment. When enough clones were available, five clones from each genotype were transferred to 3-L pots filled with a 1 : 1 mixture of potting soil and dune sand. Three grams slow-release fertilizer (N:P:K = 15:11:13 + 2 MgO; Osmocote, Marysville, OH, USA) was added to the surface of pots. In total, 94 plants were used in the experiment (see Table 1 for details of the crossing scheme). Plants were grown in a climate cell for 4 months (light 16 h, temperature 20/15 °C, relative humidity 70%), after which they were transferred to a cooled climate cell (light 8 h, temperature 4 °C, relative humidity 70%) for 13 wk of vernalization. After vernalization, plants were transferred to two climate cells (light 16 h, temperature 20/15 °C, relative humidity 70%). Throughout the experiment, plants were watered several times weekly, so that the soil was never saturated or extremely dry. Flowering began after 4 weeks, with *S. aquaticus* generally beginning to flower several days before *S. jacobaea* and hybrid individuals. In another experiment (unpublished data), we noticed that *S. aquaticus* will rarely flower without vernalization, while *S. jacobaea* absolutely requires a cold period. Only two plants, both *S. jacobaea* individuals, failed to flower. After initiation of flowering, plants were given 30 mL Steiner solution (macronutrients: N, 167 mg/L; P, 32 mg/L; K, 282 mg/L; S, 11 mg/L; Ca, 180 mg/L; Mg, 49 mg/L) weekly for 7 weeks.

Plants were paired so that for each genotype, one clone was crossed with each of the other plant groups (i.e., each *S. aquaticus* genotype was crossed with at least one *S. jacobaea* genotype and one hybrid genotype). We also tried to self F₁ genotypes (as a negative control), by pairing two clones with each other, to ensure that there was no breakdown of self-incompatibility mechanisms or contamination by pollen from nearby plants. We made five crosses between *S. jacobaea* genotypes, as a positive control. A summary of the types of crossings made is presented in Table 1. Pairs of individuals were placed adjacently in the climate cell and were crossed by rubbing flower heads together, at least every 5 days. Each plant was reciprocally crossed with only one partner.

Flower counting

In some cases, flowers could not be crossed because of pollen limitation from the partner plant or because one partner finished flowering before the other. In these cases, unpollinated flowers were removed from the plant when fully developed, and recorded separately. Number of (pollinated) flowers were counted during seed harvesting, such that the contents of each flower head was harvested, and number of flower heads were recorded.

Pollen estimation

To estimate total investment in pollen by each plant, we sampled three flower heads for pollen production. Flowers were selected randomly during mid-flowering, such

Table 1 Crossing classes (cc) resulting from pairwise crosses involving *S. jacobaea* (Sj), *S. aquaticus* (Sa), and F_1 hybrids (H). Codes listed at top and left represent genotypes used. Filled squares indicate that a cross between intersecting genotypes was made, and different shades indicate different crossing classes. For example, black squares indicate F_2 -producing crosses (H-H). Full-sibs included in the crossing scheme include H3-H4, H7-H8, and H13-H14. All other genotypes are unrelated to one another.

	H3	H4	H7	H8	H13	H14	H16	H22	H24	Sa3	Sa4	Sa6	Sa7	Sa9	Sa10	Sj2	Sj3	Sj4	Sj5	Sj6	Sj8	Sj9	Sj10
H3																							
H4																							
H7																							
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that a substantial number of flowers were open, but the majority of flowers had not begun to senesce. Stigmas emerge sequentially from the outer edge to the center of the flower, pushing pollen from the anthers to the flower surface, and flowers were sampled when stigmas at the center were freshly emerged. In such flowers, all pollen produced by the flowers was present on the flower surface.

The three flowers were dipped and shaken into one plastic 20-mL vial containing 95% ethanol. A number of flowers were examined with a microscope to ensure that all pollen was transferred to the vial. Vials were refrigerated at 4 °C until pollen analysis.

To measure the mass of pollen produced by each plant, we siphoned 15 mL of ethanol from the surface of vials and allowed the rest of the ethanol to evaporate in a fume hood at room temperature of 2 days. We then added 200 μ L of 70% ethanol in two samples to wash pollen out of the 20-mL vials and transferred the

pollen–ethanol mixture to 0.2-mL Eppendorf tubes. The pollen mixture was centrifuged for 15 min at $9.7 \times g$, so that pollen formed pellets in the tips of tubes. The ethanol was siphoned from the surface of each pellet using a micropipette, and the remaining drops of ethanol were removed with the tip of a tissue. Pollen samples were dried in a Speed-vac (Savant) at medium temperature for 60 min. After drying, the solid pellet of pollen was removed from the Eppendorf tubes using the tip of a needle, and weighed on a microbalance (Sartorius, Goettingen, Germany).

Seed counting and weighing

Total material collected from seed heads of each plant included seed, pappus, and dried petals. Seed-head material from each plant was homogeneously mixed, and three subsamples, approximately equal in volume, were taken from each plant. Subsamples, ranging from 0.1 to 0.4 g, were weighed, as was the remainder (bulk) of the seed-head material.

Seed from subsamples were separated from chaff, and the number of seeds was separately counted from each subsample. We made an estimate of the total number of seeds produced by multiplying the number of seeds per gram in subsamples by the total bulk mass of seed-head material. We then divided by the number of pollinated flowers to get an estimate of number of seeds per flower. To estimate the total potential seed production per plant, we multiplied the number of seeds per flower by the total number of flowers (including both pollinated and unpollinated flowers). In most cases, the number of unpollinated flowers ranged from 10 to 30% of the total flowers, but in rare cases, approached 50% of total flowers. We realize that there are sometimes tradeoffs between seed and flower production (and we found that this is the case here; see Results, Table 2). In these experiments, hybrids were most affected by this bias because hybrids (which produced the largest number of flowers) were more likely to have a larger proportion of unpollinated flowers. However, hybrids produced few seeds anyway, so that the trade-off was small and had a relatively tiny effect on estimates of whole plant flower and seed production.

From each plant, three replicates of 10 seeds were weighed on a microbalance. The three measurements were averaged to yield one estimate of seed mass per plant (the variation between replicates was extremely low).

Biomass measurement

After plants finished flowering and began to die back, we harvested above- and belowground plant material and carefully washed the plant roots. Plant material was dried in an oven for 2 days at 80 °C and was weighed to the nearest 10 mg.

Statistical analyses

All statistical analyses were carried out in SPSS, version 11.0 (SPSS, Chicago, IL, USA). We analyzed total seed production with regards to crossing class. Crossing classes included crosses producing F_1 s, F_2 s, backcrosses to *S. jacobaea* and *S. aquaticus*, and negative and positive controls. We analyzed plant mass, seed mass, pollen production, and flower production with regards to plant group (*S. jacobaea*, *S. aquaticus*, and F_1 hybrids), and genotypes within plant group.

Table 2 Factors (plant biomass, seed mass, and seeds per flower) that contribute significantly to regression models explaining variance in flower number within *Senecio jacobaea*, *S. aquaticus*, and F₁ hybrids.

Plant group	Independent factor	Standardized coefficient β	t^a	P^b	Model		
					R^2	F	P^c
<i>S. aquaticus</i>	Seeds/flower	-0.704	-4.209	0.001	0.468	17.718	0.001
<i>S. jacobaea</i>	Seed weight	-0.470	-2.716	0.012	0.191	7.374	0.012
F ₁ Hybrid	Seeds/flower	-0.529	-4.399	<0.000			
	Seed weight	-0.533	-4.436	<0.000	0.538	19.613	<0.000

^aStudent's t is used to test whether the standardized coefficient β differs from 0.

^bSignificance values for individual components of regression models.

^cSignificance values related to the entire regression model.

To test which kinds of crosses produce the most seeds, we summed the total (potential) number of seeds produced by both maternal plants in each cross. We defined each cross as selfed (S), F₂ producing (H × H), F₁ producing (Sj × Sa), backcross to *S. aquaticus* (Sa × H) backcross to *S. jacobaea* (Sj × H) or cross between two *S. jacobaea* genotypes (Sj × Sj). We then applied a one-way ANOVA using cross type (fixed factor) to the log-transformed value of total seeds, and used a Tukey post hoc test (Zar, 1999) to identify differences between groups.

We used multiple regression analysis (separately for each plant group) to identify the factors that influence number of flowers produced. All clones from all genotypes were included for each plant group. We tested the independent variables plant biomass, mean seed mass, and number of seeds per flower (or alternatively, total number of seeds produced). Backward elimination and forward selection methods (Zar, 1999) yielded consistent models in all cases.

We used mixed model nested ANOVAs to test for plant group and genotype differences in plant biomass, pollen production, and number of seeds per flower, and seed mass. For analysis of seed mass, we removed selfed plants from the data set, and we tested for both maternal and paternal effects of plant group (*S. jacobaea*, *S. aquaticus*, and F₁ hybrids). For analysis of seed production (number of seeds per flower), the analysis does not incorporate all the complexity in the experimental setup, because genotypes were used in multiple crosses and some F₁ genotypes were related to one another. Because the number of genotypes used here was limited, the results should be interpreted with some caution. However, we feel that that this caveat does not affect the conclusions we draw from the data presented here.

To compare the seed production of reciprocal crosses between groups, we used paired t tests on the number of seeds per flower, and on the potential seed production per plant.

RESULTS

Overall, there were large differences in potential seed production between different types of crosses ($df = 5$, $F = 9.715$, $P < 0.001$). We found that interspecific crosses (Sj

× Sa) were most successful, producing significantly more seeds even than Sj × Sj crosses (Fig. 1). The trend showed that backcrosses (Sj × H and Sa × H) were about 1/3 as successful as interspecific crosses (Sj × Sa) and that F₂-producing crosses (H × H) were about four times less successful than backcrosses (Fig. 1). The variation within cross classes is very high though, because the success of individual crosses was highly dependent on interactions between genotypes. For example, one cross between two individual *S. jacobaea* genotypes produced no seeds, while these genotypes were completely fertile in other crosses. We found similar results in all other categories, such that some backcrosses produced more seed than the most productive interspecific crosses, which suggests that at least some backcrosses, and even some F₂ producing crosses, will be highly successful, even if the mean seed production in these crossing classes is low (i.e., Fig. 1).

Although biomass significantly differs between plant groups and according to genotype (Fig. 2A, Table 3), we found that plant biomass never influenced the number of flowers produced within plant groups. This result likely arose from a low amount of variation in biomass within plant groups, which was a result of standardized growth conditions. Plant biomass was thus excluded as a covariate in further ANOVA analyses. We also found that for all plant groups, total seeds per flower explained more variance in flower number and was more significant than total seeds produced, and we therefore discarded total seeds produced from the regression analyses. For *S. jacobaea* and hybrids, total flower number was dependent on seed mass, while total seeds per flower influenced total number of flowers produced by *S. aquaticus* and hybrids (Table 2). Overall, the amount of variance in flower number explained by the best model for *S. jacobaea* (adjusted $R^2 = 0.191$) was much lower than those for *S. aquaticus* (adjusted $R^2 = 0.468$) and hybrids (adjusted $R^2 = 0.538$), suggesting that flower number is more genetically controlled in *S. jacobaea*,

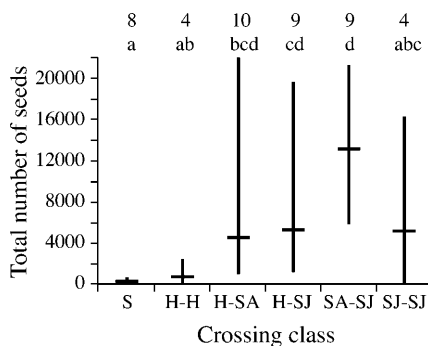


Figure 1 Total potential seed production from both maternal plants in reciprocal crosses producing selfed F₂ hybrids (S), F₁ hybrids (SA-SJ), outcrossed F₂ hybrids (H-H), backcrosses to *S. aquaticus* (H-SA), backcrosses to *S. jacobaea* (H-SJ), and *S. jacobaea* offspring (SJ-SJ). Numbers above columns indicate N, and different letters indicate significant differences between groups (Tukey test, $P < 0.05$). Vertical lines illustrate range, and horizontal bars represent means. Crosses involving genotype Sj3 were excluded because pollen production by this genotype was limited.

and less susceptible to plastic change (also see ANOVA results (Table 3), which show that flower number depends on genotype).

On average, we found that hybrids produced about 25% more flowers than parental species, followed by *S. aquaticus*, and then *S. jacobaea* (Fig. 2B, Table 3). The greater number of flowers produced by hybrids might be explained by the greater size of the plants (although we found that plant biomass never influenced flower number within plant groups, variation in biomass was greater between groups). Hybrids also likely produced more flowers because they produced fewer seeds per flower than parental species.

There was no significant effect of plant group on pollen production, but pollen production differed significantly according to genotype (Table 3). One *S. jacobaea* genotype (Sj3), for example, produced almost no pollen (consistently over five clones), and crosses with this genotype had to be removed from analyses involving seed production because pollen was extremely limiting in these crosses. The range in pollen production per flower was greater among *S. jacobaea* genotypes (0.18–2.48 mg/flower) than among *S. aquaticus* (0.56–1.88 mg/flower) or hybrids

Table 3 Results of mixed model nested ANOVA tests to identify effects of plant groups (*S. jacobaea*, *S. aquaticus*, and F_1 hybrids) and genotypes within plant groups [genotype (group)] on plant biomass, flower number, pollen production.

Dependent variable	Factor	df	MS	F	P
Biomass	Plant group	2	1168	3.734	0.040
	Genotype(group)	20	332.6	2.183	0.008
	Error	73	152.4		
Flower number	Plant group	2	6.084×10^5	9.082	0.001
	Genotype(group)	20	7.109×10^4	2.126	0.011
	Error	73	3.344×10^4		
Pollen	Plant group	2	0.177	0.104	0.902
	Genotype(group)	20	1.190	1.995	0.019
	Error	65	0.596		

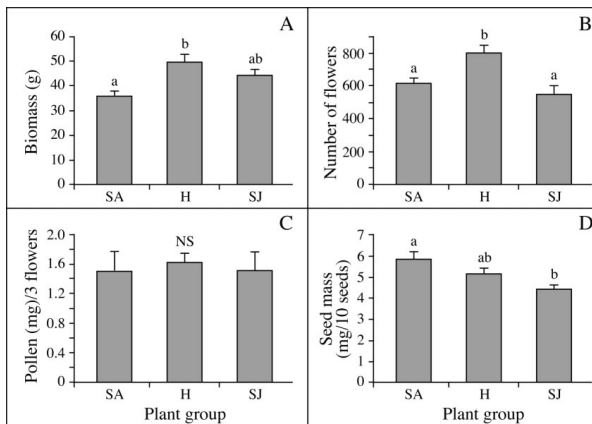


Figure 2 Total biomass, total flower production, pollen production, and seed mass of *S. aquaticus*, *S. jacobaea*, and F_1 hybrids. Different letters indicate significant differences between groups (Tukey test, $P < 0.05$), NS indicates no significant differences, and error bars represent standard error.

(0.89–1.96 mg/flower). Hybrids produce on average more pollen over the whole plant than parental species, because the number of flowers was greater in hybrids than parents (Fig. 2B), while pollen production was equal (Fig. 2C).

To test whether seed production (seeds/flower head) was asymmetrical in reciprocal crosses (Fig. 3), we used a paired *t* test to test for pairwise differences. We found no reciprocal differences in *S. aquaticus* × hybrid crosses ($t = 1.026$, $df = 9$, $P = 0.332$), or *S. jacobaea* × hybrid crosses ($t = 1.515$, $df = 9$, $P = 0.164$), but *S. aquaticus* produced significantly more seeds than *S. jacobaea* in reciprocal crosses ($t = 2.762$, $df = 8$, $P = 0.024$). Production of F_1 seeds by *S. aquaticus* vs. *S. jacobaea* is also likely increased by the trend that *S. aquaticus* produces more flowers than *S. jacobaea* (Fig. 2A).

Regarding seed mass, we found that maternal species and genotype within maternal species were significant determining factors, with no paternal species or genotype effects, and no interaction between maternal and paternal species (Table 4). *Senecio aquaticus* mothers produced the largest seeds, followed by hybrids, and lastly *S. jacobaea* (Fig. 2D). We also found an extremely low level of seed production in some selfed-hybrid crosses. The mass of such putatively selfed seeds was lower than that of outcrossed seeds, which supports the conclusion that such seeds result from selfing, and not from pollen contamination by other genotypes.

Table 4 Results of mixed model nested ANOVA tests to identify effects of maternal group (mother), paternal group (father), and genotypes within maternal and paternal groups on seed mass.

Dependent variable	Factor	df	MS	F	P
Seed mass	Mother	2	8.069	10.420	0.001
	Genotype (mother)	20	0.854	3.257	0.004
	Father	2	0.330	1.461	0.248
	Genotype (father)	20	0.219	0.835	0.656
	Mother × father	3	0.134	0.511	0.679
	Error	23	0.262		

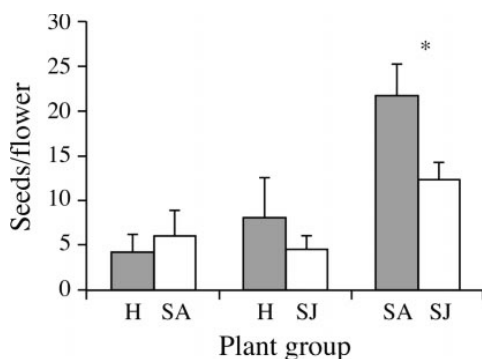


Figure 3 Seed production per flower in reciprocal crosses between F_1 hybrids (H) and *S. aquaticus* (A; $N = 10$), F_1 hybrids and *S. jacobaea* (J; $N = 10$), and *S. aquaticus* and *S. jacobaea* ($N = 9$). Error bars represent standard error. * Significant difference ($P < 0.05$, paired *t* test).

DISCUSSION

Clearly, initial crosses between *S. jacobaea* and *S. aquaticus* are highly successful, and result in the production of many seeds. Previous experiments have shown that F_1 seeds are viable, and seedlings grow heterotically (extremely vigorously) (Kirk *et al.*, 2005b). Furthermore, F_1 hybrids are fertile, and there is thus high potential for gene flow to continue to later generation hybrids after the initial crosses in nature. The lower mean success of crosses involving F_1 s does not necessarily mean that gene flow in general is restricted after the F_1 stage, since some crosses remain highly productive, and on average even F_2 producing crosses yield more than 500 seeds. Genotype dependence of cross success has been found in other studies as well; for instance, high variability in the fertility of early generation hybrids was dependent on parental genotypes of *Microseris douglasii* and *M. bigelovii* (Bachmann & Hombergen, 1997). Also, other authors have shown that lost fertility in early generation hybrids is quickly regained in subsequent generations (e.g., Wang *et al.*, 2001).

It is not surprising that on average, seed production in F_2 -producing crosses and backcrosses is lower than that of initial crosses, since backcrossing and intercrossing leads to segregation of genes, which can have many negative genetic consequences. Inability to produce embryos in hybrids (or embryo abortion) may result from, among other factors, chromosome rearrangements during meiosis in F_1 s (Grant, 1981), the breakup of co-adapted gene complexes (Dobzhansky, 1933; Muller, 1942), or incompatibility of nuclear genes with a new cytoplasmic environment (Cruzan & Arnold, 1999). Other authors have reported similar findings; Song *et al.* (2004) for example, reported that reproductive success of F_1 hybrids between *Oryza rufipogon* and *O. sativa* was lower than that of parents, while vegetative success was higher.

Male function seems to be quite robust in hybrid individuals here. While we did not explicitly test pollen viability, our results showed that it is unlikely that pollen is significantly less viable in F_1 hybrids than in parents, because seed production was equal in both hybrids and parents in reciprocal crosses. Reduced reproductive success in parent-hybrid crosses most likely resulted from genomic incompatibilities. The greater size of hybrids in general and the significantly higher number of flowers in hybrids might also lead to increased attractiveness to pollinators. Other studies have shown that frequency of pollinator visitation is often correlated with size of plants and number of flowers (Klinkhamer & de Jong, 1990). This hypothesis should be tested in the field, though, since there is significant morphological variation in flower structure between parents, and also between hybrids and parents (H Kirk, unpublished data), which may also have a strong impact on plant–pollinator interactions.

Based on the results of this experiment, we might predict that hybrids are more likely to arise from *S. aquaticus* mothers, since *S. aquaticus* produce more seeds in reciprocal crosses with *S. jacobaea*. Also, seeds produced by *S. aquaticus* mothers are larger than those from *S. jacobaea* mothers, and we might expect that offspring from *S. aquaticus* might have a fitness advantage in early life stages. Yet, advantages to hybrids derived from *S. aquaticus* mothers may be balanced out by later vegeta-

tive growth advantages for F_1 hybrids arising from *S. jacobaea* mothers (Kirk *et al.*, 2005b). However, Kirk *et al.* (2005b) controlled for seed effects by using small, equally sized clonal plants. It would be interesting to study whether selection in the natural hybrid zone favors one maternal species over the other, since reproductively, *S. aquaticus* mothers are most fit, while *S. jacobaea* mothers produce the most fit offspring with regards to vegetative growth.

These results lead to many fascinating questions about the ecology of the natural hybrid population from which the parental plants were derived. How do pollinators affect the success of interspecific hybrids in nature (e.g., Campbell *et al.*, 1997)? Can pollen competition influence the success of conspecific pollination, as found in other hybridizing species (e.g., *Brassica*, Pertl *et al.*, 2002; *Ipomopsis*, Campbell *et al.*, 2003)? Also, what is the evolutionary impact of hybridization within the genus *Senecio*? This last question may be of ultimate importance for explaining both the species and phenotypic diversity found within the genus.

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Chapter 4

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5

Comparing metabolomes: the chemical consequences of hybridization in plants

Heather Kirk, Young Hae Choi, Hye Kyong Kim, Robert Verpoorte, Ed van der Meijden

Hybridization may lead to unique phytochemical expression in plant individuals. Hybrids may express novel combinations or extreme concentrations of secondary metabolites or, in some cases, produce metabolites novel to both parental species.

Here we test whether there is evidence for extreme metabolite expression or novelty in F_1 hybrids between *Senecio aquaticus* and *Senecio jacobaea*. Hybridization is thought to occur frequently within *Senecio*, and hybridization might facilitate secondary metabolite diversification within this genus.

Parental species express different quantities of several classes of compounds known to be involved in antiherbivore defence, including pyrrolizidine alkaloids, chlorogenic acid, flavonoids and benzoquinoids. Hybrids demonstrate differential expression of some metabolites, producing lower concentrations of amino acids, and perhaps flavonoids, than either parental species. Despite evidence for quantitative hybrid novelty in this system, NMR profiling did not detect any novel compounds among the plant groups studied.

Metabolomic profiling is a useful technique for identifying qualitative changes in major metabolites according to plant species and/or genotype, but is less useful for identifying small differences between plant groups, or differences in compounds expressed in low concentrations.

Key words: chemical defence, hybridization, metabolomic profiling, pyrrolizidine alkaloids, secondary metabolites, *Senecio* (Asteraceae)

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INTRODUCTION

Secondary metabolites, defined as compounds that are biosynthetically restricted to a selection of plants (Pichersky & Gang, 2000) and are not used for growth and development, play a significant role in plant survival and fitness. Known to be particularly important for plant interactions with their biotic environment, plant secondary metabolism may mediate interactions with natural enemies including herbivores (Fraenkel, 1959), pathogens (Hol & van Veen, 2002) and competitors (Wardle *et al.*, 1998), and play a role in interactions with pollinators and seed dispersers (Adler, 2000). Furthermore, secondary metabolites are involved in a number of physiological functions including toxic nitrogen storage and transport (alkaloids and peptides), and UV-protectants (flavonoids) (summarized by Wink, 2003).

The surprising diversity of secondary metabolites in plants has been the subject of much debate and experimentation. Despite a number of theories advanced to explain metabolic diversification on an evolutionary time scale (Ehrlich & Raven, 1964; Cornell & Hawkins, 2003), secondary metabolite diversity has been poorly explained from both mechanistic (Pichersky & Gang, 2000) and functional (Wink, 2003) perspectives. However, it has been shown that plant hybridization may have a potentially large role in the evolution of novel secondary metabolites (Rieseberg & Ellstrand, 1993; Orrians, 2000), and the formation of new combinations of existing secondary metabolites within plant individuals (Orrians, 2000). Furthermore, previous reports have shown that hybridization can lead to superior resistance to herbivores and pathogens (see review by Fritz, 1999), indicating that innovative expression of secondary metabolites in hybrid individuals may be ecologically adaptive.

If hybridization often leads to the formation of unique metabolites or metabolic profiles, the implications are significant. Historical hybridization events are known to account for 30–70% of modern plant species (Ellstrand *et al.*, 1996 and references therein), indicating that the large-scale diversification of secondary metabolites through hybridization is a possibility. Furthermore, natural hybrid swarms may facilitate the introgression of fitness traits from one species to another (Rieseberg & Wendel, 1993; Rieseberg, 1995). Plant hybridization is therefore thought to have many consequences for evolutionary processes (Arnold, 1997), including the evolution of plant defence.

While hybrid inheritance of individual classes of metabolites (e.g., flavonoids, alkaloids or terpenoids) in many species has been well studied (Rieseberg & Ellstrand, 1993; Orrians, 2000), to our knowledge no study has ever examined expression of a wide range of primary and secondary metabolites within hybrid individuals. An integrated understanding of metabolic expression within hybrid individuals may be necessary for accurate estimates of frequency of novel metabolite generation, and a mechanistic understanding of the role of hybridization in phytochemical expression and/or diversification. For instance, a recent review (Orrians, 2000) has estimated that the frequency of metabolite novelty resulting from hybridization is between 5 and 20%. This estimate may be biased, as only a limited number of secondary metabolite classes have been screened for novelty in hybrids.

Until recently, large-scale studies of plant metabolites have been limited by the

time-consuming and costly nature of available technology. Metabolomics is an emerging field that encompasses the identification and quantification of the suite of metabolites within an organism, tissue or biofluid. Using techniques including nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy, it is now possible to profile the majority of organic molecules occurring within target samples. Among other uses, metabolomic studies have been applied to the elucidation of biochemical pathways (Weckwerth & Fiehn, 2002; Steuer *et al.*, 2003); to the characterization of ecotypes within species (Ward *et al.*, 2003); and to the identification of metabolomic responses to disease (Griffin, 2003; Viant *et al.*, 2003).

Senecio jacobaea and *Senecio aquaticus* are closely related, nonsister species (Pelser *et al.*, 2003) which form natural hybrid swarms (Kirk *et al.*, 2004). These species demonstrate significant differences in ecological range (Weeda *et al.*, 1991), herbivore susceptibility (personal observation), and resistance to drought and inundation (Kirk *et al.*, 2005). Additionally, *Senecio* species are well known for production of pyrrolizidine alkaloids (PAs), secondary metabolites which are highly toxic to both vertebrate (Cheeke, 1988) and invertebrate herbivores (Frei *et al.*, 1992), and which may influence growth of root-associated fungi (Hol & van Veen, 2002). Hybridization between these species may lead to the formation of novel combinations of PAs in hybrids in the wild (Kirk *et al.*, 2004). However, differences in susceptibility/resistance to specialist and generalist herbivores cannot be completely explained by differences in PA structures, diversity or concentrations (Macel *et al.*, 2002, Nico de Boer, personal communication). Additionally, we have found in herbivore choice tests between *S. jacobaea* and *S. aquaticus* that feeding preference depends on herbivore species (unpublished data). These data lead to the hypothesis that a mosaic of antiherbivore defences play a role in plant–herbivore interactions in these species.

The purpose of this study is twofold. Using a metabolomic profiling approach, we initially aim to identify differential secondary metabolite expression between *S. aquaticus* and *S. jacobaea*, which may be important in ecological interactions. Second, we aim to examine the consequences of hybridization in *Senecio* with regard to a wide range of primary and secondary metabolite expression.

MATERIALS AND METHODS

Plant material

Both *Senecio jacobaea* L. and *Senecio aquaticus* Hill are biennial to perennial, self-incompatible species. During spring 2003 we transplanted second-year rosettes of *S. jacobaea* and *S. aquaticus* from the Zwanenwater nature reserve in the Netherlands to a glasshouse at Leiden University. On flowering, eight *S. jacobaea* individuals were paired with eight *S. aquaticus* individuals, and pairs were crossed by rubbing flowers together. Seeds were subsequently collected from all plants involved in crosses. We also placed four *S. jacobaea* and four *S. aquaticus* individuals among the plants used for crossing, to control for pollen contamination and/or low levels of selfing in the glasshouse. Control plants did not set any seed, which confirms that

seeds harvested from interspecific crosses represent F_1 hybrids. Seeds of *S. jacobaea* and *S. aquaticus* were collected from the Zwanenwater nature reserve.

Seeds of F_1 hybrids, *S. jacobaea* and *S. aquaticus* were germinated, and the following week eight equally sized seedlings from each plant group (*S. jacobaea*, *S. aquaticus* and hybrids) were transplanted to pots (11 cm diameter) filled with dune sand mixed with potting soil (1 : 1). A total of 24 plants were allowed to grow for 8 wk under standard conditions, and all above-ground plant material was harvested for analysis. Plant material was flash-frozen in liquid nitrogen on harvesting and stored at $-80\text{ }^{\circ}\text{C}$ until extraction.

Extraction of plant material

Each sample was freeze-dried. Dry material (100 mg) was transferred to a 10 ml centrifuge tube, and each sample was prepared by addition of 2 ml 50% methanol- d_4 in buffer (90 mM KH_2PO_4 , apparent pH 7) containing 0.05% trimethyl silyl propionic acid sodium salt (TSP, w/v). The mixture was vortexed at room temperature for 30 s, ultrasonicated for 1 min, and centrifuged at $27.9 \times g$ at $4\text{ }^{\circ}\text{C}$ for 20 min. Each NMR sample consisted of 800 μl of the supernatant.

Nuclear magnetic resonance measurements

NMR simultaneously detects most organic compounds in a mixed sample, including carbohydrates, organic and fatty acids, amino acids, and most secondary metabolites. Proton (^1H) NMR functions by detecting protons attached to compounds in a sample, such that one compound is typically represented by multiple signals (protons) in the NMR spectrum. One-dimensional (^1H) NMR displays proton signals based on the bonding of each proton to other atoms in compound. Characterization of samples containing hundreds of metabolites, such as those analysed here, can therefore lead to complex spectra with frequent overlap of signals from different compounds. This overlap can lead to poor resolution between compounds if only one-dimensional ^1H -NMR methods are employed, as is typical of many metabolomics studies.

To improve the resolution between different signals in the spectra, we screened several kinds of two-dimensional NMR methods to improve resolution. Two-dimensional NMR collects information about the position of each proton in relation to other protons within compounds, to further resolve signals. We found that the J-resolved technique (see Appendix 1 for further details) most improved resolution of the ^1H NMR spectra. The two-dimensional spectra were reconverted to a one-dimensional data set (Fig. 1), which produced sharper peaks and better resolution than the true one-dimensional data set. We thus used the projected J-resolved spectra for data analysis.

It is not possible to report the number of compounds appearing in the NMR spectra because multiple peaks represent each compound and identification of all compounds is impractical. However, it is usually estimated that NMR detects a range of 50–100 of the most highly accumulated plant compounds. In our experience, metabolites occurring in concentrations less than $\approx 1\text{ }\mu\text{mol mg}^{-1}$ are generally not detectable using the methods described here. Also, the sensitivity of the analysis

depends on the nature of target compounds. Some areas of the spectra, such as the region where many carbohydrates are found (Fig. 1), contain many signals, and metabolites occurring in this region may be difficult to detect if present in low concentrations. Conversely, relatively few signals occur in the phenolic region of the spectra, and even weak signals may be detected here.

Identification of compounds requires further NMR analyses, described in detail in Appendix 2. Briefly, the position, intensity and splitting pattern (number of peaks representing a signal) yield information about the identities of atoms and bonds surrounding each proton. For example, protons attached to a phenolic carbon atom appear in the phenolic region of the spectrum (Fig. 1). Elucidation of structures from a complex mixture therefore requires expert knowledge of plant phytochemistry and NMR spectral patterns.

Data analysis

Initial data handling

The J-resolved projection spectra were automatically exported as a spreadsheet using AMIX software (ver. 3.7, Bruker Biospin). The spectra, which were measured in chemical shift (δ ; ppm), were divided (bucketed) into bins of equal width (0.04 ppm), and the peaks represented within each bin were integrated (the area under

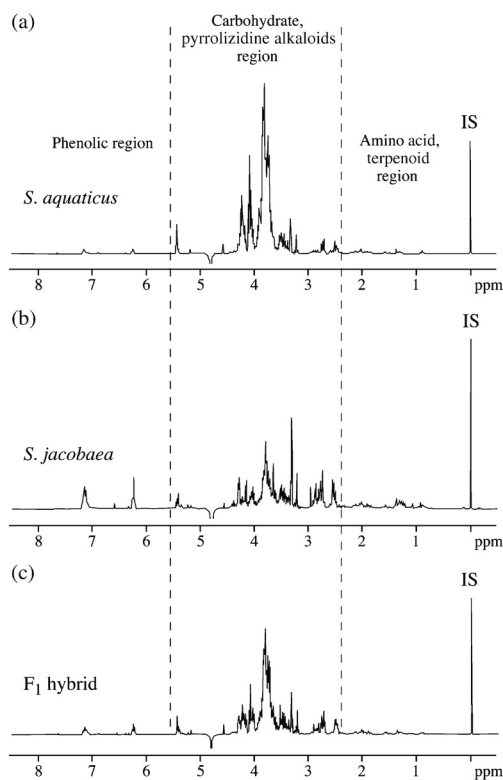


Figure 1 ^1H -NMR spectra for methanol–water extracts of individual samples of *Senecio aquaticus*, *Senecio jacobaea* and an F_1 hybrid of *S. aquaticus* and *S. jacobaea*. IS, internal standard TSP.

the spectra was calculated). Integrated signals were scaled (standardized) to the internal standard TSP (intensity = 1000), so that peak intensities represent intensity relative to the internal standard. Integrated and standardized signals were used for all further quantitative analysis of the data. The region from δ -0.4 to 10.0 (Fig. 1) was included in the analysis, and the region from δ 4.7 to 5.0 was excluded from the analysis because the water signal masked all other signals in this region. The region including citric acid, malic acid and succinic acid from δ 2.8 to 2.5 was bucketed by 0.1 ppm because chemical shift of these compounds can differ slightly according to concentration.

We compared the similarity of all pairs of individuals by matching two-dimensional J-resolved spectra using AMIX. The program calculates the percentage of signals that overlap between samples, based on the presence and absence of signals, rather than quantity.

Principal component analysis

Principal component analyses (PCA) were performed with the SIMCA-P software (ver. 10.0, Umetrics, Umeå, Sweden). Principal component analysis is an unsupervised clustering method requiring no knowledge of the data set, and acts to reduce the dimensionality of multivariate data while preserving most of the variance within it (Goodacre *et al.*, 2000). The principal components can be displayed graphically as a 'scores' plot. This plot is useful for observing any groupings in the data set. PCA models are constructed using all the samples in the study. Coefficients by which the original variables must be multiplied to obtain the PC are called loadings. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component (Massart *et al.*, 1988). Thus for NMR data, loading plots can be used to detect the metabolites responsible for the separation in the data. Generally, this separation takes place in the first three principal components (PC1, PC2 and PC3).

PCA is a visual method that facilitates separation of plant groups according to overall metabolic fingerprints. Analysis of variance (ANOVA) of individual principal components (dependent factors) provides statistical separation of plant groups. For identifying differences in expression of individual compounds, PCA is an exploratory method that indicates which compounds are most responsible for separation of different plant groups. The method therefore has no statistical power to indicate whether compounds are expressed in higher amounts in some groups than others. To test for significant differences in the expression of compounds that are important for plant group differentiation according to PCA, we applied one-way ANOVAs. Quantities of metabolites were expressed in relative intensity (based on integration under spectral peaks, and relative to the internal standard TSP), because absolute concentration is difficult to determine using the analytical techniques applied here. For ANOVAs, plant group (*S. jacobaea*, *S. aquaticus*, hybrids) were defined as fixed factors.

RESULTS

Visual inspection of the NMR spectra (Fig. 1) illustrates that *S. Jacobaea* and *S. Aquaticus* differ considerably in the overall pattern of metabolites detected. *Senecio aquaticus* exhibits much greater complexity in the carbohydrate region of the spectrum than does *S. jacobaea*, and *S. jacobaea* has stronger signals in the phenolic region of the spectra. Based on initial inspection, hybrids appeared to be intermediate.

Senecio aquaticus, *S. jacobaea* and hybrids were well differentiated based on PCA of quantitative metabolite expression (see Figs 3, 4). A variety of metabolites were responsible for the differentiation, including alanine, chlorogenic acid (Fig. 2), flavonoids (such as quercetin and kaempferol), fumaric acid, glucose, jacaranone analogues (Fig. 2), malic acid, pyrrolizidine alkaloids (Fig. 2), and sucrose. Based on matching of J-resolved spectral patterns, variation among *S. jacobaea* individuals was higher than variation among *S. aquaticus* or hybrid individuals (data not shown).

The first three principal components explained 70.3% of the variance. Plant groups could not be differentiated based on PC2. A score plot of PC1 vs. PC3 (Fig. 3) shows that species were well separated from one another by both PC1 (ANOVA; $F = 15.299$, $P < 0.000$) and PC3 (ANOVA; $F = 5.402$, $P = 0.013$). Hybrids were not clearly

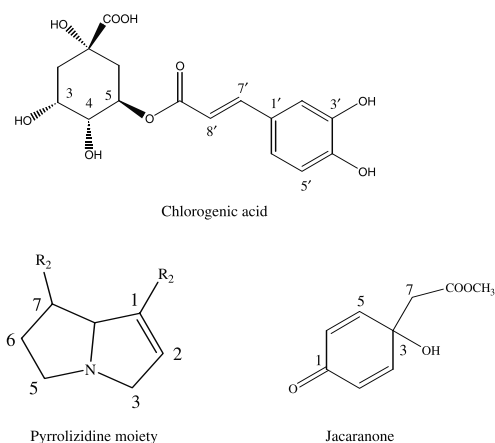


Figure 2 Chemical structures of chlorogenic acid, unsaturated pyrrolizidine moiety and jacaranone.

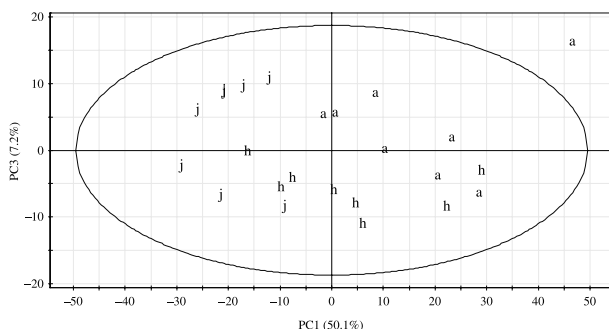


Figure 3 Score plot of principal component analysis of *Senecio aquaticus* (a), *Senecio jacobaea* (j), and F_1 hybrids (h) between the species. Ellipse represents Hotelling T^2 with 95% confidence in score plots.

intermediate to parental species based on PCA, as a number of hybrids had PC values that fell outside the range of parental individuals.

The position of peaks on the loading plots (Fig. 4) corresponds to chemical shifts of NMR signals. The intensity of peaks on the loading plots indicates the strength of the correlation between the signals and the principal component. Positive peak values indicate positive correlations, while negative peak values indicate negative correlations. Samples with higher PC values thus accumulate relatively larger amounts of metabolites represented by intense, positive signals on the loading plots, and relatively smaller amounts of metabolites represented by intense, negative signals on the loading plots. For example, *S. aquaticus* and hybrids have significantly higher PC1 values compared with *S. jacobaea* (Tukey's test, data not shown). Examination of the loading plot of PC1 (Fig. 4a) shows that chlorogenic acid, jacaranone, glucose and sucrose are positively correlated with PC1. *Senecio aquaticus* and hybrids thus contain more of these metabolites compared with *S. jacobaea*. Conversely, jacaranone analogues, fumaric acid and several oligosaccharides have negative peak values on the loading plot, and are therefore expressed in lower quantities in *S. aquaticus* or hybrids than in *S. jacobaea*.

Similarly, hybrids are significantly separated from *S. aquaticus* based on PC3. Hybrids have lower PC3 (Fig. 3) values compared with *S. aquaticus* (Tukey's test, data not shown). The loading plot (Fig. 4b) shows that chlorogenic acid, jacaranone analogues, and several oligosaccharides are negatively correlated with PC3 and thus accumulate more in hybrids, although these differences were shown to be insignificant in later statistical tests (Fig. 5). Sucrose and succinic acid were less expressed in hybrids than in *S. aquaticus*, but these differences were again insignificant in subsequent *post hoc* tests (Fig. 5).

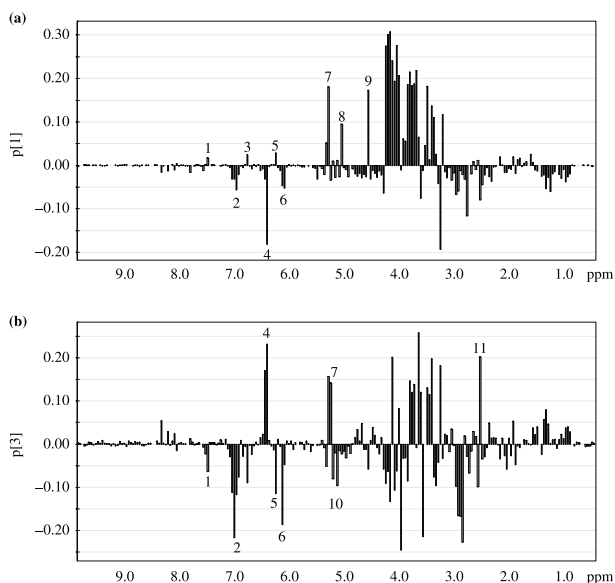


Figure 4 Loading plots of principal component analysis of *Senecio* species: (a) PC1; (b) PC3. 1, H-8' of chlorogenic acid; 2, H-2 and H-6 of jacaranone analogues; 3, H-5' of chlorogenic acid; 4, H- β of fumaric acid; 5, H-7' of chlorogenic acid; 6, H-6 of H-3 and H-5 of jacaranone analogues; 7, H-1 of sucrose; 8, H-1 of α -glucose; 9, H-1 of β -glucose; 10, H-1 of oligosaccharides; 11, H- β of succinic acid. Pyrrolizidine alkaloids and flavonoids are not shown.

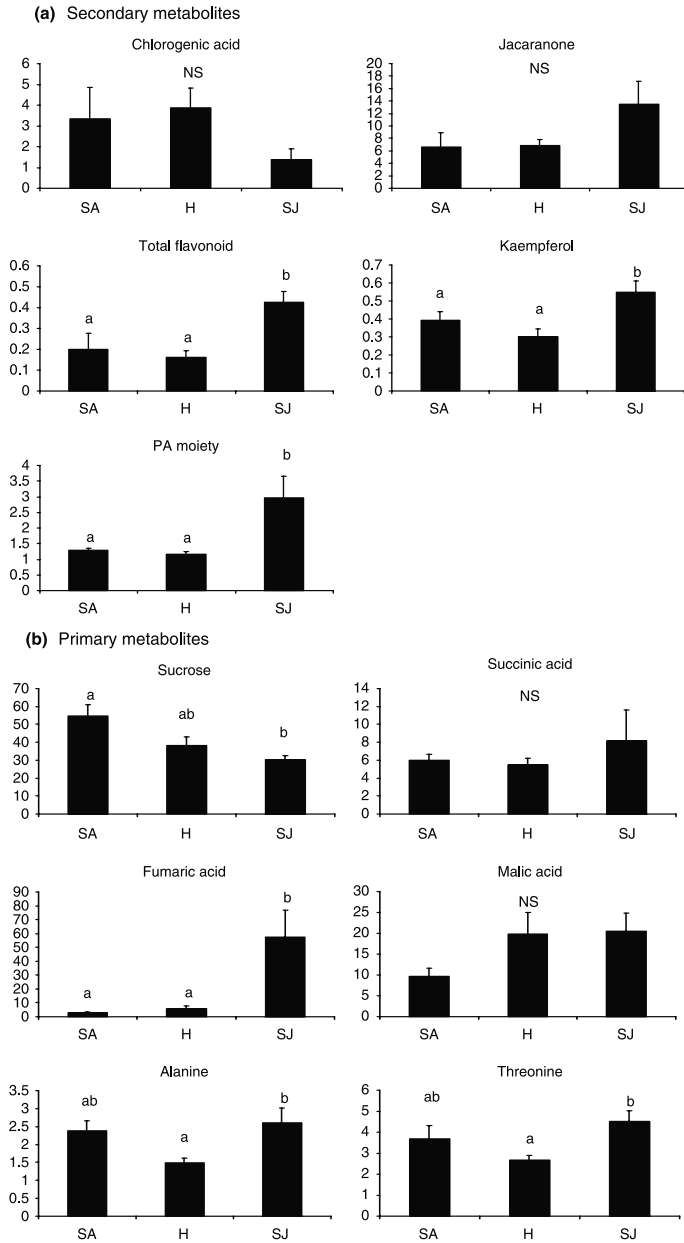


Figure 5 Quantitative expression of secondary (a) and primary (b) metabolites in *Senecio jacobaea* (SJ), *Senecio aquaticus* (SA), and F_1 hybrids (H). Total flavonoids are shown, as well as kaempferol, which was putatively identified as one of the flavonoid components. Letters indicate significant differences between groups (Tukey's test, $P < 0.05$; NS, no significant difference). The y-axis represents peak intensity relative to the internal standard TSP. Relative concentrations can be compared between plant individuals or groups, but not between different compounds. Bars represent SE.

Overall, hybrids never expressed higher concentrations of differentiating metabolites than parental species (Fig. 5), even expressing lower amounts of some metabolites, including the amino acids threonine and alanine (Fig. 5b), and phenolics including flavonoids and chlorogenic acid (Fig. 5a). There was no evidence that unique metabolites were expressed in hybrids or either parental species.

DISCUSSION

We found that parental species were well distinguished from each other based on the metabolome, although we did not identify any metabolites that are unique to either parental species in this analysis. Moreover, hybrids can be well distinguished from both parental species based on metabolite expression, and did not cluster intermediately to parents on the basis of PCA.

Hybrids demonstrated differential expression of a number of the metabolites studied here, the trend indicating that metabolite concentration was often reduced in hybrids in relation to parents. Reduction in concentration of some metabolites may result from a higher accumulation of biomass in hybrids vs. parents (Kirk *et al.*, 2005), rather than reduced production *per se*. Reduction in concentration of some metabolites might occur if the ratio of metabolite-producing organs to total plant biomass decreases (Hol *et al.*, 2003), but it is not possible to draw conclusions about whether this occurs without specific knowledge about the production and accumulation of individual compounds.

NMR proved to be a good analytical technique to detect a wide range of primary and secondary metabolites. It is generally accepted that a single analytical technique will not provide sufficient visualization of the metabolome, and therefore multiple technologies are needed for a comprehensive view (Summer *et al.*, 2003). However, sometimes limits to the amount of biological material available, time or funding limits, or metabolic instability force us to choose an optimum analytical tool for metabolomic profiling. Therefore it is preferable to use a wide-spectrum chemical analysis technique which is rapid, reproducible and stable over time, while requiring only very basic sample preparation. Nuclear magnetic resonance spectroscopy is a technique that meets those requirements.

One of the major drawbacks of NMR is that it fails to detect compounds present in relatively low concentrations. We did not identify any unique metabolites in any of the plant groups studied here. This result was probably an artefact of the NMR analysis. First, NMR necessarily detects only the most abundant metabolites in a sample. However, abundant metabolites may have a high fitness value to plants, and may be much less likely to exhibit qualitative variation in expression between genotypes and species. Second, NMR may detect general groups of secondary metabolites, but not small structural differences within such groups. For example, in this study we detected the basic structure (moiety) of unsaturated PAs, which are known to be important for plant–insect interactions in *Senecio*. Yet it is known that *S. jacobaea* alone can contain up to 10 different PA structures. Moreover, in other studies we found that *S. aquaticus* produces at least one PA that is not produced by *S. jacobaea* (Kirk *et al.*, 2004; unpublished data), and that F₁ hybrids produce one PA

that is unique to both parental species (unpublished data). In general, novel compounds produced by hybrids are likely to be present in small concentrations, and may represent structural variations within metabolite classes that cannot be detected by broad-scale metabolomic approaches using NMR. This is the major shortcoming of using metabolomic profiling techniques to study metabolite expression in hybrids vs. parental species.

NMR-based metabolomic profiling, as described here, may be best applied when there is little previous knowledge of the metabolites expressed by a group of study organisms, or when researchers are seeking to identify quantitative differences in major groups of primary and secondary metabolites. This study, for instance, provides a basis for more detailed study of a number of secondary metabolite classes, including flavonoids and jacaranone derivatives, which have never been reported from the *Senecio* species studied here. Broad-spectrum NMR profiling is a powerful approach for researchers interested in major quantitative differences between plant groups (Ward *et al.*, 2003), or between plant subjected to differing ecological conditions (Choi *et al.*, 2004).

It should also be noted that a number of secondary metabolites that may be involved in plant–herbivore interactions were detected in this study. Pyrrolizidine alkaloids have been well studied in *Senecio* species, are known to play a role in plant–herbivore interactions (Macel, 2003), and can reduce the growth of plant pathogens (Hol & van Veen, 2002). Jacaranone analogues (benzoquinoids), identified for the first time in the species studied here, have been isolated from several other *Senecio* species (Lajide *et al.*, 1996; Torres *et al.*, 2000; Xu *et al.*, 2003). Jacaranone analogues have been shown to have insecticidal activity against adult house flies (Xu *et al.*, 2003), and can act as a growth inhibitor for the generalist herbivore *Spodoptera litura* (Lajide *et al.*, 1996). Furthermore, chlorogenic acid has, to our knowledge, never been isolated from *Senecio* species, although it is a ubiquitous compound in plants. Chlorogenic acid may have a mixed effect on herbivores: Bi *et al.* (1997) found no effect of chlorogenic acid on a generalist and a specialist caterpillar feeding on tobacco; while Felton *et al.* (1992) found that the same compound reduced the nutritional quality of proteins for the generalist herbivore *Spodoptera exigua* (Felton *et al.*, 1992). Additionally, phenolic compounds such as chlorogenic acid may play a role in resistance to sucking insects (Miles & Oertli, 1993). Finally, flavonoids are known to convey herbivore resistance by inhibiting growth of Lepidopteran larvae (Mallikarjuna *et al.*, 2004), and can be sequestered by specialist herbivores (Wiesen *et al.*, 1994), indicating that these compounds can play a role in plant interactions with both specialist and generalist herbivores. Other major structural groups found in other *Senecio* species, including sesquiterpene lactones and polyacetylenes, were not apparent in the NMR spectra, and thus do not constitute a significant component of the metabolome of these species.

The presence of multiple putative defence compounds in the *Senecio* species studied here, as well as differing patterns of resistance of *Senecio* genotypes and species to different herbivores (unpublished data), confirm that these species may have a mosaic of defences that act differentially on different herbivores. It would be

interesting to continue detailed studies to determine whether structural variants of flavonoids, jacaranone and PAs occur within parental species and hybrids, and whether hybrids possess unique variants of these compounds. Furthermore, it would be interesting to combine such studies with herbivore resistance tests, to elucidate the role of these secondary metabolites in plant resistance. Overall, *Senecio* is a potentially useful genus for the study of selection on plant defences by multiple herbivores, and this study provides a basis for such future research.

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Appendix 1: Technical description of NMR measurements

^1H NMR and J-resolved spectra were recorded at 25 °C on a 400 MHz Bruker AV-400 spectrometer operating at a proton NMR frequency of 400.13 MHz. Methanol- d_4 was used as the internal lock. Each spectrum consisted of 128 scans requiring 10 min acquisition time with the following parameters: 0.25 Hz per point; pulse width = 90° (6.6 μs); relaxation delay = 5.0 s. A presaturation sequence was used to suppress the residual water signal with low-power selective irradiation at the water frequency during the recycle delay. Free induction decays were Fourier transformed with line broadening LB = 0.3 Hz and spectra were zero-filled to 32 K points. Window functions were optimized for the analysis. The resulting spectra were manually phased and baseline corrected, and calibrated to the internal standard TSP at 0.0 ppm, all using XWIN NMR (ver. 3.5, Bruker). Two dimensional J-resolved ^1H -NMR spectra were acquired using eight scans per 32 increments, collected into 16 K data points, using spectral widths of 5.208 kHz in F2 (chemical shift axis) and 50 Hz in F_1 (spin–spin coupling constant axis). A 1.0 s relaxation delay was employed, giving a total acquisition time of 14.52 min. Data sets were zero-filled to 512 points in F_1 and both dimensions were multiplied by sine-bell functions before double complex Fourier transformation (FT). J-resolved spectra were tilted by 45°, symmetrized about F_1 , and then calibrated, all using XWIN NMR. Data were exported as the one-dimensional projection (F2 axis) of the two-dimensional J-resolved spectra.

Appendix 2: Visual inspection of NMR spectra and assignment of metabolites

^1H - ^1H -COSY (correlated spectroscopy), TOCSY (total COSY), HSQC (heteronuclear single quantum correlation) and HMBC (heteronuclear multiple bond correlation) were measured at 600 MHz Bruker DMX-600 spectrometer operating at a proton NMR frequency of 600.13 MHz. The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. The HSQC spectra were obtained with 1.0 s relaxation delay, 6361 Hz spectral width in F2 and 27 164 Hz in F₁. The HMBC spectra were recorded with the same parameters as the HSQC spectrum, except for 30 183 Hz spectral width in F2. The TOCSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions.

The major differences between the *Senecio* species are observed in the aromatic region of the ^1H -NMR spectra (Figs 3, 5). The cluster of signals distinguished at δ 7.1 and 6.1 was found to be a doublet ($J = 9.6$ Hz) in ^1H - ^1H -2DJ-resolved spectra, and assigned as H-2, H-3, H-5 and H-6 of jacaranone analogues. HMQC spectra showed that H-2It was confirmed by ^1H - ^1H -COSY and HMBC spectra in which H-2, H-6 at δ 7.1 and H-3, H-5 at δ 6.1 correlated with C-1 at δ 189.2 and C-4 at δ 69.4. Other phenolic metabolites, such as chlorogenic acids and flavonoids, were detected. Two characteristic *trans* olefinic protons of chlorogenic acids were shown at δ 7.64 (H-8', $d, J = 15.9$ Hz) and δ 6.39 (H-7', $d, J = 15.9$ Hz) (Choi *et al.*, 2004). As a minor signal, H-6 and H-8 of flavonoids were observed at δ 6.32 ($d, J = 2.0$ Hz) and δ 6.50 ($d, J = 2.0$ Hz). A signal at δ 7.94 was putatively identified to belong to kaempferol, which has a 4' hydroxyl group. The singlet at δ 6.52 was assigned to be an olefinic proton of fumaric acid, which was confirmed by HMBC in which the proton correlated with the carbonyl signal at δ 176.2.

There was also significant difference in the anomeric signals of carbohydrates such as δ 5.42 ($d, J = 3.8$ Hz); δ 5.18 ($d, J = 3.7$ Hz); and δ 4.57 ($d, J = 9.5$ Hz). These were assigned to be the anomeric protons of sucrose, α -glucose, and β -glucose, respectively (Agrawal, 1992). Another anomeric signal obtained from the fructose moiety of sucrose is also easily distinguishable at δ 4.22 ($d, J = 8.8$ Hz). The residual proton signals of the sugars shown in the crowded region (δ 3.0–4.0) were assigned by comparison of ^1H -NMR spectra of the reference compounds ^1H - ^1H -COSY and TOCSY spectra. Other anomeric signals detected at δ 5.35–5.45 were assigned as anomeric signals of oligosaccharides, which was confirmed by HMBC and TOCSY spectra. In the region δ 5.40–5.90, H-2 of unsaturated pyrrolizidine alkaloids was observed as minor signals.

The complexity in the range of δ 2.5–3.0 was also clearly resolved by two-dimensional J-resolved spectra. The splitting pattern of this region was deconvoluted by the J-resolved spectrum, indicating signals belonging to malic acid at δ 2.80 ($dd, J = 16.6$ Hz, 4.7 Hz) and δ 2.61 ($dd, J = 16.6$ Hz, 6.6 Hz), and succinic acid at δ 2.54 (s).

6

Plant hybridization and secondary metabolite expression: a case study of pyrrolizidine alkaloids in the genus *Senecio*

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Among other consequences in plants, hybridization may influence the expression and evolution of resistance to natural enemies. Plant resistance to parasites, including both microbial pathogens and herbivores, is often mediated by the composition of secondary metabolites expressed by the plant. Here, we focus on pyrrolizidine alkaloids (PAs), a class of secondary metabolites that exhibits high structural diversity among species within the plant genus *Senecio*. We examine PA composition in *Senecio jacobaea*, *S. aquaticus*, artificially generated F₁ hybrids, and also later generation natural hybrids between these two species. We test the hypothesis that hybridization may contribute to PA diversity within plants, by comparing PA expression in hybrids to that in parents across a range of water and nutrient treatments. We report that hybrids produce a putatively novel PA, and that this PA is conserved in natural hybrids, which are highly backcrossed to *S. jacobaea*. Also, the range of PA concentration and diversity is more extreme in the roots and shoots of artificial hybrids over various environmental conditions, and high ranges are preserved in the shoots of natural hybrids. These results suggest that hybridization may partially explain the diversity of PAs found within the *Senecio* genus. Also, hybridization may increase the variation upon which selection can act in hybrid populations, such that some hybrids have the potential to be more resistant to natural enemies than parental individuals.

Key words: hybridisation, pyrrolizidine alkaloids, *Senecio jacobaea*, *Senecio aquaticus*, secondary metabolite diversity, plant resistance

INTRODUCTION

Over the past decade it has become evident that hybridization may play a much larger role in organic evolution than earlier anticipated. Especially in plants, research into the role of hybridization in the evolution of novel traits and new species is gaining momentum (Arnold, 1997; Buerkle, 2000; Rieseberg, 2003; Vellend *et al.*, 2007; Abbott *et al.*, 2008). It is now clear that hybridization can have various roles in plant evolution, from the homogenization of divergent species (McCarthy *et al.*, 1995), to the generation of novel, sometimes adaptive traits (Rieseberg *et al.*, 1999), to speciation (Rieseberg *et al.*, 2003; Seehausen, 2004; Abbot *et al.*, 2008). Yet, it is not yet clear how often hybridization plays an evolutionary role, or under what circumstances hybridization is most likely to be adaptive.

Among other consequences, hybridization may be involved in the evolution of resistance to natural enemies in plants. Many studies, especially in *Eucalyptus* (e.g., Dungey *et al.*, 2000) and willows (Hochwender & Fritz, 2004), have been directed at the effects of plant hybridization on phytophagous insect communities (Whitham *et al.*, 1999). Such studies have reported high variation in expression of secondary metabolites within hybrid zones and classes. Also, resistance among hybrids to herbivores is often highly variable, and hybrid zones may thus support highly diverse herbivore communities (Hochwender & Fritz, 2004; Drew *et al.*, 2005; Bangert *et al.*, 2006).

From a plant's perspective, attack by natural enemies can constitute an enormous selection pressure (Kover & Caicedo, 2001), and hybridization may provide a mechanism for rapid evolution of resistance to parasites, including herbivores and pathogens. Evolution of resistance through hybridization could function via a number of mechanisms. Fritz *et al.* (1994, 1999), showed that F_1 hybrids can demonstrate extreme levels of resistance in relation to parental species. Yet, it was not clear whether extreme resistance could be found in later generation hybrids, and in natural hybrid populations. In 1999, Rieseberg *et al.* showed that transgressive segregation, or the expression of extreme traits in later generation hybrids, occurs in an overwhelming majority of hybrid populations. More recently, Fritz & Hochwender (2004) have shown that F_1 and F_2 hybrids between two willow species have different patterns of resistance against 14 different herbivores, such that resistance to some herbivores is greater in hybrids than parental species.

Increased resistance in hybrids may be attributable to a number of different genetic and molecular processes (Fritz, 1999). Firstly, epistatic interactions between genes combined from both parents may create unique resistance traits, such as unique secondary metabolites. If hybridization can generate entirely new resistance traits, or new combinations of resistance traits to which natural enemies have never been exposed, resistance may be increased, and hybrid lines or hybrid genes may be selectively favored. For example, Ehrlich & Raven (1964) proposed that the diversity of secondary metabolites in plants can be explained if production of novel secondary metabolites increases plant resistance to specialist herbivores, and thus allows plants to escape attack. This hypothesis has received strong support by the wild parsnip and parsnip webworm system (Berenbaum *et al.*, 1989; Berenbaum &

Zangerl, 1998), in which different furanocoumarins have different specificity, and therefore different deterrent effects on the specialist herbivore. Similar effects have been found in a number of systems (Linhart & Thompson, 1999; Shonle & Bergelson, 2000; Adler & Kittelson, 2004; Macel *et al.*, 2005; Albrechtsen *et al.*, 2007). If hybridization leads to the production of novel secondary metabolites, which in turn leads to increased resistance to natural enemies, hybridization may be adaptive.

Also, if resistance traits exhibit higher genetic variation among hybrid individuals than among parental species, hybrids may be more responsive to high selection pressure than pure parental species. This hypothesis has been supported empirically (though not in relation to parasite resistance) in *Drosophila*, for which several studies have shown that variation in abiotic stress resistance can be higher in hybrid lines than in parental lines (Hercus & Hoffman, 1999; Wisseman, 2007), such that hybrids may evolve more quickly in response to stress than parental species.

In general, secondary metabolites provide good examples of anti-herbivore/pathogen systems that can be strongly affected by hybridization. Many classes of secondary metabolites, including alkaloids, flavonoids, terpenoids, etc. are known to mediate resistance to natural enemies. Studies from various plant genera have shown that in both artificial, and later generation natural hybrids, secondary metabolites can be expressed in extreme concentrations in relation to parental species (O'Reilly-Wapstra *et al.*, 2005; Kirk *et al.*, 2005). Furthermore, Orians (2000) showed that hybridization indeed often facilitates the production of secondary metabolites that are novel (not present in either parental species).

The *Senecio* genus is a useful system for studies of hybridization, herbivore resistance, and secondary metabolite diversity. *Senecio* contains more than 1500 species, and hybridization may play an important role in the species diversity within the genus (e.g., Harris & Ingram, 1992; Abbott *et al.*, 2000; Kirk *et al.*, 2004). Moreover, the genus is notorious for the production of pyrrolizidine alkaloids (PAs), secondary metabolites that are highly toxic to generalist mammalian (Cheeke, 1988) and insect (Frei *et al.*, 1992; Macel *et al.*, 2005) herbivores, and which may play a role in pathogen resistance (Hol & van Veen, 2002).

PAs are an interesting and diverse example of secondary metabolites, which occur in a number of families including Asteraceae, Boraginaceae, and Orchidaceae (Hartmann & Witte, 1995). These nitrogen containing compounds have been particularly well studied in the genus *Senecio* (Asteraceae), in which PAs are synthesized as senecionine N-oxide in plant roots (Hartmann & Toppel, 1987; Toppel *et al.*, 1987). Senecionine N-oxide is transported through the phloem to the shoots (Hartmann & Dierich, 1998) where diversification into a number of related PA structures occurs. PA diversification is species specific, such that the suite of PAs within a plant species is generally unique. Furthermore, significant variation in PA composition and concentration within *Senecio* species is observed (Macel *et al.*, 2004). Also, PA composition, including concentration and diversity, can alter in response to plant developmental cues (Schaffner *et al.*, 2003), abiotic environment (Macel *et al.*, 2009), and interactions with natural enemies (Hol *et al.*, 2004).

Senecio jacobaea (Asteraceae) has been particularly well studied, since this species is an invasive pest in North America, Australia, and New Zealand. *Senecio jacobaea* individuals can contain more than 10 PAs (Witte *et al.*, 1992), which play a role in interactions with specialist (Macel & Vrieling, 2003) and generalist herbivores (Macel, 2003), and with root-associated fungi (Hol & van Veen, 2002). Selection pressure by specialist herbivores on *S. jacobaea* may be quite high, since a number of specialist herbivores, including *Tyria jacobaeae* and *Longitarsus jacobaeae*, cause extreme damage to above ground and below ground plant parts.

Senecio jacobaea hybridizes in nature with *Senecio aquaticus* (Kirk *et al.*, 2004), a species that is much less susceptible to specialist herbivore attack in nature (personal observation). *Senecio aquaticus* is usually found in seasonally water-logged, humic, chalk-poor, and nutrient-rich soils (Weeda & Deursen, 1994), while *S. jacobaea* prefers sunny environments with sparse vegetation (Weeda & Deursen, 1994). Natural hybrids, first observed at the Zwanenwater in 1979 (R van der Meijden, personal communication) can be found in a narrow zone spanning a bank at the edge the lake, which appears to be intermediate to parental sites with regards to soil organic content and humidity. Very little overlap seems to occur between the local distributions of these species (personal observation). Genotype $\times$ environment interactions may be relevant in this study because *S. jacobaea* and *S. aquaticus* inhabit substantially different abiotic and biotic environments, and environmental factors can have a significant impact on PA expression in at least *S. jacobaea* (Macel, 2003; Hol *et al.*, 2004; Macel & Klinkhamer, 2009).

Here, we investigate the quantitative and qualitative expression of PAs in both natural and artificial hybrids of *S. jacobaea* and *S. aquaticus*. We ask (1) is there evidence that novel PAs are produced in hybrids? (2) Do hybrids express extreme concentrations of PAs compared to parental species? (3) Do hybrids produce a higher diversity of PAs than parents? (4) Do genotype $\times$ environment factors affect PA expression of hybrids in relation to parental species.

MATERIALS AND METHODS

Study system

Viable hybrids between *S. jacobaea* L. and *S. aquaticus* Hill have been reported from a number of locations including the United Kingdom (Stace, 1975), Germany (Christian Düring, personal communication), and The Netherlands (Kirk *et al.*, 2004). In this investigation, we studied natural *S. jacobaea* $\times$ *S. aquaticus* hybrids from the Zwanenwater reserve (The Netherlands). Composed mostly of sand dunes, the Zwanenwater reserve contains a small lake around which a hybrid population exists (see Kirk *et al.* 2005 for diagram). *Senecio jacobaea* are abundant in the dunes surrounding the lake, while *S. aquaticus* occurs infrequently at the lake fringe. *Senecio jacobaea* is susceptible to severe herbivory by the specialist lepidopteran *T. jacobaeae*, which is stimulated to oviposit by PAs (Macel & Vrieling, 2003). *Senecio aquaticus* is not subject to attack by *T. jacobaeae*, and other specialists that pupate in the soil around the plant, because pupae do not survive in the moist environments

where *S. aquaticus* is found (personal observation). Thrips appear to be common in the *S. aquaticus* population (personal observation). Preliminary tests show that in climate chamber experiments, *S. aquaticus* and *S. jacobaea* are equally susceptible to *T. jacobaeae* (Macel *et al.*, 2002), and the generalist lepidopteran herbivore *Spodoptera exigua* (personal observation), but *S. aquaticus* is much more resistant to the generalist thrips species *Thrips tabaci* than is *S. jacobaea* (Kirk *et al.*, 2005).

Seeds of *S. jacobaea*, *S. aquaticus*, and natural hybrids were collected from plants in the field during 2001 and 2002. Putative hybrids were identified in the field based on leaf lobe and flower morphology, and were later confirmed to be hybrids based on diagnostic amplified fragment length polymorphism (AFLP) markers (Kirk *et al.*, 2004).

F₁ hybrids were produced by collecting second year rosettes of parental plants, exhibiting the development of flowering stems, from the field. To minimize chances that introgressive genes were present in experimental parents, *S. aquaticus* individuals were collected from a marshy agricultural grassland approximately 500 meters from the hybrid zone, and *S. jacobaea* individuals were collected from dunes located approximately 300 meters from the hybrid zone. Plants from both species were placed in a greenhouse, allowed to flower, and were crossed in pairs of *S. jacobaea* × *S. aquaticus* by rubbing flower heads together. Seeds were harvested from both parental plants.

Plant growth

We selected five *S. aquaticus* genotypes, five *S. jacobaea* genotypes, and five natural hybrid genotypes for experimental use. We also included 10 genotypes from F₁ producing crosses, from both parental plants in the reciprocal cross, such that genotypes originated from five crosses, and ten parental plants (five *S. aquaticus* mothers, and five *S. jacobaea* mothers). F₁ genotypes were unrelated to parental genotypes used in this experiment.

One equal sized clonal plantlet from most genotypes was transplanted into each of six experimental columns (1 m length, 15 cm diameter), yielding a total of 136 experimental plants. We aimed at 150 plants but due to variance in sizes and difficulties with cloning 136 plants were used. One plant from each genotype was thus subjected to each of six experimental treatments.

The experiment was established to test a combination of two nutrient and three water treatments. We used sieved dune sand to fill all columns. In half the columns, the dune sand was mixed with 'Osmocote' slow release fertilizer (N:P:K = 15:11:13 + 2 MgO) at a concentration of 1.3 g/L sand to provide a nutrient rich medium. After establishment of seedlings, columns were partially submerged in water of three different depths: 5, 50, and 100 cm.

All columns were given sufficient water at the beginning of the experiment to allow for seedling establishment. Therefore at the beginning of the experiment, soil throughout the total length of the column was moist. Experimental conditions were established two weeks after seedlings were transplanted to columns, and the experiment was subsequently continued for ten weeks. Roots and shoots were harvested separately, and were dried in an oven for three days at 50 °C.

PA extraction

All dried leaves and roots from each plant were separately milled to a fine powder and homogenized. Milled samples were stored in a freezer at -80°C until use. For extraction, approximately 15 mg of plant material was extracted according to a modified version (de Boer, 1999) of the acid-base extraction method (Hartmann & Zimmer, 1986). Extractions were dissolved in methanol containing heliotrine (Latoxan, France) as an internal standard and analysed using gas chromatography (GC). Conditions (injector 250°C , temperature programme 0-22-5-250, split mode 1-30, carrier gas N_2 0.9 ml min^{-1} , pressure 56 kPa; detector NPD) were controlled by a Hewlett Packard gas chromatographer (model 6890). GC traces were compared with known references to identify sample composition.

Statistical analysis

We tested PA concentrations and diversity separately for roots and shoots, since PAs may interact both with root and with shoot pathogens and herbivores. We quantified PA diversity by counting the number of PAs present in each sample. Differences in both diversity and total PA concentration were analyzed using ANOVAs, for which we identified water, nutrient and plant group's treatments as fixed factors. Plant group denotes the both parental species, the F_1 and the natural hybrids. Shoot to root ratio was used as a covariate because it is assumed that root biomass is proportional to PA production and the size of the shoots is not related to PA production. Relatively larger shoots therefore may lead to a dilution of PAs. We first tested for maternal effect on PA expression in F_1 hybrids, by including only F_1 hybrids in the analysis. Since we found that maternal parent was never a significant factor in the analyses, we treated all F_1 hybrids as one group for subsequent ANOVAs using the entire data set.

We also applied principal component analysis (PCA) to quantitative PA data. PCA reduces a large number of variables into a smaller number of uncorrelated principal components (PCs), while preserving most of the variance in the data set. We applied ANOVAs to each PC, to test whether nutrient and water treatments and plant group had significant effects on PA composition. We then identified individual PAs that were highly correlated with each PC.

RESULTS

Eleven PAs were identified during our analysis (Table 1). We found eight PAs in *S. jacobaea* (eight in the shoots, and eight in the roots). Additionally, PA1 was present in low amounts in two plants of *S. jacobaea* and florosenine in one plant. To our knowledge, neither of these PAs have previously been reported from *S. jacobaea* individuals, and may represent PAs introgressed from the Zwanenwater hybrid zone. Ten PAs were present in *S. aquaticus*, of which otosenine appeared to be specific to *S. aquaticus* compared to *S. jacobaea* although it is also rare in *S. aquaticus*. In contrast to findings for *S. jacobaea*, PA1 is almost always present in *S. aquaticus* shoots. The reverse is true for jacobine. All eleven PAs were present among both natural

Table 1 Presence of 11 pyrrolizidine alkaloids in the roots (R), and shoots (Sh) of *S. jacobaea*, *S. aquaticus*, natural hybrid, and F₁ hybrid genotypes

Retention time	Alkaloid	<i>Senecio jacobaea</i>	<i>Senecio aquaticus</i>	Natural hybrid	F ₁ hybrid
3.97	PA1	Sh/R	Sh/R	Sh/R	Sh/R
7.07	Senecivernine	Sh/R	Sh/R	Sh/R	Sh/R
8.015	Senecionine	Sh/R	Sh/R	Sh/R	Sh/R
8.257	Seneciphylline	Sh/R	Sh/R	Sh/R	Sh/R
8.874	Integerrimine	Sh/R	Sh/R	Sh/R	Sh/R
10.386	Jacobine	Sh/R	Sh	Sh/R	Sh/R
10.852	Jacoline	Sh/R	Sh/R	Sh/R	Sh/R
11.30	PA2	R	Sh/R	Sh	R
12.01	Erucifoline	Sh/R	Sh/R	Sh/R	Sh/R
12.50	Otosenine		R	R	R
14.31	Floroseneine	R		Sh/R	Sh

hybrid and artificial hybrid genotypes. Florosenine (Fig. 1) appeared to be specific to hybrids, though trace amounts were expressed by one *S. jacobaea* plant.

Some hybrid individuals had extreme PA concentrations compared to parents, though on average, PA concentrations were not extreme in hybrids compared to parental species (Table 2). There was no significant effect of, or interaction involving plant group on either shoot or root PA concentrations (Table 3, Fig. 2). PA concentrations in *S. jacobaea* and in the natural hybrids were very similar in both roots and shoots. In wet sandy conditions low PA concentrations in the shoot were found as indicated by the significant main effect of water and the interaction between nutrients and water. The roots in sandy soils have higher PA concentrations. Generally, PA concentrations in the roots are lower in dryer soils (Table 3, Fig. 2).

PA diversity in the shoots differed significantly among groups. Averaged over all treatments the number of PAs per plant ($\pm$ SE) were respectively, 3.96 ± 0.35 , 3.59 ± 0.33 , 4.13 ± 0.33 , 4.80 ± 0.24 for *S. jacobaea*, *S. aquaticus*, natural hybrids and F₁ hybrids. In shoots, PA diversity was affected by an interaction between nutrient and water treatments and interactions between nutrient treatment and plant group (Table 2 and 4). The latter interaction is mainly caused by extreme low diversity of *S. aquaticus* in medium and dry nutrient rich conditions. In the roots, water was the only significant factor; PA diversity was higher in wetter conditions (Fig. 3).

PCA yielded two components (Table 5) for PAs in plant shoots, which cumulatively explained 85.6% of the variation in PA expression. The first component mostly explained variation in the least metabolically derived alkaloids identified in this experiment (i.e., significantly correlated with) senecionine ($r = 0.68$), seneciphylline

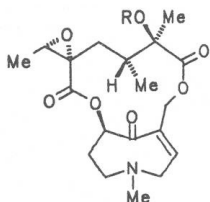
**Figure 1** Chemical structures of otosenine (R = H) and florosenine (R = Ac).

Table 2 Effect of nutrient and water treatment, and plant group (fixed factors) on pyrrolizidine alkaloid concentration in the roots and shoots of *S. jacobaea*, *S. aquaticus*, natural hybrids, and F_1 hybrids. Shoot to root ratio (SRratio) of the plant was used as a covariate

Source	Type III SS	df	MS	F	P
Shoots					
Covariate SRratio	0.640	1	0.640	1.528	0.219
group	0.726	3	0.242	0.578	0.631
nutrient	1.465	1	1.465	3.497	0.063
water	6.632	2	3.316	7.917	0.001
group*nutrient	0.408	3	0.136	0.325	0.807
group*water	4.152	6	0.692	1.652	0.140
nutrient*water	10.123	2	5.062	12.085	0.000
group*nutrient*water	2.846	6	0.474	1.132	0.348
Error	46.490	111	0.419		
Roots					
Covariate SRratio	0.794	1	0.794	1.292	0.258
group	4.059	3	1.353	2.202	0.092
nutrient	6.884	1	6.884	11.204	0.001
water	6.549	2	3.275	5.329	0.006
group*nutrient	1.900	3	0.633	1.031	0.382
group*water	1.507	6	0.251	0.409	0.872
nutrient*water	0.639	2	0.320	0.520	0.596
group*nutrient*water	7.858	6	1.310	2.132	0.055
Error	68.203	111	0.614		

Table 3 Effect of nutrient and water treatment, and plant group (fixed factors) on pyrrolizidine alkaloid diversity in the roots and shoots of *S. jacobaea*, *S. aquaticus*, natural hybrids, and F_1 hybrids. Shoot to root ratio (SRratio) of the plant was used as a covariate

Source	Type III SS	df	MS	F	P
Shoots					
Covariate SRratio	1.758	1	1.758	0.577	0.449
group	30.530	3	10.177	3.339	0.022
nutrient	0.439	1	0.439	0.144	0.705
water	5.421	2	2.711	0.889	0.414
group*nutrient	31.988	3	10.663	3.499	0.018
group*water	24.382	6	4.064	1.333	0.248
nutrient*water	67.319	2	33.660	11.044	0.000
group*nutrient*water	8.652	6	1.442	0.473	0.827
Error	338.292	111	3.048		
Root					
Covariate SRratio	2.364	1	2.364	0.806	0.371
group	28.433	3	9.478	3.233	0.025
nutrient	1.727	1	1.727	0.589	0.444
water	42.868	2	21.434	7.312	0.001
group*nutrient	22.180	3	7.393	2.522	0.061
group*water	21.237	6	3.539	1.207	0.308
nutrient*water	1.975	2	0.988	0.337	0.715
group*nutrient*water	7.419	6	1.236	0.422	0.863
Error	325.386	111	2.931		

($r = 0.99$) and integerimine ($r = 0.74$) and to a lesser extent variation in jacobine ($r = 0.21$), jacoline ($r = 0.47$) and erucifoline ($r = 0.38$) (Table 5). The second component explained variation in jacobine ($r = 0.98$), florosenine ($r = 0.44$), PA1 ($r = -0.29$) and senecionine ($r = -0.31$) (Table 5). Component 1 was significantly affected by all abiotic conditions and their interaction. Scores for component 1 were higher under sandy conditions and were low in wet and high in intermediate wet conditions. The second component (PC2) was significantly affected by group with the parents showing the most extreme scores (*S. jacobaea* $0.34 \pm 0.17a$), *S. aquaticus* ($-0.61 \pm 0.17b$) and hybrids intermediate (natural hybrids $-0.04 \pm 0.17b$, F_1 hybrids $0.16 \pm 0.12a$)

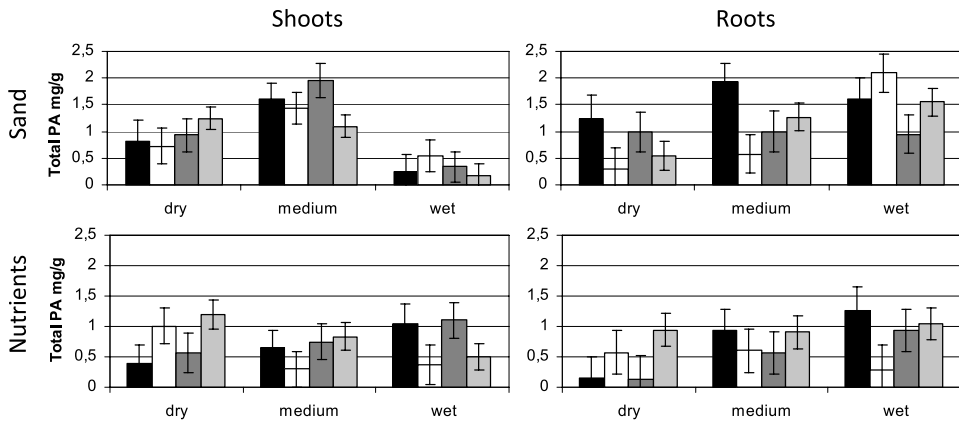


Figure 2 PA concentration in the shoots and roots of *S. jacobaea* (black), *S. aquaticus* (white), natural hybrids (dark grey), and F_1 hybrids (light grey) in a factorial design with two nutrient levels and three water levels. Vertical bars represent standard error.

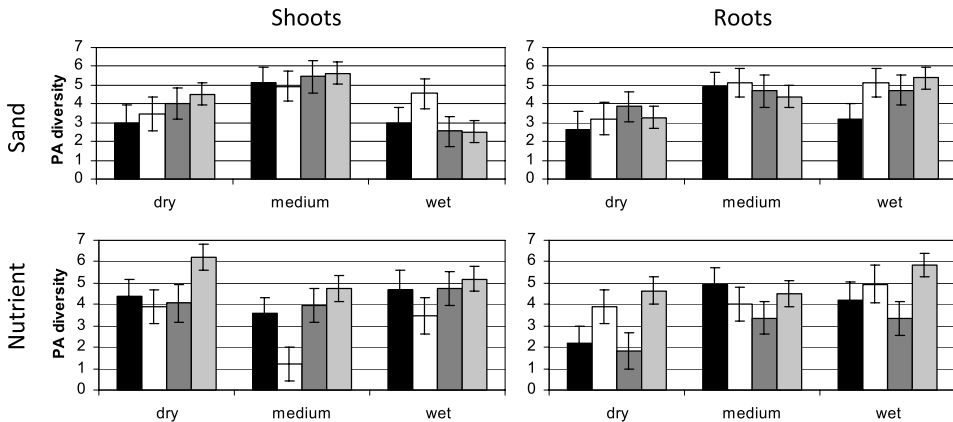


Figure 3 PA diversity, defined as the number of PAs detected within each sample, in the shoots and roots of *S. jacobaea* (black), *S. aquaticus* (white), natural hybrids (dark grey), and F_1 hybrids (light grey) in a factorial design with two nutrient levels and three water levels. Vertical bars represent standard error.

Table 4 Range of PA diversity and concentration among individuals within plant groups. Bold numbers in natural and F₁ hybrids indicate values that are extreme in relation to both parental ranges

Treatment	<i>S. jacobaea</i>	<i>S. aquaticus</i>	Natural hybrid	F ₁ hybrid
Shoot diversity				
Dry sand	2-4	1-8	0-5	2-8
Medium sand	4-6	4-6	3-8	4-8
Wet sand	2-4	1-5	2-6	2-8
Dry nutrients	4-6	3-4	3-7	2-8
Medium nutrients	1-6	0-2	3-5	2-8
Wet nutrients	3-6	3-5	3-5	1-10
Shoot concentration				
Dry sand	0.03-0.51	0.09-1.79	0-1.45	0-1.02
Medium sand	0.79-1.85	0.49-2.39	0.23-3.45	0.21-3.26
Wet sand	0.60-1.26	0.12-1.54	0.16- 2.35	0.27-2.29
Dry nutrients	0.25-0.91	0.23-0.57	0.31-2.64	0.16-0.91
Medium nutrients	0.25-1.46	0.10-0.52	0.45-1.16	0.23-2.44
Wet nutrients	0.03-0.82	0.53-1.62	0.27-0.96	0.02-3.02
Root diversity				
Dry sand	0-5	4-7	4-7	2-8
Medium sand	4-6	3-7	3-7	3-7
Wet sand	2-3	2-4	2-5	2-7
Dry nutrients	3-5	4-6	0-5	4-8
Medium nutrients	3-7	2-7	2-5	0-9
Wet nutrients	0-7	3-4	0-3	2-8
Root concentration				
Dry sand	0-3.03	0.60-4.69	0.35-2.23	0.35-3.16
Medium sand	1.10-3.36	0.10-0.85	0.43-1.59	0.57-2.91
Wet sand	0.09-3.01	0.29-0.48	0.24-2.41	0.04-1.86
Dry nutrients	0.36-1.98	0.21-0.40	0-1.80	0.23-2.18
Medium nutrients	0.05-1.82	0.01-2.06	0.05-1.63	0-2.31
Wet nutrients	0-0.49	0.02-1.19	0-0.28	0.11-2.18

(different letters indicate significant differences). Another significant main effect was found for nutrients with scores for nutrient rich conditions (0.31 ± 0.11) higher than for sandy soils (-0.38 ± 0.11).

Overall PA composition in the shoots was often extreme in both natural and artificial hybrids, as revealed by extreme PC scores from the PCA (Fig. 4).

Two PCs accounted for 88.9% of the variation in root PA expression. Like in the shoots, the first component mostly explained variation in the least metabolically derived alkaloids (i.e., senecionine ($r = 0.95$), seneciphylline ($r = 0.66$) and integerimine ($r = 0.77$)) and to a lesser extent variation in jacoline ($r = 0.47$) and erucifoline ($r = 0.25$) (Table 5). The second component explained variation in senecionine ($r = -0.31$), seneciphylline ($r = 0.75$), integerimine ($r = 0.26$), jacoline ($r = 0.6$), PA2 ($r = 0.5$) and erucifoline ($r = 0.48$). All three main effects (group, water, nutrients) were significant for component 1. Again parents showed the most extreme scores (*S.*

Table 5 Effects of nutrient treatment, water treatment, and plant group on principal components (PC) that are correlated with PA expression in roots and shoots

	PC	% variation ¹	Correlated PAs ²	Significant factors ($P < 0.05$) ³
Shoots	1	53.0	(+)Senecionine	Nutrient
			(+)Seneciphyllin	Water
			(+)Integerrimine	Water*nutrient
			(+)Jacobine	
			(+)Jacoline	
			(+)Erucifoline	
Roots	2	32.6	(-)PA1	Group
			(-)Senecionine	Nutrient
			(+)Jacobine	
			(+)Florosene	
			(+)Senecionine	Group
	1	64.7	(+)Seneciphyllin	Water
			(+)Integerrimine	Nutrient
			(+)Jacobine	
			(+)Erucifoline	
			(-)Senecionine	Group
	2	24.2	(+)Seneciphyllin	Nutrient
			(+)Integerrimine	Water*nutrient
			(+)Jacobine	
			(+)PA2	
			(+)Erucifoline	

¹Indicates percent of total PA variation explained by each PC.

²Indicates PAs that are highly correlated with each PC ($P < 0.05$).

³Significant factors represent single factors and/or interactions that had significant effects on PC values in ANOVA analyses.

jacobaea $0.53 \pm 0.18a$, *S. aquaticus* $-0.31 \pm 0.17b$) and hybrids intermediate (natural hybrids $-0.21 \pm 0.17b$, F_1 hybrids $0.01 \pm 0.12ab$) (different letters indicate significant differences). Scores for component 1 decreased with dryer conditions and were lower in nutrient rich soils. Component 2 showed significant main effects of group and nutrients and a significant interaction of water and nutrients. For 'group' the parents showed the most extreme scores (*S. jacobaea* $-0.84 \pm 0.17a$, *S. aquaticus* $0.42 \pm 0.16b$) and hybrids intermediate (natural hybrids $-0.01 \pm 0.16b$, F_1 hybrids $0.15 \pm 0.11b$) (different letters indicate significant differences).

Analysis of PC1 and PC2 for roots and shoots yield that group factor is significant in both shoots and roots indicating that PA diversity is influenced by the group factor. In contrast total PA concentration is not influenced by plant group (Table 3).

DISCUSSION

It has recently been stated that the possibility that 'synergistic host resistance result[ing] from formation of F_2 hybrids... is unlikely because recombination is not predicted to enhance plant defences against herbivores' (Hochwender & Fritz, 2004). This statement may be inaccurate if epistatic interactions lead to the forma-

tion of novel structural variations of secondary metabolites, to which herbivores are unadapted. We cannot say with 100% certainty that novel secondary metabolites arose from hybridization here. However, our findings strongly indicate that hybridization may indeed lead to unique patterns of secondary metabolite expression, which may very well lead to increased resistance to herbivores after recombination.

Both F_1 and natural hybrids produce the PA florosenine, which may be a novel product of hybridization in the Zwanenwater population. Florosenine has been previously reported from the South American *Senecio* species *S. glaber* (Reina *et al.*, 1993) and *S. leptolobus* (Mendez *et al.*, 1990), as well as from a Swiss population of *S. aquaticus* (Pelser *et al.*, 2005). Florosenine was never found in *S. aquaticus* from the Zwanenwater nature reserve during this current study, or during previous (Kirk *et al.*, 2004) studies. Trace amounts of florosenine were found in one *S. jacobaea* individual here, but this occurrence may represent introgression, since this PA has never been reported from other *S. jacobaea* populations, and was not found in either Zwanenwater *S. jacobaea* genotypes, or control populations from a variety of European locations analysed in a previous study (Kirk *et al.*, 2004).

Florosenine is the O-acetyl derivative of otosenine. Mechanistically, it is possible that hybridization combines the ability of *S. jacobaea* to acetylate PAs, and the ability of *S. aquaticus* to synthesize otosenine. If such inter-specific epistatic interactions between enzymes and substrates can result in the production of unique PA structures, then hybridization within the genus *Senecio* may be a mechanism for structural PA diversification

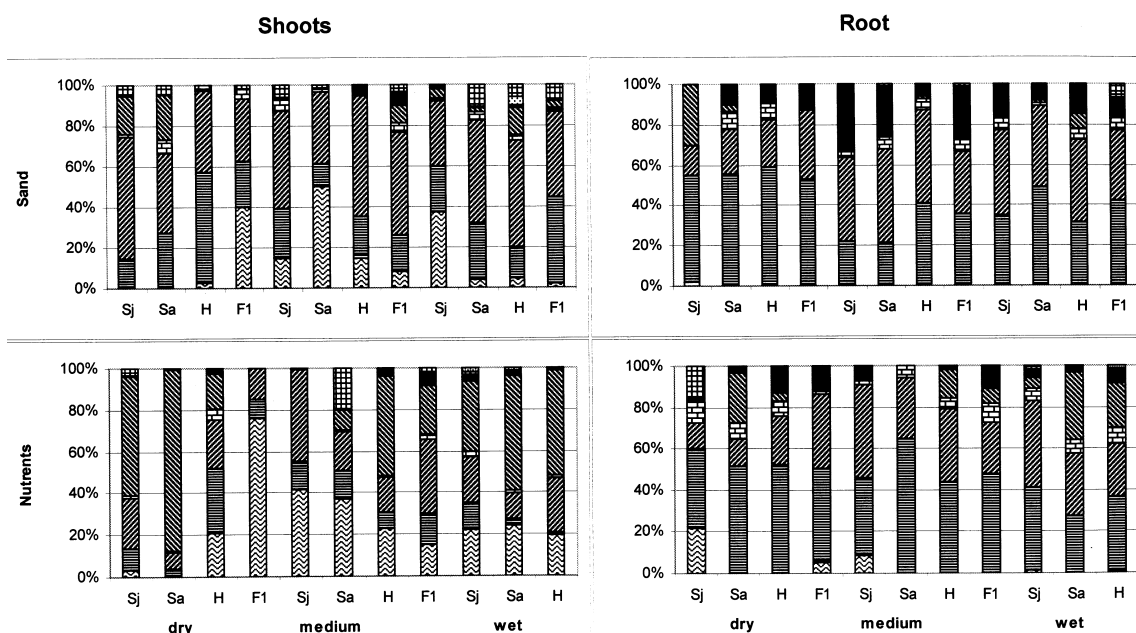


Figure 4 Relative PA composition in the shoots and roots of *S. jacobaea*, *S. aquaticus*, natural hybrids, and F_1 hybrids in a factorial design with two nutrient levels and three water levels.

Regardless of whether florosenine is a novel PA resulting from hybridization, hybridization clearly alters patterns of PA expression in hybrids compared to parental individuals. On average, hybridization did not lead to higher PA concentration and diversity within individual hybrids compared to parental individuals. Yet, there was greater variation in PA concentration and diversity in artificial hybrids, and this variation seemed to be preserved in the shoots, though not in the roots of natural hybrid individuals. This high variation suggests that there is more adaptive potential among both natural and artificial hybrids than in parental species.

Interestingly the ANOVA's of the factors of the PCA analyses (Table 5) show that the natural hybrids which are backcrossed several times to *S. jacobaea* (Kirk *et al.*, 2004) are with regard to PA diversity significantly different from *S. jacobaea* but not from *S. aquaticus*. The F_1 s are in all cases more similar to *S. jacobaea*. This suggests that PA diversity is under the influence of natural selection.

Although it is not yet clear whether increased PA diversity is adaptive, hybridization may provide a partial explanation for the high diversity of PAs within *Senecio*, even if such increased diversity is selectively neutral. In order to test whether unique PA patterns generated by hybridization provide selective advantages to plants, it will be interested to test whether hybrid individuals that express unique PA compositions are more resistant to natural enemies than parental individuals.

High variation in PA expression within hybrid classes supports the conclusion that it most useful to study hybrid ecology and evolution by focusing on genotypic differences between individuals in hybrid populations, rather than fitness differ-

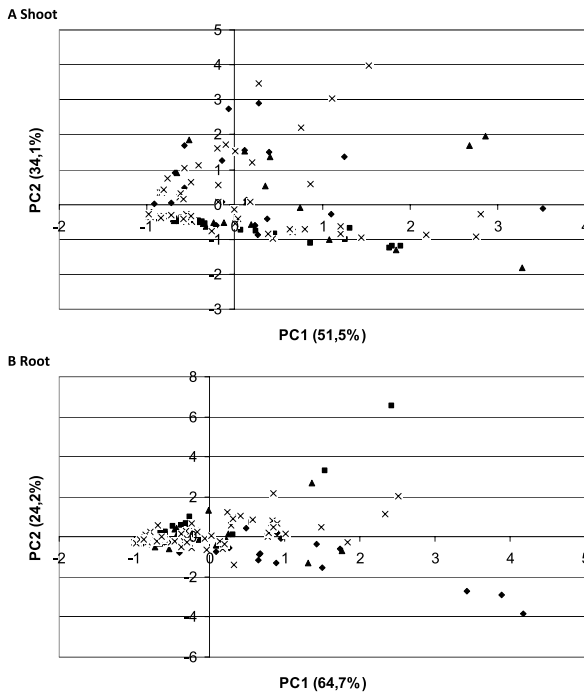


Figure 5 Plots of the two principal components accounting for most of the variation in PA composition in the shoots (A) and roots (B) of in a factorial design with two nutrient levels and three water levels. *Senecio jacobaea* (diamonds), *S. aquaticus* (squares), natural hybrids (triangles) and F_1 hybrids (crosses) are shown.

ences between hybrid classes (i.e., F_1 , F_2 , BC, etc.). Variation between hybrid individuals can be extremely high, and the fate of hybrid individuals and/or hybrid gene combinations may differ widely. In future studies, evaluating natural selection on different hybrid genotypes possessing different combinations of PAs may be extremely useful for elucidating the role of PA diversity in plants (i.e., Lexer *et al.*, 2003).

Also, hybridization between *S. jacobaea* and *S. aquaticus* may have different effects on herbivore/pathogen resistance in different environments, since PA concentrations are plastic depending species-specific reaction to soil nutrient and water content. For example, natural hybrids seemed to have lower PA concentrations than parental species in nutrient rich soils, which may reflect negative selection on PA concentration in nutrient rich habitats. It is well known that abiotic environmental factors such as light, water, and nutrient availability can alter the defensive ability of plants with regard to insect and pathogen resistance (Kytö *et al.*, 1996; Glynn *et al.*, 2003). Also, different pathogens and herbivores are present in different environments, and may thus constitute significantly different selection pressures in differing habitats. Researchers studying the effects of hybridization on secondary metabolite expression should incorporate abiotic factors occurring in parental and hybrid habitats, especially when abiotic factors differ greatly across habitats.

Overall, our findings indicate that hybridization can putatively lead to the production of novel PAs, and to the expression of extreme and/or unique PA compositions within hybrid individuals. Environmental conditions may play an important role in the resistance of hybrids to natural enemies, since species interact with environmental condition to determine expression of secondary metabolites. Though PAs are clearly involved with plant-parasite interactions, the consequences of increased PA diversity, concentration, and novelty through hybridization are not yet understood. Research into the ecological and adaptive consequences of unique PA patterns in hybrids may lead to an increased understanding of the role of hybridization in the evolution of plant resistance.

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7

Summary and conclusions

Heather Kirk

Exactly 150 years from the time that Darwin (1859) published his intriguing questions about the role of hybridization in evolutionary change (Chapter 1), the scientific community has come a long way towards finding answers. Natural hybridization is increasingly recognized as a process that may have important consequences for the ecology and evolution of natural plant populations and associated biological communities, and for the evolution of new traits and species. For example, recent studies of cottonwoods (*Populus*) have shown that hybrid genotypes have strong impacts on associated arthropod and mollusc communities (Wimp *et al.*, 2005; Albrechtsen *et al.*, 2007), such that hybrid zones may be sinks of biological diversity (Bangert *et al.*, 2005; Blair *et al.*, 2008). Furthermore, studies of plant genera including sunflowers (*Helianthus*) have shown that hybridization can generate key adaptations that can lead species diversification (Rieseberg *et al.*, 2003; Rieseberg, 2009).

Many reports of interspecific hybridization have arisen from the genus *Senecio*, and hybridization may play a particularly important role in the evolution of *Senecio* species. For example, the annual species *Senecio squalidus* is believed to have arisen from hybrids between *S. aethensis* and *S. chrysanthemifolius*. Hybrids between the latter species were transferred from a natural hybrid population on Mount Etna (Sicily) to the British Isles, where they established as a hybrid species (Abbott *et al.*, 2000, 2002). Similarly, hybridization between *S. vulgaris* and *S. squalidus* led to the formation of the polyploid species *S. cambrensis* and *S. eboracensis* in the British Isles (Abbott & Lowe, 2004). An elegant study by Kim *et al.* (2008) showed that a specific trait was passed on from one species to another. The introduction of *S. squalidus* in the UK led not only to the formation of two new hybrid species (*S. eboracensis* and *S. cambrensis*), but also passed on the trait ray florets to *Senecio vulgaris* (Kim *et al.*, 2008). Many other reports of hybridization between various *Senecio* species (e.g., *S. vulgaris* and *S. vernalis*; Comes, 1994; *S. germanicus*, *S. hercynicus*, and *S. ovatus*; Hodalova & Marhold, 1996; Hodalova, 2002; *S. keniodendron* and *S. keniensis*; Beck *et al.*, 1992) suggest that evolution via hybridization may be widespread within the genus.

In this thesis, I initiated a study on possible ecological and evolutionary consequences of hybridization between *S. jacobaea* and *S. aquaticus*. These species were selected because reports of hybridization between them are common, although their ecological distributions are quite different. *Senecio jacobaea* typically exhibits weedy growth, and inhabits disturbed, nutrient poor habitats. Conversely, *S. aquati-*

cus inhabits nutrient rich habitats that are subject to winter flooding and contain dense vegetation. Also, these species interact with different specialist and generalist herbivores in nature, and produce different compositions of pyrrolizidine alkaloids, secondary metabolites that are involved in plant interactions with natural enemies.

In order to test whether hybridization between these species could have significant ecological and/or evolutionary outcomes, I first undertook to confirm the existence of a natural hybrid population in the Netherlands (Chapter 2). I subsequently estimated the potential for natural hybrids to persist and reproduce in the natural population by estimating vegetative and reproductive fitness of artificial and natural hybrids in climate chamber experiments (Chapters 3 and 4). Lastly, I tested whether hybridization can potentially impact the expression, and ultimately the evolution of primary and secondary metabolites that may be involved in interactions with natural enemies (Chapters 5 and 6). Below, I summarize my findings regarding the ecological success and evolutionary potential of natural hybrids between *S. jacobaea* and *S. aquaticus*.

The ecology of natural *Senecio jacobaea* × *aquaticus* hybrids in the Zwanenwater reserve

Natural hybrids between *S. jacobaea* and *S. aquaticus* are present in the Zwanenwater reserve in the Netherlands (Chapter 2), and have likely existed as a significant population for at least 25 years (R van der Meijden, personal communication). Naturally high levels of back-crossing to *S. jacobaea*, as observed from molecular studies (Chapter 2), may result from passive mating of hybrids to *S. jacobaea*, which are more abundant than *S. aquaticus* in the vicinity of the hybrid swarm. Alternatively, selection may favour hybrid offspring that arise from *S. jacobaea* mothers, since such offspring may be more fit over a range of environmental than hybrid offspring arising from *S. aquaticus* mothers (Chapter 3). I also demonstrate that even highly back-crossed hybrids can possess a unique mixture of defence chemicals which combine PA's that are found only in either of the parental species (Chapter 2).

While I have not undertaken to study the dynamics of this population over the course of several years, it is clear that natural hybrids are, on average, at least as fit as *S. jacobaea* individuals in habitats in which they occur (Chapter 3). Reasonable levels of fitness in natural hybrids, combined with the ability of even early generation hybrids to reproduce successfully (Chapter 4), suggests that negative selection does not act strongly against hybrids in the hybrid zone, and that the hybrid population can potentially persist over a longer time period, or even expand to compete with parental individuals in dune areas and marshes where the latter occur.

The potential for hybrids to exceed their parents in terms of vegetative growth is high in F_1 hybrids, which are quite vigorous in relation to both parental species, and natural hybrids in most growth conditions (Chapter 3). If vigorous F_1 hybrids are formed frequently in nature, then their presence may have a significant impact on population dynamics of both parental species in the Zwanenwater reserve.

However, molecular studies indicated that intermediate, F_1 -like hybrid genotypes are relatively rare in the natural population (Chapter 2).

Maternal effects may also play a large role in the dynamics of the hybrid populations for at least two reasons. Firstly, (F_1) hybrids that arise from *S. jacobaea* mothers exhibit more vigorous growth than those that arise from *S. aquaticus* mothers (Chapter 3). If such maternal effects carry forward to later generations, backcrosses to *S. jacobaea* may be more successful than those to *S. aquaticus*, which may partially explain the high level of backcrossing to the former parental species observed in molecular studies (Chapter 2). Secondly, measurements of reproductive success in F_1 hybrids compared to parental species indicate that F_1 backcrosses to *S. aquaticus* produce more and larger seeds than do those to *S. jacobaea* (Chapter 4), which may partially counteract vegetative advantages enjoyed by hybrid offspring from *S. jacobaea* mothers. The possible role of maternal effects on hybrid swarm dynamics warrants additional field study.

Overall, the above results indicate that the hybrid zone in the Zwanenwater is not maintained by a balance between dispersal of hybrids into the zone followed by negative selection, as postulated by Barton & Hewitt's (1985) tension zone model. Although parental species are clearly distributed in habitats to which they are most adapted, there is little evidence that hybrids are relatively more fit compared to parental species in some environments than others (Chapter 3). Evidence from the studies described here indicates that ephemeral genetic effects, such as heterosis and maternal effects (Chapter 3), combined with passive dispersal and possibly genotype-specific selection on hybrid individuals (Chapter 4), may determine the genetic composition and geographical distribution of the hybrid population.

Evolutionary consequences

Variability among hybrid genotypes has been one of the recurring themes from the results presented in this thesis. Classically, many authors have sought to compare broad hybrid classes (i.e., F_1 , F_2 , BC) to each other and to parental species, without considering genotypic differences within classes (Campbell & Waser, 2001; Johnston *et al.*, 2001a; Hochwender & Fritz, 2004). However, from classical Mendelian genetics it is clear that within F_2 and BC plants segregation takes place. The variability in F_2 and BC increased by segregation, and through recombination new combinations of genes are produced. Therefore the variation in segregating F_2 populations and to a lesser extent BC populations are very interesting from evolutionary point of view. Indeed, authors have pointed out that variation among hybrid individuals can be quite high, and that such variation can form the basis of high adaptive potential among hybrid genotypes (Johnston *et al.*, 2001; Gross *et al.*, 2004).

Observations regarding reproductive success and phytochemistry in *S. jacobaea* $\times$ *aquaticus* hybrids support the conclusion that high variation precludes the ability to make wide-sweeping conclusions about the fitness of hybrids in relation to parental species (Chapter 4). The reproductive success of early generation hybrids is dependent on genetic interactions between crossing partners, such that reproductive success of an individual can only be measured with relation to the available mating pool. Furthermore, reproductive success of genotypes within hybrid classes

may be dependent on additional factors such as flower morphology (which is highly variable among genotypes (Kim *et al.*, 2008), and pollen competition (not investigated in this thesis).

Chapters 5 and 6 demonstrated that even F_1 hybrid genotypes can be highly variable suggesting that with regards to primary and especially secondary metabolite production, there is already a lot of genetic variation in both of the parents species. It may be expected that variation becomes even greater in segregating generations, due to transgressive segregation. Primary (Zangerl & Berenbaum, 2004) and secondary metabolites (O'Reilly-Wapstra *et al.*, 2005) play roles in interactions with a variety of natural enemies, in addition to being involved with numerous other processes. Our findings that hybridization can alter expression of several primary metabolites, as well as secondary metabolites including pyrrolizidine alkaloids, suggest that hybridization can potentially play a role in the evolution of metabolic expression.

Additionally, variation in ecologically important traits such as PA expression, flower morphology, and seed size can provide the basis for studies of natural selection on, or genetic regulation of such traits (i.e., Lexer *et al.*, 2003; Kim *et al.*, 2008; Leiss *et al.*, 2009). Interspecific hybrids can thus be a useful tool for further evolutionary studies.

Hybridization in *Senecio*

Reports of hybridization between species in the genus are common, and several *Senecio* species have directly arisen from hybridization events. Especially studies by Richard Abbott and colleagues have been instrumental in demonstrating the importance of hybridization between different species within this genus. Yet many other researchers have been unaware of, or have only paid lip service to, the possibility that hybridization can mediate ecological interactions or influence evolutionary diversification of *Senecio* species. While the overall role of hybridization in diversification within the genus has not been directly addressed by this thesis, it may be central to understanding the success of *Senecio* species compared to other plant genera.

A major barrier to identifying hybridization events leading to speciation in phylogenetic/evolutionary studies is that the tools for identifying the molecular footprints of hybridization have been, until recently, unavailable. Recent developments in this area have been perfected, and provide the tools for methodical identification of interspecific gene flow in speciation studies (Seehausen, 2004; Linder & Rieseberg, 2004; Kim *et al.*, 2008).

This study has initiated research on the hybrids between *S. jacobaea* and *S. aquaticus* in the Plant Ecology group in Leiden. P, F_1 and 130 F_2 lines of the cross between *S. jacobaea* and *S. aquaticus* have been established to study the variation in chemical, physiological and morphological characters. The evolutionary consequences of such variability and recombination in the F_2 can subsequently be studied in field and laboratory (Leiss *et al.*, 2009). A genetic map is under construction which will allow the study of the relationship between traits and to determine the number of underlying genes controlling such traits.

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Samenvatting

Precies 150 jaar nadat Darwin (1859) zijn fascinerende vragen over de rol van hybridisatie bij evolutionaire veranderingen (Hoofdstuk 1) heeft gepubliceerd, zijn wetenschappers goed op weg hierop de antwoorden te vinden. Natuurlijke hybridisatie wordt in toenemende mate gezien als een proces, met belangrijke gevolgen voor de ecologie en evolutie van natuurlijke plantenpopulaties en voor de levensgemeenschappen waarvan ze deel uitmaken, en bovendien voor de evolutie van nieuwe kenmerken en soorten. Recent onderzoek aan populieren toont bijvoorbeeld aan dat hybride genotypen een grote invloed hebben op de levensgemeenschappen van geleedpotigen en weekdieren die er in voorkomen (Wimp *et al.*, 2005; Albrechtsen *et al.*, 2007), en wel zodanig dat de zones met hybriden een reservoir kunnen zijn van biologische diversiteit (Bangert *et al.*, 2005; Blair *et al.*, 2008). Bovendien heeft onderzoek aan plantensoorten, waaronder zonnebloemen (*Helianthus*), aangetoond dat hybridisatie sleutelaanpassingen kan opleveren die tot soortsvorming kunnen leiden (Rieseberg *et al.*, 2003; Rieseberg, 2009).

Er zijn veel gevallen bekend van interspecifieke hybridisatie binnen het genus *Senecio*. Hybridisatie zou wel eens een zeer belangrijke rol kunnen spelen bij de evolutie van *Senecio*-soorten. Van de eenjarige soort *Senecio squalidus* wordt bijvoorbeeld gedacht dat zij is ontstaan uit een hybride van *S. aethensis* en *S. chrysanthemifolius*. Hybriden van de laatst genoemde soorten werden vanuit een natuurlijke populatie op de berg Etna (Sicilië) overgebracht naar de Britse eilanden waar ze zich als een hybridesoort vestigden (Abbott *et al.*, 2000, 2002). Op de zelfde wijze heeft hybridisatie tussen *S. vulgaris* en *S. squalidus* geleid tot het ontstaan van de polyploïde soorten *S. cambrensis* en *S. eboracensis* op de Britse eilanden (Abbott & Lowe, 2004). Een mooi onderzoek van Kim *et al.* (2008) heeft aangetoond dat een specifiek kenmerk van de ene naar de andere soort kan worden doorgegeven. De introductie van *S. squalidus* in Engeland heeft niet alleen de vorming van twee nieuwe hybridesoorten (*S. eboracensis* en *S. cambrensis*) tot gevolg gehad, maar heeft ook lintbloemen als eigenschap aan *S. vulgaris* doorgegeven (Kim *et al.*, 2008). Diverse andere artikelen over hybridisatie tussen verschillende *Senecio*-soorten (e.g., *S. vulgaris* en *S. vernalis*; Comes, 1994; *S. germanicus*, *S. hercynicus* en *S. ovatus*; Hodalova & Marhold, 1996; Hodalova, 2002; *S. keniodendron* en *S. keniensis*; Beck *et al.*, 1992) geven de indruk dat evolutie via hybridisatie binnen dit genus zeer veel voorkomt.

In dit proefschrift rapporteer ik over mijn studie naar de mogelijk ecologische en evolutionaire consequenties van hybridisatie tussen *S. jacobaea* en *S. aquaticus*.

Voor deze soorten is gekozen omdat, ondanks de grote verschillen in ecologische verspreiding, er veel gevallen van hybridisatie tussen beide soorten bekend zijn. *Senecio jacobaea* komt voornamelijk voor in verstoorde en voedselarme habitats. *Senecio aquaticus* daartegen groeit voornamelijk in voedselrijke habitats die in de winter kunnen overstromen en een dichte vegetatie bezitten. Daarnaast worden deze soorten belaagd door verschillende specialistische en generalistische herbivoren en maken ze verschillende pyrrolizidine alkaloiden. Dat zijn secundaire plantenstoffen die een rol spelen bij hun interactie met natuurlijke vijanden.

Om te kunnen nagaan of hybridisatie tussen deze soorten belangrijke ecologische en/of evolutionaire gevolgen heeft, ben ik begonnen om vast te stellen of natuurlijke populaties van hybriden in Nederland voorkomen (Hoofdstuk 2). Vervolgens heb ik het vermogen van natuurlijke hybriden om zich blijvend te vestigen en te reproduceren in de natuurlijke populatie bepaald door een schatting te maken van de vegetatieve en reproductieve fitness van gekweekte en natuurlijke hybriden in een klimaatcel (Hoofdstuk 3 en 4). Tenslotte heb ik getest of hybridisatie van invloed is op de expressie, en uiteindelijk op de evolutie van primaire en secundaire metabolieten die een rol kunnen spelen bij hun interactie met natuurlijke vijanden (Hoofdstuk 5 en 6). Hieronder vat ik samen wat ik heb gevonden over het ecologische succes en de evolutionaire potentie van natuurlijke hybriden tussen *S. jacobaea* en *S. aquaticus*.

De ecologie van natuurlijke *Senecio jacobaea* × *aquaticus* hybriden in het natuurreservaat het Zwanenwater

Natuurlijke hybriden tussen *S. jacobaea* en *S. aquaticus* komen voor in het Zwanenwater (Hoofdstuk 2), en naar alle waarschijnlijkheid is er al ruim 25 jaar een aanzienlijke populatie aanwezig (R van der Meijden, persoonlijke mededeling). Een van nature hoog niveau van terugkruising naar *S. jacobaea*, zoals blijkt uit moleculair onderzoek (Hoofdstuk 2), kan het resultaat zijn van passieve kruising van de hybriden met *S. jacobaea*, die algemener zijn dan *S. aquaticus* in de buurt van de hybridenzwermen. Maar het is ook mogelijk dat er selectie plaatsvindt van hybride nakomelingen met een *S. jacobaea* moeder, omdat deze misschien een hogere fitness bezitten onder de heersende omgevingsfactoren dan hybriden met een *S. aquaticus* moeder (Hoofdstuk 3). Verder toon ik aan dat zelfs sterk teruggekruiste hybriden over een unieke mix van afweerstoffen kunnen beschikken die een combinatie vormen van PA's van beide oudersoorten (Hoofdstuk 2).

Hoewel ik geen onderzoek naar de dynamiek van deze populatie gedurende meerdere jaren heb gedaan, is het wel duidelijk dat natuurlijke hybriden over het algemeen op zijn minst een even hoge fitness hebben als de *S. jacobaea* individuen waarmee zij in een habitat voorkomen (Hoofdstuk 3). Een redelijk niveau van fitness van natuurlijke hybriden in combinatie met het vermogen van vroege-generatie hybriden om succesvol tot reproductie te komen (Hoofdstuk 4), suggereert dat er geen sterke negatieve selectie plaatsvindt tegen hybriden in de hybridenzone. Het suggereert ook dat de hybridenpopulatie zich potentieel over een langere periode kan handhaven of zelfs uitbreiden in concurrentie met individuen van de oudersoorten in duinen en natte weilanden.

Het vermogen van hybriden om het beter te doen dan hun ouders wat betreft vegetatieve groei is duidelijk aanwezig bij de F_1 hybriden. Het zijn onder de meeste omstandigheden krachtige groeiers in vergelijking met beide oudersoorten en zelfs de natuurlijke hybriden (Hoofdstuk 3). Als groeikrachtige F_1 hybriden in de natuur vaak gevormd worden, dan zal hun aanwezigheid een significant effect op de populatiedynamiek van beide oudersoorten in het Zwanenwater hebben. Uit ons moleculaire onderzoek blijkt echter dat intermediaire, op F_1 hybriden lijkende genotypen, relatief zeldzaam zijn in de natuurlijke populatie (Hoofdstuk 2).

Er zijn minstens twee redenen waarom maternale effecten een grote rol kunnen spelen bij de dynamiek van hybride populaties. Ten eerste zijn (F_1) hybride nakomelingen van *S. jacobaea*-moederplanten veel groeikrachtiger dan die van *S. aquaticus*-moederplanten (Hoofdstuk 3). Als zulke maternale effecten worden overgedragen naar volgende generaties, dan kunnen terugkruisingen naar *S. jacobaea* succesvoller zijn dan die naar *S. aquaticus*. Dit kan een verklaring zijn voor het hoge niveau van terugkruising naar de oorspronkelijke oudersoort zoals blijkt uit de moleculaire studies (Hoofdstuk 2). Ten tweede hebben metingen van het reproductieve succes van F_1 hybriden in vergelijking tot hun oudersoorten laten zien dat F_1 terugkruisingen naar *S. aquaticus* meer en grotere zaden produceren dan die naar *S. jacobaea* (Hoofdstuk 4), wat de vegetatieve voordelen die een hybride nakomeling van een *S. jacobaea* moeder ondervindt gedeeltelijk tegenwerkt. Voor vaststellen van de mogelijke rol van maternale effecten op de dynamiek van hybride zwermen is dan ook aanvullend veldonderzoek nodig.

Samenvattend laten de bovenstaande resultaten zien dat de hybridenzone in het Zwanenwater niet in stand wordt gehouden via een evenwicht tussen verspreiding van hybriden in de zone gevolgd door een negatieve selectie zoals wordt voorgesteld door Barton & Hewitt's (1985) in hun 'tension zone model'. Hoewel de oudersoorten duidelijk voorkomen in het voor hen meest geschikte habitat, zijn er weinig aanwijzingen dat hybriden in vergelijking met de oudersoorten in sommige omgevingen relatief fitter zijn dan in andere. De hier beschreven studies geven de indicatie dat efemere genetische effecten, zoals heterose en maternale effecten (Hoofdstuk 3), in combinatie met passieve verspreiding en mogelijk genotype-specifieke selectie op hybride individuen (Hoofdstuk 4), de genetische compositie en geografische distributie van de hybridenpopulatie bepalen.

Evolutionaire consequenties

Variabiliteit in hybride genotypen is één van de terugkerende thema's in de resultaten die in dit proefschrift worden gepresenteerd. Veel auteurs hebben geprobeerd om klassen van hybriden (bijvoorbeeld F_1 , F_2 , BC) onderling en met hun oudersoorten te vergelijken, zonder rekening te houden met genotypische variatie binnen deze klassen (Campbell & Waser, 2001; Johnston *et al.*, 2001a; Hochwender & Fritz, 2004). Vanuit de klassieke Mendeliaanse genetica gezien, is het duidelijk dat binnen F_2 en BC planten segregatie plaatsvindt. De variatie binnen de F_2 en BC wordt vergroot door segregatie, en door recombinatie ontstaan nieuwe combinaties van genen. Dat maakt de variatie in segregerende F_2 -populaties, en in mindere mate in BC populaties vanuit een evolutionair oogpunt zeer interessant. Zo hebben diverse

auteurs er op gewezen dat de variatie tussen hybride individuen erg groot kan zijn en dat deze variatie de basis van het grote adaptatievermogen van hybride genotypen kan zijn (Johnston *et al.*, 2001; Gross *et al.*, 2004).

Waarnemingen met betrekking tot het reproductieve succes en de fytochemie van *S. jacobaea* × *S. aquaticus* hybriden ondersteunen de conclusie dat het door de grote variatie onmogelijk is om een allesomvattende conclusie over de fitness van hybriden in relatie tot de oudersoorten te trekken (Hoofdstuk 4). Het reproductieve succes van vroege-generatie hybriden is afhankelijk van de genetische interactie tussen de kruisingsouders, in die zin dat het reproductiesucces van een individu alleen gemeten kan worden in relatie tot de beschikbare pool van individuen waarmee kruising kan plaatsvinden. Verder kan het reproductiesucces van genotypen binnen een hybride klasse afhangen van aanvullende factoren zoals bloem-morfologie (die zeer variabel is tussen genotypen; Kim *et al.*, 2008) en concurrentie om pollen (in dit proefschrift niet onderzocht).

De hoofdstukken 5 en 6 tonen aan dat zelfs F_1 -hybride genotypen zeer variabel kunnen zijn, wat er op wijst dat in de productie van primaire en vooral secundaire metabolieten, al veel genetische variatie bij de beide oudersoorten aanwezig moet zijn. Verwacht mag worden dat de variatie in segregerende generaties zelfs nog groter zal worden door middel van transgressieve segregatie. Behalve dat primaire (Zangerl & Berenbaum, 2004) en secundaire metabolieten (O'Reilly-Wapstra *et al.*, 2005) een belangrijke rol spelen bij de interactie met veel verschillende natuurlijke vijanden, zijn zij betrokken bij een groot aantal andere processen. We hebben gevonden dat hybridisatie de expressie van verschillende primaire en secundaire metabolieten, waaronder pyrrolizidine alkaloiden, kan veranderen. Dit wijst erop dat hybridisatie een belangrijke rol kan spelen bij de evolutie van metabolische expressie.

Aanvullend hierop, kan de variatie in ecologisch belangrijke kenmerken zoals PA expressie, bloemmorfologie en zaadgrootte, de basis vormen voor studies naar de natuurlijke selectie of genetische regulatie van deze kenmerken (bijvoorbeeld Lexer *et al.*, 2003; Kim *et al.*, 2007; Leiss *et al.*, 2009). Inter-specifieke hybriden kunnen zo een belangrijk instrument voor verdere evolutionaire studies vormen.

Hybridisatie in *Senecio*

Er zijn talrijke meldingen van hybridisatie tussen soorten binnen het genus *Senecio* en diverse *Senecio*-soorten zijn ontstaan als gevolg van hybridisatie. De studies van Richard Abbott en zijn collega's in het bijzonder, zijn zeer nuttig geweest om het belang van hybridisatie tussen verschillende soorten binnen dit genus te demonstreren. Veel andere onderzoekers zijn zich echter niet of onvoldoende bewust geweest van het belang van hybridisatie voor ecologische interacties en voor de diversificatie van *Senecio*-soorten. Hoewel de totale rol die hybridisatie speelt in de soortsvorming binnen het genus hier niet besproken is, zou die rol wel eens cruciaal kunnen zijn om te begrijpen waarom *Senecio*-soorten zo succesvol zijn in vergelijking met andere genera.

Een grote barrière om de rol van hybridisatie vast te stellen bij soortsvorming in fylogenetische/evolutionaire studies, was dat er tot voor kort geen instrumenten

beschikbaar waren om moleculaire aanwijzingen van hybridisatie te herkennen. Door recente ontwikkelingen op dit gebied zijn er nu sterk verbeterde methoden beschikbaar die systematische identificatie van interspecifieke geneflow in speciatie studies mogelijk maken (Seehausen, 2004; Linder & Rieseberg, 2004; Kim *et al.*, 2008).

Deze studie is de aanzet geweest voor onderzoek naar hybriden tussen *S. jacobaea* en *S. aquaticus* door de onderzoeksgroep Plantenecologie (Ecology and Phytochemistry) in Leiden. Van de kruising tussen *S. jacobaea* en *S. aquaticus* zijn P, F₁ en 130 F₂-lijnen gemaakt die worden aangehouden om de variatie in chemische, fysiologische en morfologische kenmerken te bestuderen. De evolutionaire consequenties van een dergelijke variabiliteit en recombinatie in de F₂-generatie kunnen op deze manier zowel in het veld als in het laboratorium worden bestudeerd (Leiss *et al.*, 2009). Er wordt een genetische kaart gemaakt die het mogelijk zal maken om de relatie tussen verschillende kenmerken te bestuderen en om het aantal genen dat bepalend is voor deze kenmerken te achterhalen.

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Curriculum vitae

I was born on July 18, 1980, in Toronto, Ontario, in Canada. After graduating from Ajax High School (Ajax, Ontario, Canada) in 1998, I attended both the University of Guelph and the University of Toronto for undergraduate studies, where I developed an interest in ecology and evolution. A field ecology course that took me to Nicaragua especially stimulated my interests. I graduated with high distinction from the University of Toronto (BSc) in 2001.

After moving to the Netherlands in 2001, I applied to the Masters program at Leiden University as an international student. After carefully considering the available programs, I chose to undertake my studies in the Plant Ecology section, which offered a variety of fascinating student projects. I started my Masters studies in September 2002, and completed the requirements of the program in March, 2004. I had the good fortune of having three supervisors for my Masters project, including Klaas Vrieling, Peter Klinkhamer, and Ed van der Meijden, who exposed me to a variety of knowledge regarding plant ecology, molecular biology, and plant chemical defense mechanisms. During the summer of 2003, I was offered a three-month internship at CABI Biosciences, where I worked under Urs Schaffner to test the specificity of several specialist flea beetle species on *Senecio jacobaea* and a number of its close relatives. The aim of this project was to verify the usefulness of these specialist insects as biological control agents.

Upon completion of my MSc. studies in 2004, I was offered a position as a PhD-student in the plant ecology section at Leiden University, which allowed me to carry on the work that I initiated during my MSc. studies. This work was very diverse in nature, and allowed me to develop my skills in various aspects of biology. I conducted AFLP studies in the molecular lab, and learned various chemical techniques including gas chromatography, and metabolomic profiling using NMR spectroscopy.

From the fall of 2009, I am employed on a part-time basis as a college instructor at Sir Sanford Fleming College (Lindsay, Ontario, Canada) in the school of Environmental and Natural Resource Sciences. I also run my own freelance business for coaching equestrian sports, and currently coach approximately 40 students.

SCIENTIFIC CONTRIBUTIONS

Publication list

- Kirk H, Macel M, Klinkhamer PGL, Vrieling K. 2004. Natural hybridization between *Senecio jacobaea* and *Senecio aquaticus*: molecular and chemical evidence. *J Mol Ecol* 13: 2267-2274.
- Kirk H, Vrieling K, Klinkhamer PGL. 2005. Heterosis and maternal effects influence the fitness of plant hybrids. *New Phytol* 166: 685-694.
- Kirk H, Choi YH, Kim HK, Verpoorte R, van der Meijden E. 2005. Comparing Metabolomes: a search for evidence that hybridization can lead to adaptive change in plant chemistry. *New Phytologist* 167: 613-622.
- Kirk H, Vrieling K, Klinkhamer PGL. 2005. Reproductive fitness of hybrids between *Senecio jacobaea* and *S. aquaticus*. *Am J Bot* 92: 1467-1473.

Conferences and workshops

- Schaffner U, Kirk H, Pelser P, Vrieling K. 2004. What determines resistance to specialist herbivores? A case study with the plant genus *Senecio* and *Longitarsus* flea beetles. Entomological Society of America Meeting in Salt Lake City.
- Kirk H, Vrieling K, Klinkhamer PGL. 2005. Heterosis and maternal effects influence the fitness of plant hybrids. Workshop on Natural Hybridization and Introgression in Skodobo, Denmark.

Scholarships

2004. Natural Sciences and Engineering Research Council of Canada (NSERC) postgraduate (doctoral) scholarship. Value: \$21 000 yearly for three years.
2003. Three month fellowship with the Max Planck Institute of Chemical Ecology (Jena, Germany). Value: 715 Euros monthly plus living expenses.
2001. University of Toronto, Woodsworth College Graduate Book Prize. Value: \$100.
1998. Canadian Federation of University Women Achievement Award. Value: \$1000.
1998. University of Guelph Entrance Award. Value: \$5000.
1998. Monarch Youth Scholarship. Value: \$5000.