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

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Abstract

Hybridization is known to be involved in a number of evolutionary processes, including species formation, and the generation of novel defence characteristics in plants. The genus *Senecio* of the Asteraceae family is highly speciose and has historically demonstrated significant levels of interspecific hybridization. The evolution of novel chemical defence characteristics may have contributed to the success of *Senecio* hybrids. Chemical defence 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 amplified fragment length polymorphisms (AFLPs) and pyrrolizidine alkaloid (PA) composition to confirm that natural hybridization occurs between *S. jacobaea* and the closely related species *S. aquaticus*. AFLPs are also used to estimate the ancestry of hybrids. We also demonstrate that even highly back-crossed hybrids can possess a unique mixture of defence 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 defence and resistance.

Keywords: amplified fragment length polymorphisms (AFLPs), chemical defence, hybridization, pyrrolizidine alkaloids, *Senecio aquaticus*, *Senecio jacobaea*

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Introduction

Early theories regarded hybridization events as counterproductive to speciation, creating gene flow between otherwise divergent clades. However, recent estimates indicate that plant hybridization events are at the root of 30–80% 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 defences (Fritz *et al*. 1994; Orians 2000) and tolerance to damage (Hochwender *et al*. 2000), and in broader studies of species formation (see Barton & Hewitt 1985; Arnold 1997).

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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 interspecific 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). In addition, 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 defence 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), defence compounds that are known for their toxic and repellant effects on vertebrate (Cheeke 1988) and invertebrate

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(Macel 2003) herbivores. In particular, *S. jacobaea* L. has been used as a model organism in studies of the effects of PA 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 defence evolution.

A candidate hybrid system includes *S. jacobaea* and *S. aquaticus* Hill. *Senecio 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). *Senecio 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 back-crossing between a number of plant and animal species (see Pooler & Riedel 2002; Teo *et al*. 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 & Ellstrand 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 defence 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. *Senecio jacobaea* individuals were collected from sand dunes at least 300 m 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 (Chéreng, $n = 1$; second population, $n = 2$), Switzerland (L'Himelette, $n = 2$), and Germany ($n = 2$). *Senecio 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 16 h, temperature 20 °C, relative humidity 100%). Approximately 1 week after germination, seedlings were planted in potting soil and were grown a climate room for 6–10 weeks (light 16 h, temperature 20 °C/15 °C, relative humidity 70%).

Molecular analyses

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

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

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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 plc) 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 *Mse*I and *Eco*RI 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 pre-amplified using one selective nucleotide with each AFLP primer (A for *Eco*RI primer, and C for *Mse*I 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, reference *S. aquaticus* and *S. jacobaea* were analysed (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 milligrams 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) as an internal standard and analysed using gas chromatography. Conditions (injector 250 °C, temperature program 0-22-5-250, split mode 1–30, carrier gas N_2 0.9 mL/min, pressure 56 kPa; Nitrogen-Phosphor vs detector) were controlled by a Hewlett Packard gas chromatographer (model 6890). Gas chromatograph 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 to 500 bases with a fluorescent intensity greater than 50 were scored as present. Bands were scored 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 henceforth 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 the 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 analysis of variance (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 (d.f. = 1, $F = 3.846$, $P = 0.189$), and no interaction between sampling location and analysis (d.f. = 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

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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 *Senecio 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, eight were confirmed as hybrids based on diagnostic AFLP bands (Fig. 1B).

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 the ancestry of each individual, expressed as the proportion derived from each parental cluster (which we will refer to from here 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

A

Hybrid individual	H10a	H7a	H7ia	H3a	H12a	H5a*	H6a*	H9a*	H1a*	H15a*	H5ia*	H7iia*	H3ia*	H8a*	H4ia*	H13a*	H6ia*	H8ia*	H3iia*	H2a*
Inferred proportion from *S. jacobaea* 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																				

Fig. 1 Presence and absence of *Senecio aquaticus* and *S. jacobaea* diagnostic pyrrolizidine alkaloids (PA) and amplified fragment length polymorphism (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 the proportion of individuals derived from *S. jacobaea*, is indicated for each individual (columns). Rows represent markers. Note that PA composition was not measured for analysis 1. *indicates a hybrid on the basis of AFLP bands; **a hybrid on the basis of PAs; and ***a hybrid on the basis of both AFLP bands and PAs.

B

Hybrid Individual	H1b	H2b	H3b**	H4b	H5b	H6b	H7b**	H9b*	H10b***	H11b*	H14b*	H15b*	H12b***	H13b*	H8b***
Inferred proportion from *S. jacobaea* 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															
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CTG/ACA 250															
CTG/ACA 357															
CTG/ACA 355															
CTG/ACA 322															
CTG/AGG 307															
CTG/AGG 210															

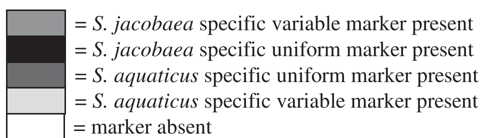

Fig. 1 *Continued*

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

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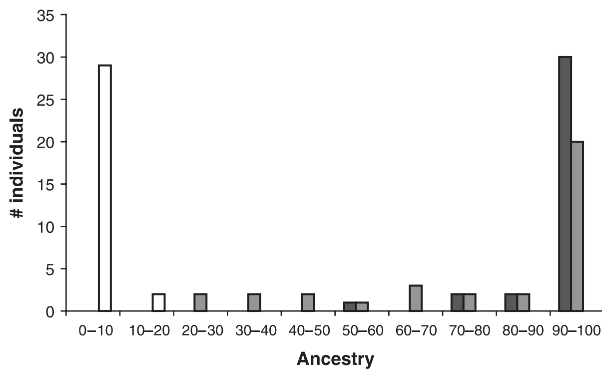

Fig. 2 Histogram of ancestry of putative *Senecio jacobaea* (black bars), hybrids (grey bars), and *S. aquaticus* (white bars). Ancestry (*x*-axis) is expressed as percentage of genome derived from the *S. jacobaea* cluster.

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) were missing uniform *S. jacobaea*-specific markers and/or possessed *S. aquaticus*-specific markers but were almost entirely derived from *S. jacobaea* (> 99%). Conversely, H9b was mostly derived from *S. aquaticus* according to Bayesian analysis but possessed a considerable number of *S. jacobaea*-specific markers and no *S. aquaticus*-specific markers. Such results may occur as a result of stochastic inheritance of the diagnostic markers identified in our study.

PA analysis

We considered 17 different PAs in our analysis (Table 1). Eleven potential PAs were discarded from our analysis because they were very rare among sampled individuals and therefore not informative, or because only trace amounts were detected by gas chromatography 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

Table 1 Pyrrolizidine alkaloids (PA) (with gas chromatograph retention times) included in PA analysis

Retention time	Pyrrolizidine alkaloid	*Senecio jacobaea*	*Senecio aquaticus*	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

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.

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 the 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, because 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 the 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 *Senecio jacobaea* and *S. aquaticus* are common in the 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 300 m 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 interspecific 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 the 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 Orian (2000) indicates, both qualitative and quantitative variation in the 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 back-crossed hybrid individuals is also significant from the perspective of hybrid ecology and the evolution of chemical defences. Many authors postulate that enhanced defence 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 the causes of the Zwanenwater hybrid swarm persistence and the evolutionary potential of *Senecio* hybrids for the development of new defence characteristics.

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References

Abbott RJ, James JK, Irwin JA, Comes HP (2000) Hybrid origin of the Oxford Ragwort, *Senecio squalidus* L. *Watsonia*, **23**, 123–138.

Andersson S (2001) The genetic basis of floral variation in *Senecio jacobaea* (Asteraceae). *Journal of Heredity*, **92**, 409–414.

Arnold ML (1997) *Natural Hybridization and Evolution*. Oxford University Press, New York.

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. *Annual Review of Ecology, Evolution, and Systematics*, **16**, 113–148.

Beck E, Scheibe R, Schlutter I, Sauer W (1992) *Senecio* × *Saundersii* Sauer and Beck (Asteraceae), an intermediate hybrid between *S. keniodendron* and *S. keniensis* of Mt Kenya. *Phyton-Annales Rei Botanicae*, **32**, 9–38.

Benoit PM, Crisp PC, Jones BMG, Stace CA (1975) *Hybridization and the Flora of the British Isles*, pp. 404–405. Academic Press for the Botanical Society of the British Isles, London.

Boecklen WJ, Howard DJ (1997) Genetic analysis of hybrid zones: numbers of markers and power of resolution. *Ecology*, **78**, 2611–2616.

Chater AO, Walters SM (1976) In: *Flora Europaea* (eds Tutin TG, Heywood VH, Burgers NA, Moore DM, Valentine DH, Walters SM, Webb DA), Vol. 4, pp. 191–205. Cambridge University Press, Cambridge.

Cheeke PR (1988) Toxicity and metabolism of pyrrolizidine alkaloids. *Journal of Animal Science*, **66**, 2343–2350.

Christov VS, Mikhova BP, Evstatieva LN (2002) Alkaloids from *Senecio aquaticus*. *Fitoterapia*, **73**, 171–173.

Comes HP (1994) The occurrence of *Senecio vulgaris* L. var *vulgaris* and *S.* × *helwingii* (*S. vulgaris* × *S. vernalis* Waldst *et* Kit) in Israel. *Israel Journal of Plant Sciences*, **42**, 195–213.

De Boer NJ (1999) Pyrrolizidine alkaloid distribution in *Senecio jacobaea* minimizes losses to generalist feeding. *Entomologia Experimentalis et Applicata*, **91**, 169–173.

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation, Version II. *Plant Molecular Biology Reporter*, **1**, 19–21.

Ellstrand NC, Whitkus R, Riesberg LH (1996) Distribution of spontaneous plant hybrids. *Proceedings of the National Academy of Sciences of the USA*, **93**, 5090–5093.

Fritz RS (1999) Resistance of hybrid plants to herbivores: genes, environment, or both? *Ecology*, **80**, 382–391.

Fritz RS, Nichols-Orians CM, Brunsfeld SJ (1994) Interspecific hybridization of plants and resistance to herbivores: hypotheses, genetics, and variable responses in a diverse herbivore community. *Oecologia*, **97**, 106–117.

Fritz RS, McDonough SE, Rhoads AG (1997) Effects of plant hybridization on herbivore–parasitoid interactions. *Oecologia*, **110**, 360–367.

Fritz RS, Moulia C, Newcombe G (1999) Resistance of hybrid plants and animals to herbivores, pathogens, and parasites. *Annual Review of Ecology and Systematics*, **30**, 565–591.

Harris SA, Ingram R (1992) Molecular systematics of the genus *Senecio*-L. 1. Hybridization in a British polyploid complex. *Heredity*, **69**, 1–10.

Hartmann T, Zimmer M (1986) Organ specific distribution and accumulation of pyrrolizidine alkaloids during the life history of two annual *Senecio* species. *Plant Physiology*, **112**, 67–80.

Hartmann T, Witte L (1995) Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In: *Alkaloids: Chemical and Biology Perspectives* (ed. Pelletier SW), Vol. 9, pp. 156–233. Pergamon Press, Elmsford, New York.

Hochwender CG, Fritz RS, Orians CM (2000) Using hybrid systems to explore the evolution of tolerance to damage. *Evolutionary Ecology*, **14**, 509–521.

Hodalova I (2002) A new hybrid *Senecio* × *slovacus* from the *S-nemorensis* group (Compositae) in the West Carpathians. *Biologia*, **57**, 75–82.

Hodalova I, Marhold K (1996) Sympatric populations of *Senecio ovatus* subsp *ovatus*, *S. germanicus* subsp *germanicus* (Compositae) and their hybrid in the Carpathians and the adjacent part of Pannonia. 1. Multivariate morphometric study. *Flora*, **191**, 283–290.

Hol WHG, van Veen JA (2002) Pyrrolizidine alkaloids from *Senecio jacobaea* affect fungal growth. *Journal of Chemical Ecology*, **28**, 1763–1772.

Lowe AJ, Abbott RJ (2000) Routes of origin of two recently evolved hybrid taxa: *Senecio vulgaris* var. *Hibernius* and Your radiate groundsel (Asteraceae). *American Journal of Botany*, **87**, 1159–1167.

Macel M (2003) On the evolution of the diversity of pyrrolizidine alkaloids: the role of insects as selective forces. Phd Thesis, University of Leiden.

Macel M, Klinkhamer PGL, Vrieling K, van der Meijden E (2002) Diversity of pyrrolizidine alkaloids in *Senecio* species does not affect the specialist herbivore *Tyria jacobaea*. *Oecologia*, **133**, 541–550.

Orians CM (2000) The effects of hybridization in plants on secondary chemistry: implications for the ecology and evolution of plants and herbivores. *American Journal of Botany*, **87**, 1749–1756.

Pelser PB, Gravendeel B, van der Meijden R (2003) Phylogeny reconstruction in the gap between too little and too much divergence: the closest relatives of *Senecio jacobaea* (Asteraceae) according to DNA sequences and AFLPs. *Molecular Phylogenetics and Evolution*, **29**, 613–628.

Pooler MR, Riedel LGH (2002) Molecular markers used to verify interspecific hybridization between hemlock (*Tsuga*) species. *Journal of American Horticultural Science*, **127**, 623–627.

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

Rieseberg LH, Ellstrand NC (1993) What can molecular and morphological markers tell us about plant hybridization. *Critical Reviews in Plant Sciences*, **12**, 213–241.

Teo LL, Kiew R, Set O, Lee K, Gan YY (2002) Hybrid status of kuwini, *Mangifera odorata Griff.* (Anacardiacaea) verified by amplified fragment length polymorphism. *Molecular Ecology*, **11**, 1465–1469.

Vos P, Hogers R, Bleeker M, *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Vrieling K, de Vos H, van Wijk AM (1993) Genetic analysis of the concentrations of pyrrolizidine alkaloids in *Senecio jacobaea*. *Phytochemistry*, **32**, 1141–1144.

Vrieling K, Saumitou-Laprade P, Cuguen J, van Dijk H, de Jong TJ, Klinkhamer PGL (1999) Direct and indirect estimates of the selfing rate in small and large individuals of the bumblebee pollinated *Cynoglossum officinale* L. (Boraginaceae). *Ecology Letters*, **2**, 331–337.

Research presented here is part of a Phd. thesis project conducted by Heather Kirk. Klaas Vrieling is primarily interested in molecular population genetics and plant-herbivore interactions. Peter Klinkhamer is principally interested in the evolution of plant reproduction and plant defense systems. Mirka Máčel is involved in research relating to plant-insect interactions and soil ecology.