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)


**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 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). 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 and 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 & Ellstrand, 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 & 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 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 (Cham, n = 1), France (Chereng , 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 Msel 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 Msel 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 N₂ 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).

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**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 (***)
### Natural hybridization

#### Hybrid individual

<table>
<thead>
<tr>
<th>Inferred proportion from S. jacobaea ancestry</th>
<th>HIa⁺</th>
<th>HIb⁺</th>
<th>HIf⁺</th>
<th>HIa⁻</th>
<th>HIb⁻</th>
<th>HIf⁻</th>
<th>HIa⁺⁺</th>
<th>HIb⁺⁺</th>
<th>HIf⁺⁺</th>
<th>HIa⁻⁻</th>
<th>HIb⁻⁻</th>
<th>HIf⁻⁻</th>
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<td>997</td>
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</tbody>
</table>

**APFL Markers:** Primer combination and marker identity (# bases)

- CAG/AACA 109
- CAG/AACA 218
- CAG/AACA 327
- CAG/AACA 336
- CAG/AACA 445
- CAG/AACA 466
- CAG/AACA 637
- CAG/AACA 111
- CAG/AACA 164
- CAG/AACA 325
- CAG/AACA 564
- CAG/AACA 125
- CAG/AACA 144
- CAG/AACA 234
- CAG/AACA 424
- CAG/AACA 426
- CAG/AACA 449
- CAG/AACA 546
- CAG/AACA 457
- CAG/AACA 483

#### Hybrid Individual

<table>
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<tr>
<th>Inferred proportion from S. jacobaea ancestry</th>
<th>HIa⁺</th>
<th>HIb⁺</th>
<th>HIf⁺</th>
<th>HIa⁻</th>
<th>HIb⁻</th>
<th>HIf⁻</th>
<th>HIf*</th>
<th>HIa⁺⁺</th>
<th>HIb⁺⁺</th>
<th>HIf⁺⁺</th>
<th>HIa⁻⁻</th>
<th>HIb⁻⁻</th>
<th>HIf⁻⁻</th>
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<tr>
<td>992</td>
<td>996</td>
<td>993</td>
<td></td>
<td>994</td>
<td>995</td>
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<td>993</td>
<td>994</td>
<td>995</td>
<td>993</td>
<td></td>
</tr>
</tbody>
</table>

**PA Markers:** Retention time and PA location in plant (roots r, shoot s)

- 8.15 s    r     -     -     -     -     -     -     -     -     -     -     -     -
- 10.20 s   r     -     -     -     -     -     -     -     -     -     -     -     -
- 10.53 s   r     -     -     -     -     -     -     -     -     -     -     -     -
- 13.18     r     -     -     -     -     -     -     -     -     -     -     -     -

**APFL Markers:** Primer combination and marker identity (# bases)

- CAG/AACA 481
- CAG/AACA 470
- CAG/AACA 507
- CAG/AACA 437
- CAG/AACA 405
- CAG/AACA 370
- CAG/AACA 352
- CAG/AACA 262
- CAG/AACA 255
- CAG/AACA 241
- CAG/AACA 218
- CAG/AACA 209
- CAG/AACA 177
- CAG/AACA 146
- CAG/AACA 140
- CAG/AACA 334
- CAG/AACA 213
- CAG/AACA 200
- CAG/AACA 156
- CAG/AACA 126
- CAG/AACA 106
- CAG/AACA 381
- CAG/AACA 315
- CAG/AACA 253
- CAG/AACA 132
- CAG/AACA 124
- CAG/AACA 335
- CAG/AACA 250
- CAG/AACA 357
- CAG/AACA 387
- CAG/AACA 355
- CAG/AACA 322
- CAG/AACA 307
- CAG/AACA 210

Legend:
- = S. jacobaea specific variable marker present
- = S. jacobaea specific uniform marker present
- = S. aquaticus specific uniform marker present
- = S. aquaticus 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
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, shoots, or both (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.

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**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

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