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The Relationship between Structurally Different Pyrrolizidine Alkaloids and Western Flower Thrips Resistance in F₂ Hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*

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Segregating plant hybrids often have more ecological and molecular variability compared to parental species, and are therefore useful for studying relationships between different traits, and the adaptive significance of trait variation. Hybrid systems have been used to study the relationship between the expression of plant defense compounds and herbivore susceptibility. We conducted a western flower thrips (WFT) bioassay using a hybrid family and investigated the relationship between WFT resistance and pyrrolizidine alkaloid (PA) variation. The hybrid family consisted of two parental (*Jacobaea vulgaris* and *Jacobaea aquatica*) genotypes, two F₁ genotypes, and 94 F₂ hybrid lines. The *J. aquatica* genotype was more susceptible to thrips attack than the *J. vulgaris* genotype, the two F₁ hybrids were as susceptible as *J. aquatica*, and susceptibility to WFT differed among F₂ hybrid lines: 69 F₂ lines were equally susceptible compared to *J. aquatica*, 10 F₂ lines were more susceptible than *J. aquatica* and 15 F₂ lines were as resistant as *J. vulgaris* or were intermediate to the two parental genotypes. Among 37 individual PAs that were derived from four structural groups (senecionine-, jacobine-, erucifoline- and otosenine-like PAs), the N-oxides of jacobine, jaconine, and jacoline were negatively correlated with feeding damage caused by WFT, and the tertiary amines of jacobine, jaconine, jacoline, and other PAs did not relate to feeding damage. Total PA concentration was negatively correlated with feeding damage. Among the four PA groups, only the total concentration of the jacobine-like PAs was negatively correlated with feeding damage. Multiple regression tests suggested that jacobine-like PAs play a greater role in WFT resistance than PAs from other structural groups. We found no evidence for synergistic effects of different PAs on WFT resistance. The relationship between PA variation and WFT feeding damage in the *Jacobaea* hybrids suggests a role for PAs in resistance to generalist insects.

Key Words Hybridization, *Jacobaea vulgaris*, *Jacobaea aquatica*, secondary metabolite diversity, chemical defense, *Frankliniella occidentalis*

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1. Introduction

In plants, research into the role of hybridization in the evolution of novel traits and new species is gaining momentum (Barton, 2001; Seehausen, 2004; Abbott et al., 2008). Hybrids have been used increasingly in experimental studies in ecology and evolution in part because interspecific hybrids (specifically segregating generations) often show greater variation in traits compared to parental species. Furthermore, segregating hybrids frequently show greater independence between different traits than the parental species (Hochwender et al., 2000; Lexer et al., 2003; Orians et al., 2010). Interspecific hybrids can have novel patterns of secondary chemical expression or accumulation compared to parental species, and sometimes can be more resistant or susceptible to herbivores than parental species (Rieseberg and Elstrand, 1993; Orians 2000; Fritz 1999). This makes hybrids useful for studying the relationship between secondary metabolite variation and herbivores (Hallgren et al., 2003; Leiss et al., 2009).

Hybridization occurs frequently in the Jacobaea (syn. Senecio, Asteraceae) genus (Vincent, 1996). Members of this genus have been used extensively to study plant-herbivore interactions, which are largely mediated by a diverse group of pyrrolizidine alkaloids (PAs; see reviews by Hartmann, 1999; Macel, 2011). Twenty-six PAs have been reported from 24 Jacobaea species (Pelser et al., 2005). PAs are ester alkaloids composed of a necine base (amino alcohol moiety) and an alkyl, or rarely aralkyl, necic acid (Hartmann, 1999). PAs can occur in two forms in vivo, the tertiary amine (free base) or the N-oxide form (Hartmann et al., 1989; Rizk, 1991; Wiedenfeld et al., 2008; Chapter 3). In Jacobaea species, all PAs except for senecivernine are derived from senecionine N-oxide, which is synthesized in the roots, transported to the shoots, and diversified into other PA structures (Hartmann and Toppel, 1987). Variation in PA structure and form can lead to variation in the performance of generalist insect and other plant enemies such as nematodes (van Dam et al., 1995; Macel et al., 2005; Dominguez, 2008; Thoden et al., 2009).

Jacobaea vulgaris (tansy ragwort or common ragwort, syn. Senecio jacobaea) is native to Europe and west Asia but invasive in North America, Australia and New Zealand. Jacobaea aquatica (marsh ragwort, syn. Senecio aquaticus) is closely related to, but not a sister species of, J. vulgaris (Pelser et al., 2003). Natural hybrids between these species occur in at least one location in The Netherlands (Kirk et al., 2004). The two parental species are attacked by different suites of specialist and generalist herbivores (personal observation). A previous study showed that artificial hybridization between these two species can be used to produce F1 lines that are in some cases extremely susceptible, and in other cases extremely resistant, to generalist herbivores (Leiss et al., 2009).

Western flower thrips, Frankliniella occidentalis (hereafter WFT), is a key insect pest on a wide range of agricultural and horticultural crops globally (Kirk and Terry, 2003). Since this species is highly polyphagous and infests about 200 wild and cultivated host species (Yudin et al., 1986), F. occidentalis is often used as a representative generalist herbivore in studies of plant-insect interactions (e.g., Macel et al., 2005; Leiss et al., 2009). Previous studies investigated the effects of PAs on WFT with experiments that used artificial diets (Macel et al., 2005), or demonstrated the relationship between PAs and WFT resistance in host plants (Macel, 2003; Leiss et al., 2009). These studies showed that PAs are toxic to WFT and play a role in the plant resistance against this insect. However, these authors incorporated only a limited number of PAs in their studies. Some PAs were not easily acquired for experiments. Only the major PAs were quantified in host plants, and PAs were measured without discrimination between the two forms due to technical limitations in analytical methods. The effects of PA variation in host plant on WFT resistance have not yet been tested. This study aimed to overcome the challenges associated with isolating many PA variants for diet studies by measuring WFT resistance in a segregating hybrid family, which is expected to demonstrate great variation in composition and concentration of secondary metabolites such as PAs. Additionally, technological advances now permit the detection of PAs that are present in extremely low concentrations or that demonstrate only slight structural variations compared to other PAs, which allows us to test the relationship between WFT resistance and PA composition using a comprehensive set of PAs in vivo.

In this study, we carried out WFT bioassays with an artificial hybrid family including one J. vulgaris genotype, one J. aquatica genotype, two F1 offspring, and 94 different F2 hybrid lines. We measured WFT feeding damage in the shoots of these genotypes, and investigated the relationship between PA variation and susceptibility to attack by WFT in the segregating F2 generation. We addressed the following questions: 1) Is there variation in WFT resistance among segregating Jacobaea hybrids? 2) Is WFT resistance explained by PA concentration and composition, and if so, 3) Do different structural PA variants affect WFT resistance differently? 4) Are there any interactions between the effects of different PAs on WFT resistance?

2. Methods and Material

2.1. Study system and plant growth

Jacobaea vulgaris seeds (collected at Meijendel Nature Reserve, 52° 7’ 54” N, 4° 19’ 46” E, The Netherlands) and J. aquatica seeds (collected at the Zwanenwater Reserve, 52° 48’ 38” N, 4° 41’ 7” E, The Netherlands) were germinated in glass vials. Clones were produced from tissue cultured seedlings, and several clones were subsequently grown in pots in climate rooms under standard conditions (20°C, 70% relative humidity, light: dark 16: 8h). Potted plants were vernalized at 4°C with the standard light and humidity conditions for approximately 10 weeks to facilitate flowering. Both species are self-incompatible and crosses were performed by rubbing flower heads together (Kirk et al., 2005). Two rayed F1 offspring were selected from this initial cross, and were reciprocally crossed with each other to produce two sets of F2 offspring. One F1 set consisted of 56 individuals and the other consisted of 46 individuals. The parental, F1, and F2 individuals were maintained in tissue culture and were cloned to perform experiments using replicate genotypes.

We grew about 6 cloned replicates per F2 genotype and about 12 cloned replicates per parental F1 genotype for the WFT bioassay. In addition we grew the same number of replicates of the genotypes for PA analysis. The PA data for these genotypes were used both to study WFT resistance as described in this paper and for an analysis of patterns of PA profiles in Jacobaea hybrid plants that was published elsewhere (Chapter 2). The clones were individually potted in 1.3 liter pots filled with 95% sandy soil (collected from Meijendel), 5% potting soil (Singerland Potgrond company, Zoeterwoude, The Netherlands) and 1.5 g/1 Osmocote slow release fertilizer (N:P:K=15:9:11, Scotts®, Scotts Miracle-Gro, Marysville, Ohio, USA). Plants were kept in a climate room under standard conditions described above for six weeks before the bioassay was initiated.

2.2. WFT bioassay

We used 12 replicates of each parental and F1 genotype, and three to six replicates of each of 94 F2
hybrid genotypes (six replicates were used for most genotypes, though less than six were used in cases where plants died or were too small compared to other plants of the same genotype). A total of 587 plants were randomly placed in a climate room and grown under standard conditions. About 5870 adult WFT, previously reared on chrysanthemum (Dendanthea grandiflora), were released at evenly spaced points in the climate room. During the three week feeding period the plants were watered every two days without wetting or disturbing the leaves. After three weeks, silver damage caused by feeding from WFT on both upper and lower leaf surfaces was visually scored in mm² for each leaf, according to the methods developed by Leiss et al (2009). Above ground plant parts (shoots) were harvested just above the root crown and dried for three days in an oven at 50°C before establishing the dry masses of the shoots.

2.3. PA data acquisition
A Waters Acquity ultra performance liquid chromatographic system coupled to a Waters Quattro Premier XE tandem mass spectrometer (LC-MS/MS) (Waters, Milford, MA, USA) was used for PA analysis. Analysis was performed using a different set of the same tissue culture-derived clonal plants consisting of the same genotypes and number of clones as those used in the WFT bioassay, and grown under identical conditions. The plant shoots were harvested, stored at -80°C and freeze-dried for one week under vacuum with a collector temperature of -55°C. The dried plant material was ground to a fine powder and about 10 mg was extracted with 2% formic acid in a mass to volume ratio of 1:100.

For extraction, 975 μl of water was added to the plant material (PRISNA, Leiden, The Netherlands). The identity of the isolated standards was confirmed by ‘H-NMR and LC-MS analysis and by comparison with literature data (Logie et al, 1994). Acetyl-seneciphylline was obtained by acetylation of seneciphylline with acetic anhydride and pyridine, according to the procedure described by He et al (2010). Integerrimine N-oxide, jacobine, erucifoline N-oxide, and acetyseneciphylline N-oxide were obtained from Phytolab, Vestenbergsgreuth, Germany; senkirkine was obtained from Phytoplan, Heidelberg, Germany. Riddelline and its N-oxide were obtained as authentic standards from Dr M. Chou (NCTR, Jefferson, AR, USA) and integerrimine was a gift of Dr. J. Trigo (UNICAMP, Campinas, Brasil). Jacobine and erucifoline were isolated from J. vulgaris plant material (PRISNA, Leiden, The Netherlands). The identity of the isolated standards was confirmed by ‘H-NMR and LC-MS analysis and by comparison with literature data (Logie et al, 1994). Acetyl-seneciphylline was obtained by acetylation of seneciphylline with acetic anhydride and pyridine, according to the procedure described by He et al (2010). Integerrimine N-oxide, jacobine, erucifoline N-oxide, and acetyseneciphylline N-oxide were prepared by N-oxidation of the corresponding tertiary amine PAs according to the procedure described by Christie et al (1949) and adapted by Chou et al (2003). The purity of the obtained standards was checked by the procedure described by LC-MS analysis and was at least 90%.

The other PAs listed in Table 1 were tentatively identified on the basis of their retention time, molecular mass and fragmentation pattern and on comparison with PA standards and literature data. The presence of PA N-oxides was confirmed by selective reduction to the corresponding tertiary amines according to the method of Joosten et al (2010). All PAs included in this study have been reported before as constituents of J vulgaris and/or J aquatica (Langel et al, 2011; Hartmann and Witte, 1995, Chapter 3) and no new PAs were identified.

### Table 1 Pyrrolizidine alkaloids (PAs) detected in Jacobaea aquatica, Jacobaea vulgaris and hybrids

| Group | PA | Retention time (min) | Precursor mass (m/z) | Fragment mass 1/2 (m/z) | Collision energy 1/2 (eV) | Standard PA used for quantification |
|-------|----|----------------------|----------------------|-------------------------|--------------------------|-----------------------------------|
| Senecionine-like PAs (senecionine-related derivatives) | senecionine | 9.93 | 316.2 | 94.0; 120.0 | 40; 10 | senecionine |
| | senecionine N-oxide | 9.72 | 316.2 | 94.0; 120.0 | 40; 10 | senecionine N-oxide |
| | integerrimine | 9.80 | 316.2 | 94.0; 120.0 | 40; 10 | integerrimine |
| | integerrimine N-oxide | 9.83 | 316.2 | 94.0; 120.0 | 40; 10 | integerrimine N-oxide |
| | retrorsine | 8.49 | 352.2 | 94.0; 120.0 | 40; 10 | retrorsine |
| | retrorsine N-oxide | 8.01 | 360.2 | 94.0; 120.0 | 40; 10 | retrorsine N-oxide |
| | usaroline | 8.29 | 352.2 | 94.0; 120.0 | 40; 10 | usaroline |
| | usaroline N-oxide | 5.89 | 368.2 | 94.0; 120.0 | 40; 10 | usaroline N-oxide |
| | radilaline | 7.91 | 350.2 | 94.0; 130.0 | 40; 10 | radilaline |
| | radilaline N-oxide | 5.48 | 366.2 | 94.0; 130.0 | 40; 10 | radilaline N-oxide |
| | seneciphylline | 9.16 | 314.2 | 94.0; 120.0 | 40; 10 | seneciphylline |
| | seneciphylline N-oxide | 6.36 | 350.2 | 94.0; 130.0 | 40; 10 | seneciphylline N-oxide |
| | spartioidine | 6.86 | 334.2 | 120.0; 130.0 | 30; 10 | spartioidine |
| | spartioidine N-oxide | 6.56 | 350.2 | 94.0; 130.0 | 40; 10 | spartioidine N-oxide |
| | acetyl-seneciphylline | 11.30 | 376.2 | 94.0; 130.0 | 30; 10 | acetyl-seneciphylline |
| | acetyl-seneciphylline N-oxide | 8.86 | 392.2 | 94.0; 110.0 | 40; 10 | acetyl-seneciphylline N-oxide |
| | jacobine | 7.89 | 352.2 | 120.0; 155.0 | 30; 10 | jacobine |
| | jacobine N-oxide | 5.49 | 368.2 | 120.0; 196.0 | 30; 10 | jacobine N-oxide |
| | jacobine | 6.13 | 370.2 | 94.0; 130.0 | 40; 10 | jacobine |
| | jacobine N-oxide | 4.39 | 386.2 | 94.0; 120.0 | 40; 10 | jacobine N-oxide |
| | jacobine | 6.75 | 388.2 | 94.0; 120.0 | 40; 10 | jacobine |
| | jacobine N-oxide | 5.77 | 404.2 | 94.0; 130.0 | 40; 10 | jacobine N-oxide |
| | jacinthe | 7.23 | 350.2 | 94.0; 130.0 | 40; 10 | jacinthe |
| | jacinthe N-oxide | 8.37 | 366.2 | 94.0; 110.0 | 40; 10 | jacinthe N-oxide |
| | dehydroyacizine | 7.66 | 350.2 | 94.0; 120.0 | 40; 10 | dehydroyacizine |
| | dehydroyacizine N-oxide | 7.56 | 350.2 | 94.0; 120.0 | 40; 10 | dehydroyacizine N-oxide |
| | erucifoline | 4.80 | 366.2 | 94.0; 110.0 | 40; 10 | erucifoline |
| | erucifoline N-oxide | 10.18 | 392.2 | 94.0; 110.0 | 40; 10 | erucifoline N-oxide |
| | acetyl-erucifoline | 7.17 | 408.2 | 94.0; 120.0 | 40; 10 | acetyl-erucifoline |
| | acetyl-erucifoline N-oxide | 7.17 | 408.2 | 94.0; 120.0 | 40; 10 | acetyl-erucifoline N-oxide |
| | senkirkine | 7.31 | 366.2 | 122.0; 164.0 | 30; 10 | senkirkine |
| | senkirkine | 5.60 | 352.2 | 122.0; 164.0 | 30; 10 | senkirkine |
| | senkirkine | 4.35 | 400.2 | 122.0; 164.0 | 30; 10 | senkirkine |
| | senkirkine | 6.26 | 418.2 | 122.0; 164.0 | 30; 10 | senkirkine |
| | florosenine | 5.51 | 388.2 | 94.0; 130.0 | 40; 10 | florosenine |
| | florosenine | 5.01 | 460.2 | 122.0; 164.0 | 30; 10 | florosenine |
Data were recorded in multiple monitoring mode (MRM) using two selected precursor ions to produce ion transitions per compound. The MS settings are shown in Table 1. For quantification, the sum of the two peak areas obtained for each compound was normalized against the peak area of the internal standard. Quantification was performed against a standard solution (100 μg/l) of the PAs in an extraction of tansy (Tanacetum vulgare), a plant known to be free of PAs. The use of a PA standard solution in blank plant extract was considered to be a more reliable approach than quantification against a PA solution in solvent only. This PA standard extraction was injected every 30 samples and the averaged response was used for quantification. For those PAs without standards available, a semi-quantitative (indicative) value was obtained by comparison with the most closely related analogue (e.g. an isomer) as indicated in Table 1. Data processing was conducted with Masslynx 4.1 software (Waters, Milford, MA, USA).

PA expression is genetically controlled under standard growth conditions, and PA production is not induced in shoots by aboveground herbivory in Jacobaea plants (Vrieling and Bruin, 1987; van Dam et al., 1993; Vrieling et al., 1993). Therefore, we averaged the concentration of each PA across all clones of each genotype and used the genotypic mean concentrations in the analyses presented here. The 37 PAs identified from the Jacobaea hybrids could be classified into four types, according to their structural characteristics, biosynthetic pathways and expression pattern: senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs and otonecine-like PAs (Pelser et al., 2005; Chapter 2). We followed this classification in this study (Table 1–2). The total PA concentration as well as the amount for each structural group was calculated by summing the concentrations of the individual PAs.

### Table 2
Pearson/Spearman correlation tests between western flower thrips (WFT) feeding damage and the concentrations of individual pyrrolizidine alkaloids (PAs) in the 94 F2 hybrid genotypes from Jacobaea aquatica and Jacobaea vulgaris.

| Group          | PA               | r          | P     | Adjusted P* |
|----------------|------------------|------------|-------|-------------|
| Senecionine-like PAs | n -oxide        | -0.247     | *     | ns          |
| Senecionine-like PAs | n -oxide        | -0.247     | *     | ns          |
| Intermonine     | -0.292           | **         |       |             |
| Intermonine n -oxide |              | -0.241     | *     | ns          |
| Retrione        | -0.201           | +          | ns    |             |
| Retrione n -oxide |               | -0.104     | m     | ns          |
| Usaramine       | -0.011           | m          | ns    |             |
| Usaramine n -oxide |              | 0.11       | m     | ns          |
| Senecionine     | 0.217            | *          | ns    |             |
| Riddelliine     | -0.197           | *          | ns    |             |
| Retrione        | -0.17            | +          | ns    |             |
| Senecionine     | -0.109           | m          | ns    |             |
| Usaramine       | -0.081           | m          | ns    |             |
| Usaramine n -oxide |              | 0.044      | m     | ns          |
| Usaramine n -oxide |              | 0.062      | m     | ns          |
| Senecionine     | -0.123           | **         |       |             |

Note: *P* values of the correlation tests were adjusted by Bonferroni method.

Significance codes: *P* ≤ 0.05; **P* ≤ 0.01; ***P* ≤ 0.001.

underlying relationships between WFT resistance and PA expression. We were not able to test for differences in these relationships between the different genotypes described in this study because only a limited number of genotypes were included from the parental and F1 generations. However the parental and F1 plants provided reference points for WFT resistance comparison. We used log-transformed genotypic mean values of feeding damage and PA concentrations to carry out correlation analyses. Either Spearman (for six minor PAs that did not have normally distributed concentrations) or Pearson correlation tests were carried out to test the relationship between feeding damage and the concentrations of individual PAs, pooled concentrations of each of the four PA groups and total PA.

For correlation tests and principal components analysis, we included only data from F1 genotypes, since we were interested in using the variation from this segregating generation to search for

### Chapter 5.

The Relationship between Structurally Different Pyrrolizidine Alkaloids and Western Flower Thrips Resistance
PAs from within structural groups were closely correlated with each other, and it was therefore not possible to investigate the interactions between them. The PAs from different structural groups, however, were generally expressed independently. The sum concentrations of the PAs from the four groups were not correlated with one another (Chapter 2). We used a multiple-regression model to test for interactions between the effects of different PA structural classes on feeding damage. In this model, feeding damage (represented by log-transformed genotypic mean values) was defined as the dependent variable, and the sum concentrations of each of the four PA structural groups (log-transformed and centered genotypic mean concentrations) were defined as independent variables.

The principal component analysis (PCA) was carried out by using log-transformed genotypic mean concentrations of all individual PAs except the six minor PAs that did not have normally distributed concentrations. Compared to the major PAs these six PAs were present at very low concentrations (on average less than 1% of total PA concentration). Pearson correlation tests were carried out between the first six principle components (PCs) from the PCA and feeding damage. In order to evaluate the contribution of each PA to each PC (in other words the loading), Pearson correlation tests were adjusted using the sequential Bonferroni method when multiple tests were carried out. All analyses were conducted in R version 2.10.0 (R Development Core Team, 2009).

3. Results

3.1. Variation in feeding damage

Feeding damage was genotype-dependent (df = 97,488; F = 5.30; P < 0.001). Plant mass also had effect on feeding damage (df = 1,488; F = 18.44; P < 0.001). Among the two parental genotypes, J. aquatica suffered more feeding damage than J. vulgaris (df = 1, 22; t = 6.18; P < 0.001). Both of the F1 lines were as susceptible as J. aquatica. Among the 94 F2 hybrids, 69 were as susceptible as J. aquatica, 10 were more susceptible than J. aquatica, 15 showed intermediate resistance, 9 were as resistant as J. vulgaris, and none were more resistant than J. vulgaris (Fig.1, see statistical details in Table S1).

3.2. Relationship between feeding damage and PA concentration

Correlation tests between feeding damage and individual PAs showed that feeding damage was negatively correlated with the concentrations of the N-oxides of two jacobine-like PAs (jacozine and jacoline). Jacobine N-oxide concentration was marginally correlated with feeding damage, and the correlations between the free bases of jacobine-like PAs and feeding damage were not significant after correction for multiple testing. No other individual PAs were correlated with feeding damage (Table 2). Total PA concentration was also correlated with feeding damage (Fig.2a). Of the four structural groups of PAs, only the sum concentration of jacobine-like PAs was significantly correlated with feeding damage by WFT (Fig.2c). The sum concentrations of the other three groups were not correlated with feeding damage (see the statistical results for senecionine-and erucifoline-like PAs in Fig 2b,d; for otosenine-like PAs: df = 92, r = 0.35, P = 0.77).

The multiple regression models showed that among the four PA groups only jacobine-like PAs had significant negative effects on feeding. There were no two-way interactions between the groups.

A three-way interaction between senecionine-like, jacobine-like and erucifoline-like PAs and an interaction between the four PA groups were present. However these were only marginally significant (0.05 < P < 0.1, Table 3).

3.3. Relationships between feeding damage and PA composition

We used principal component analysis (PCA) to reduce the PA data set to a smaller number of uncorrelated axes. PC1 explained 44%, PC2 explained 19% and PC3 explained 12% of the variation in the data. More than 90% of the total variation was accounted for by the first 6 PCs. Among first 6 PCs, PC1 was negative correlated (df = 92, r = -0.32, P = 0.002) and PC3 was positively correlated with feeding damage (df = 92, r = 0.03, P = 0.04). No other PCs were correlated with feeding damage (data not shown). Correlation tests between each PC and individual PAs concentrations allowed us to identify which PAs were associated with each PC. Jacobine-like PAs (except jacozine and its N-oxide) were strongly correlated with PC1, such that individuals with high PC1 scores had high concentrations of jacobine-like PAs. Variation in some senecionine-like, erucifoline-like PAs and otosenine-like PAs contributed strongly to PC3 (individuals with high PC3 scores had high concentrations of these PAs; Table S2). A plot of PC1 versus PC2 (Fig.3) shows that F2 hybrids can roughly be divided into different feeding damage (mm2) in Jacobina aquatica, Jacobina vulgaris, 2 F1 and 94 F2 hybrids. (a) Mean feeding damage for one of the genotypes only (b) Distribution frequency for genotypic mean WFT feeding damage of 94 F2 hybrids. N = 3-6 for each genotype. In total, 587 plants were used in WFT bioassay.
Fig 2. Relationship between feeding damage by western flower thrips (WFT) (mm²) and the concentration of total pyrrolizidine alkaloid (PA), senecionine-like, jacobine-like and erucifoline-like PAs (μg/g dw) of F₂ hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris*. The data for WFT feeding damage and concentrations are the log-transformed genotypic mean values. In each panel the results of the Pearson correlation tests between feeding damage and the PA concentrations are provided; in all cases, df = 92.

Fig. 3 Principle component analysis (PCA) of the pyrrolizidine alkaloid (PA) profiles of F₂ hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris*. PCA was performed on the log-transformed genotypic mean concentrations of all individual PAs excluding six minor PAs that did not have normally distributed concentrations (see Table 2). One dot represents one of 94 F₂ hybrid genotypes. Size of each dot represents mean WFT feeding damage for that genotype. The genotypic mean concentrations are the average value of the three to six replicates from the same genotype.

Table 3 Results of multiple regression of western flower thrips (WFT) feeding damage (mm²) against the sum concentration of four structural groups of pyrrolizidine alkaloids (PAs, μg/g dw) in the 94 F₂ hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris* (For the regression model: adjusted \( R^2 = 0.1655; \) df = 15, 78; \( F = 2.23; \) \( P = 0.012 \))

| Predictors | Estimate | t value |
|------------|----------|---------|
| senecionine-like PAs | -0.22 | -1.45 |
| jacobine-like PAs | -0.26 | -0.95 |
| erucifoline-like PAs | 0.05 | 0.93 |
| otose-n type PAs | -0.03 | -0.50 |
| senecionine:jacobine | -0.43 | -1.07 |
| senecionine:erucifoline | -0.26 | -0.49 |
| jacobine:erucifoline | -0.27 | -0.95 |
| senecionine:otose-n type PAs | 0.05 | 0.93 |
| jacobine:otose-n type PAs | 0.15 | 0.93 |
| erucifoline:otose-n type PAs | -0.03 | -0.50 |
| senecionine:jacobine:erucifoline | 1.59 | 1.89 |
| senecionine:jacobine:otose-n type PAs | 0.43 | 1.07 |
| jacobine:erucifoline:otose-n type PAs | -1.19 | -1.29 |
| jacobine:otose-n type:eto-n type PAs | 1.11 | 1.63 |
| senecionine:jacobine:erucifoline:otose-n type PAs | -3.02 | -1.71 |

Significance codes: + \( P < 0.1 \), * \( P < 0.05 \), ** \( P < 0.01 \).

4. Discussion

Segregating hybrids are sometimes used to study correlations and trade-offs between different ecologically important traits in plants, because they exhibit greater variation than parental species, and greater independence between traits (e.g. Orians et al, 2010). We showed that there is high variation in the WFT susceptibility among F₂ hybrids of *J. vulgaris* and *J. aquatica*. Although most F₂ hybrids were as susceptible as or even more susceptible than *J. aquatica* (73% and 11% among all F₂ hybrids respectively), there were still some hybrids with resistance similar to *J. vulgaris* (10%) or intermediate to the two parents (6%). The expression of PAs among the F₂ hybrid generation was highly variable (Chapter 2, and also in Fig 2 and Fig 3), and this variation provided an excellent opportunity to investigate the *in vivo* effects of PA composition on plant resistance to a generalist herbivore.

We demonstrated that concentrations of total PA and jacobine-like PAs were negatively correlated with feeding damage using correlation tests. The multiple regression and PCA also indicated that concentrations of jacobine-like PAs were more closely related to WFT resistance than concentrations of the other PAs. The important role of jacobine-like PAs in WFT resistance of *Jacobaea* plants has also been supported by previous studies. Macel (2003) found that WFT feeding damage was negatively correlated with total PA concentration and with jacobine (both \( N \)-oxide and free base) concentration in *J. vulgaris* plants. Leiss et al (2009) found that resistant *Jacobaea* hybrids had higher concentrations of jacobine \( N \)-oxide and jaconine \( N \)-oxide than susceptible hybrids. To develop a better understanding of the deterrent effects of different PAs on WFT, bioassays should be conducted using pure samples.
of different PAs.

Macel et al (2005) tested WFT larval survival on artificial diets containing six individual PAs including senecionine, seneciphylline, retoresine, senkirkine, heliotroline and monochrotiline, or mixtures of senecionine, seneciphylline and retrosine. The experiment indicated that toxic effects of PAs on WFT larva differed among the individual PAs. Furthermore, higher PA concentrations had more potent toxic effects, and no synergistic effects resulted from PA mixtures. These findings support the results of our study, with the caveat that our analysis revealed a potential weak interaction between the different kinds of PAs. However, the interactions were slight (0.05 < P < 0.1, Table 3), and it is difficult to interpret interactions between more than two predictors.

PA variation accounted for a relatively low proportion of the variation in feeding damage ($R^2 = 0.17$, Table 3). Therefore, other factors likely play roles in plant susceptibility to WFT. These factors may include plant physical characteristics such as plant size, which was found to be a significant covariate in this study. Total PA concentration and plant size together explained a slightly higher proportion of the total variation ($R^2 = 0.20$). Other secondary metabolites have been reported from these species and their hybrids, including flavonoids, kaempferol glucoside, and chlorogenic acid (Leiss et al, 2009; Kirk et al, 2011), and other phytochemicals such as sesquiterpene lactones may be present but remain unreported. These metabolites may also play a role in resistance to herbivores, individually or in interaction with PAs.

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### Supplementary Material

- **Table S1** General linear models of the thrips resistance indicator (thrips feeding damage, mm², for the models: df = 97, 489; F = 5.30; P < 0.001)

| Genotype | Intercept Estimate ± Std. Error df F P |
|----------|---------------------------------------|
|           |                                       |
| The other parent | -1.16 ± 0.18 -6.75 <0.001 1.18 ± 0.18 6.73 <0.001 -- |
| F1-A      | -0.25 ± 0.17 -3.46 0.146 0.93 ± 0.17 5.32 <0.001 Ds |
| F1-B      | -0.31 ± 0.18 -1.77 ± 0.07 0.87 ± 0.17 5.07 <0.001 Ds |
| 60127     | -0.83 ± 0.21 -3.80 ± 0.01 0.35 ± 0.21 1.68 0.093 Dr |
| 60129     | -0.93 ± 0.21 -4.35 ± 0.01 0.25 ± 0.21 1.20 0.232 Dr |
| 60152     | -0.86 ± 0.27 -3.12 ± 0.02 0.53 ± 0.27 1.20 0.231 Dr |
| 60161     | -1.13 ± 0.21 -5.26 ± 0.01 0.86 ± 0.21 0.27 0.784 Dr |
| 60223     | -0.92 ± 0.21 -4.20 ± 0.01 0.27 ± 0.21 1.28 0.202 Dr |
| 60260     | -0.91 ± 0.21 -4.23 ± 0.01 0.28 ± 0.21 1.12 0.187 Dr |
| 60270     | -0.95 ± 0.21 -4.45 ± 0.01 0.23 ± 0.21 1.10 0.270 Dr |
| 70120     | -0.87 ± 0.21 -4.06 ± 0.01 0.32 ± 0.21 1.50 0.135 Dr |
| 70139     | -0.76 ± 0.27 -2.77 ± 0.06 0.42 ± 0.27 1.56 0.120 Dr |
| 60113     | 0.50 ± 0.21 2.31 0.021 1.68 ± 0.21 7.97 <0.001 A |
| 60145     | 0.49 ± 0.21 2.29 ± 0.022 1.68 ± 0.21 7.94 <0.001 A |
| 60156     | 0.56 ± 0.21 2.64 ± 0.009 1.75 ± 0.21 8.29 <0.001 A |
| 60268     | 0.46 ± 0.21 2.13 ± 0.013 1.64 ± 0.21 7.78 <0.001 A |
| 70202     | 0.48 ± 0.21 2.27 ± 0.024 1.67 ± 0.21 7.92 <0.001 A |
| 70217     | 0.84 ± 0.27 5.07 ± 0.002 2.03 ± 0.27 7.48 <0.001 A |
| 60102     | -0.24 ± 0.21 -1.10 ± 0.272 0.95 ± 0.21 4.50 <0.001 Ds |
| 60104     | -0.07 ± 0.21 -0.34 ± 0.716 1.11 ± 0.21 5.27 <0.001 Ds |
| 60106     | -0.19 ± 0.21 -0.87 ± 0.382 1.00 ± 0.21 4.73 <0.001 Ds |
| 60109     | 0.34 ± 0.21 1.60 ± 0.110 1.51 ± 0.21 7.24 <0.001 Ds |
| 60110     | -0.42 ± 0.21 -1.94 ± 0.013 0.77 ± 0.21 3.65 <0.001 Ds |
| 60116     | -0.42 ± 0.21 -1.98 ± 0.049 0.76 ± 0.21 3.61 <0.001 Ds |
| 60125     | 0.50 ± 0.21 0.47 ± 0.636 1.29 ± 0.21 6.50 <0.001 Ds |
| 60137     | 0.25 ± 0.21 1.18 ± 0.217 1.44 ± 0.21 6.62 <0.001 Ds |
| 60140     | 0.12 ± 0.21 0.55 ± 0.582 1.30 ± 0.21 6.68 <0.001 Ds |
| 60141     | 0.01 ± 0.21 0.14 ± 0.885 1.22 ± 0.21 5.76 <0.001 Ds |
| 60146     | -0.19 ± 0.21 -1.83 ± 0.068 0.79 ± 0.21 3.76 <0.001 Ds |
| 60157     | 0.26 ± 0.21 1.20 ± 0.213 1.44 ± 0.21 6.63 <0.001 Ds |
| 60159     | -0.18 ± 0.21 -0.83 ± 0.407 1.01 ± 0.21 4.77 <0.001 Ds |
| 60168     | -0.32 ± 0.21 -3.10 ± 1.134 0.86 ± 0.21 4.09 <0.001 Ds |
| 60183     | 0.39 ± 0.21 1.83 ± 0.086 1.58 ± 0.21 7.47 <0.001 Ds |
| 60184     | 0.12 ± 0.21 0.54 ± 0.586 1.30 ± 0.21 6.67 <0.001 Ds |
| 60185     | 0.13 ± 0.21 0.60 ± 0.548 1.31 ± 0.21 6.23 <0.001 Ds |
| 60205     | -0.23 ± 0.21 -1.10 ± 0.273 0.93 ± 0.21 4.30 <0.001 Ds |
| 60215     | -0.27 ± 0.21 -1.25 ± 0.211 0.92 ± 0.21 4.15 <0.001 Ds |
| 60217     | 0.26 ± 0.21 1.20 ± 0.212 1.44 ± 0.21 6.63 <0.001 Ds |
| 60220     | 0.32 ± 0.21 1.50 ± 0.114 1.51 ± 0.21 7.54 <0.001 Ds |
| 60229     | -0.16 ± 0.21 -0.76 ± 0.446 1.02 ± 0.21 4.84 <0.001 Ds |
| 60230     | -0.31 ± 0.27 -1.52 ± 0.264 0.88 ± 0.27 3.22 <0.001 Ds |

Continue on next page
### Table 5.2

Statistics results of correlation tests between the first three PCs and individual Pyrrolizidine alkaloids (PAs) from PCA in the shoots of 94 F1 hybrid genotypes of *Jacobaea aquatica, Jacobaea vulgaris* and the hybrids.

| Group          | PC1  | PC2  | PC3  |
|----------------|------|------|------|
|                | R    | P    | P    |
| semerine        | 0.05 | 0.82 | 0.17 |
| semerine-N-oxide| 0.05 | 0.86 | 0.25 |
| jacobine        | 0.24 | 0.73 | 0.14 |
| jacobine-N-oxide| 0.10 | 0.75 | 0.26 |
| retrorsine      | 0.54 | 0.14 | 0.04 |
| retrorsine-N-oxide| -0.06| 0.49 | 0.20 |
| jasamine        | 0.39 | 0.10 | -0.12|
| jasamine-N-oxide| 0.16 | 0.55 | 0.60 |
| retevamine      | 0.54 | 0.14 | 0.15 |
| jasamine-N-oxide| -0.03| 0.02 | 0.79 |
| acetylenephrine| 0.26 | 0.46 | 0.48 |
| semerine        | 0.96 | -0.10 | -0.11 |
| jacobine        | 0.96 | -0.09 | 0.08 |
| jacobine        | 0.97 | -0.12 | -0.10 |
| jacobine-N-oxide| 0.96 | -0.13 | -0.10 |
| jasamine        | 0.96 | 0.13 | 0.08 |
| jasamine-N-oxide| 0.54 | 0.14 | 0.15 |
| jasamine-N-oxide| -0.03| 0.02 | 0.79 |
| jasamine-N-oxide| 0.55 | 0.13 | <0.05 |
| jasamine-N-oxide| 0.01 | 0.16 | 0.56 |
| jasamine        | 0.14 | -0.04 | 0.14 |
| jasamine-N-oxide| 0.20 | 0.00 | 0.30 |
| jasamine-N-oxide| 0.26 | 0.04 | 0.46 |
| jasamine        | 0.31 | 0.75 | -0.45 |
| jasamine        | 0.37 | -0.71 | -0.47 |

* Excluding six minor PAs that did not show normally distributed correlations (see details of six minor PAs in Table 5).

* The R values are the correlation coefficients from the Pearson correlation tests.

* The P values were assessed using the Bonferroni method.

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**Note:**
- Dr: Dominant to resistant parent (9 F1 genotypes, 95.7% among all F2 hybrids); A: additive (resistance intermediate to parents; 6 F1 genotypes, 6.58%); Ds: Dominant to susceptible parent (71 F1 genotypes, 73.40%); S: More susceptible than both of the parents (10 F1 genotypes, 10.62%).
- The estimate coefficient of a genotype indicates whether it suffered more or less damage than the reference (one of the parents).
- *F1* = A and *F2* = B represent *F1* hybrids; other genotypes represent *F2* hybrids.

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**Chapter 5.**

The relationship between structurally different pyrrolizidine alkaloids and western flower thrips resistance.
