Pyrrolizidine alkaloid variation in Jacobaea hybrids : influence on resistance against generalist and specialist insect herbivores

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Chapter 5

The Relationship between Structurally Different Pyrrolizidine Alkaloids and Western Flower Thrips Resistance in F_2 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 F_1 genotypes, and 94 F_2 hybrid lines. The *J. aquatica* genotype was more susceptible to thrips attack than the *J. vulgaris* genotype, the two F_1 hybrids were as susceptible as *J. aquatica*, and susceptibility to WFT differed among F_2 hybrid lines: 69 F_2 lines were equally susceptible compared to *J. aquatica*, 10 F_2 lines were more susceptible than *J. aquatica* and 15 F_2 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 insects 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 F_2 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 (Marcel 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 WTF 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 F_1 offspring, and 94 different F_2 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 F_2 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 16h: 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 F_1 offspring were selected from this initial cross, and were reciprocally crossed with each other to produce two sets of F_2 offspring. One F_2 set consisted of 56 individuals and the other consisted of 46 individuals. The parental, F_1 and F_2 individuals were maintained in tissue culture and were cloned to perform experiments using replicate genotypes.

We grew about 6 cloned replicates per F_2 genotype and about 12 cloned replicates per parental and F_1 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 (Slingerland Potgrond company, Zoeterwoude, The Netherlands) and 1.5 g/l Osmocote slow release fertilizer (N:P:K=15:9:11, Scott®, 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 F_1 genotype, and three to six replicates of each of 94 F_2

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 (*Dendranthema 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 mm^2 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. Heliotrine (Latoxan, Valence, France) was added as internal standard to the extraction solvent at a concentration of 1 µg/ml. The extract was filtered through a 0.2 µm nylon membrane filter (Acrodisc, Pall Life Sciences, Ann Arbor, MI, USA). An aliquot (25 µl) of the filtered PA extract was diluted with water (975 µl) and injected in to the LC-MS/MS system.

Seventeen individual PA standards were available for this study, representing the major PAs present in the plant extracts (Table 1). Senecionine, seneciphylline, retrorsine and their corresponding *N*-oxides were obtained from Phytolab, Vestenbergsgreuth, Germany; senkirkine was obtained from Phytoplan, Heidelberg, Germany. Riddelliine 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 ^{1}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 *N*-oxide, erucifoline *N*-oxide and acetylseneciphylline *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 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 (simple senecionine-related derivatives)	senecionine	9.93	336.2	94.0; 120.0	40; 30	senecionine
	senecionine *N*-oxide	6.97	352.2	94.0; 120.0	40; 30	senecionine *N*-oxide
	integerrimine	9.72	336.2	94.0; 120.0	40; 30	integerrimine
	integerrimine *N*-oxide	6.83	352.2	94.0; 120.0	40; 30	integerrimine *N*-oxide
	retrorsine	8.49	352.2	94.0; 120.0	40; 30	retrorsine
	retrorsine *N*-oxide	6.01	368.2	94.0; 120.0	40; 30	retrorsine *N*-oxide
	usaramine	8.29	352.2	94.0; 120.0	40; 30	retrorsine
	usaramine *N*-oxide	5.89	368.2	94.0; 120.0	40; 30	retrorsine *N*-oxide
	riddelliine	7.91	350.2	94.0; 138.0	40; 30	riddelliine
	riddelliine *N*-oxide	5.48	366.2	94.0; 118.0	40; 30	riddelliine *N*-oxide
	seneciphylline	9.16	334.2	94.0; 120.0	40; 30	seneciphylline
	seneciphylline *N*-oxide	6.36	350.2	94.0; 138.0	40; 30	seneciphylline *N*-oxide
	spartioidine	8.96	334.2	120.0; 138.0	30; 30	seneciphylline
	spartioidine *N*-oxide	6.36	350.2	94.0; 138.0	40; 30	seneciphylline *N*-oxide
	acetylseneciphylline	11.80	376.2	120.0; 138.0	30; 30	acetylseneciphylline
	acetylseneciphylline *N*-oxide	8.86	392.2	94.0; 118.0	40; 30	acetylseneciphylline *N*-oxide
	senecivernine	10.09	336.2	94.0; 120.0	40; 30	integerrimine
Jacobine-like PAs (jacobine-related derivatives)	jacobine	7.89	352.2	120.0; 155.0	30; 30	jacobine
	jacobine *N*-oxide	5.49	368.2	120.0; 296.0	30; 25	jacobine *N*-oxide
	jacoline	6.13	370.2	94.0; 138.0	40; 30	jacobine
	jacoline *N*-oxide	4.39	386.2	94.0; 120.0	40; 30	jacobine *N*-oxide
	jaconine	8.75	388.2	94.0; 120.0	40; 30	jacobine
	jaconine *N*-oxide	5.77	404.2	94.0; 138.0	40; 30	jacobine *N*-oxide
	jacozine	7.23	350.2	94.0; 138.0	40; 30	jacobine
	jacozine *N*-oxide	5.11	366.2	94.0; 118.0	40; 30	jacobine *N*-oxide
	dehydrojaconine	7.86	386.2	94.0; 120.0	40; 30	jacobine
Erucifoline-like PAs (erucifoline-related derivatives)	erucifoline	7.56	350.2	94.0; 120.0	40; 30	erucifoline
	erucifoline *N*-oxide	4.80	366.2	94.0; 118.0	40; 30	erucifoline *N*-oxide
	acetylerucifoline	10.18	392.2	94.0; 118.0	40; 30	erucifoline
	acetylerucifoline *N*-oxide	7.17	408.2	94.0; 120.0	40; 30	erucifoline *N*-oxide
Otosenine-like PAs (otosenine-related derivatives)	senkirkine	7.31	366.2	122.0; 168.0	30; 25	senkirkine
	otosenine	5.60	382.2	122.0; 168.0	30; 25	senkirkine
	onetine	4.35	400.2	122.0; 168.0	30; 30	senkirkine
	desacetyldoronine	6.26	418.2	122.0; 168.0	30; 30	senkirkine
	florosenine	8.35	424.2	122.0; 168.0	35; 30	senkirkine
	floridanine	6.79	442.2	122.0; 168.0	30; 30	senkirkine
	doronine	9.01	460.2	122.0; 168.0	30; 30	senkirkine

Data were recorded in multiple monitoring mode (MRM) using two selected precursor ions to product 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.

2.4. Data analysis

We used general linear models to determine whether WFT resistance differed according to plant genotype. Feeding damage (dependent variable) was log- transformed to achieve normality, and plant genotype was defined as the independent variable with plant dry mass as covariate. Normal distributions and homogenous variances were confirmed by testing the residuals of the models using Shapiro tests and Bartlett tests respectively. Two models were set up: in the first model, *J. vulgaris* was used as a reference, and in the second, *J. aquatica* was used as a reference. All other genotypes were compared to the reference in the model. Differences between the hybrid and parental genotypes were evaluated using the regression coefficient matrices of the two models. In each matrix, the estimated coefficient of a hybrid indicated whether it had suffered more or less damage than the reference genotype, and the *P* value showed whether the difference was significant (Crawley, 2005). This is similar to a post-hoc test of an ANOVA model, however such a post-hoc test includes all pair-wise comparisons between groups, and we were only interested in testing for differences between hybrid and parental genotypes. The difference between the two parental genotypes was also tested using the same regression coefficient matrices. WFT resistance of each hybrid genotype was categorized according to these definitions: ND - no difference, leaf damage area of the hybrid was not different from that of both parents; A - additive, damage was intermediate between that of the parents; Ds - susceptible-dominant, damage was similar to that of the susceptible parent; Dr - resistant-dominant, damage was similar to that of the resistant parent, S - susceptible, damage was greater than that of the susceptible parent; R - resistant, damage was less than that of the resistant parent (Table S1).

For correlation tests and principal components analysis, we included only data from F_2 genotypes, since we were interested in using the variation from this segregating generation to search for

Table 2 Pearson/Spearman correlation tests between western flower thrips (WFT) feeding damage and the concentrations of individual pyrrolizidine alkaloids (PAs) in the 94 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*.

Group	PA	r/r_s	*P*	Adjusted *P* [b]
Senecionine-like PAs	senecionine	-0.247	*	ns
	senecionine *N*-oxide	-0.247	*	ns
	integerrimine	-0.292	**	ns
	integerrimine *N*-oxide	-0.243	*	ns
	retrorsine	-0.201	+	ns
	retrorsine *N*-oxide	-0.104	ns	ns
	usaramine	-0.013	ns	ns
	usaramine *N*-oxide [a]	0.11	ns	ns
	riddelliine [a]	0.217	*	ns
	riddelliine *N*-oxide	-0.109	ns	ns
	seneciphylline	-0.282	**	ns
	seneciphylline *N*-oxide	-0.214	*	ns
	spartioidine	-0.17	+	ns
	spartioidine *N*-oxide	-0.15	ns	ns
	acetylseneciphylline	0.044	ns	ns
	acetylseneciphylline *N*-oxide	0.062	ns	ns
	senecivernine	-0.323	**	+
Jacobine-like PAs	jacobine	-0.281	**	ns
	jacobine *N*-oxide	-0.322	**	+
	jacoline	-0.296	**	ns
	jacoline *N*-oxide	-0.331	**	*
	jaconine	-0.278	**	ns
	jaconine *N*-oxide	-0.325	**	*
	jacozine	-0.141	ns	ns
	jacozine *N*-oxide	-0.057	ns	ns
	dehydrojaconine	-0.102	ns	ns
Erucifoline--like PAs	erucifoline	-0.113	ns	ns
	erucifoline *N*-oxide	-0.081	ns	ns
	acetylerucifoline	-0.209	*	ns
	acetylerucifoline *N*-oxide	-0.195	+	ns
Otosenine--like PAs	senkirkine [a]	0.088	ns	ns
	otosenine	-0.056	ns	ns
	onetine	-0.086	ns	ns
	desacetyldoronine	-0.019	ns	ns
	florosenine [a]	0.252	*	ns
	floridanine [a]	0.234	*	ns
	doronine [a]	0.218	*	ns

[a.] PAs with concentrations that were not normally distributed, for which Spearman correlation tests were carried out, while Pearson correlation tests were carried out for all other PAs.
[b.] *P*-values of the correlation testes were adjusted by Bonferroni method.
Significance codes: [ns] not significant, [+] $P < 0.1$, [*] $P < 0.05$, [**] $P < 0.01$.

underlying relationships between WTF resistance and PA expression. We were not able to test for differences in these relationships between the different generations described in this study because only a limited number of genotypes were included from the parental and F_1 generations. However the parental and F_1 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(details

in Table 2 and Fig.3).

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 carried out between individual PAs and the first 3 PCs, since PCs four to six accounted for a low proportion of the total variation and were not correlated with WFT feeding damage. The *P*-values 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 F_1 lines were as susceptible as *J. aquatica*. Among the 94 F_2 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 (jaconine 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 Fig2 b,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).

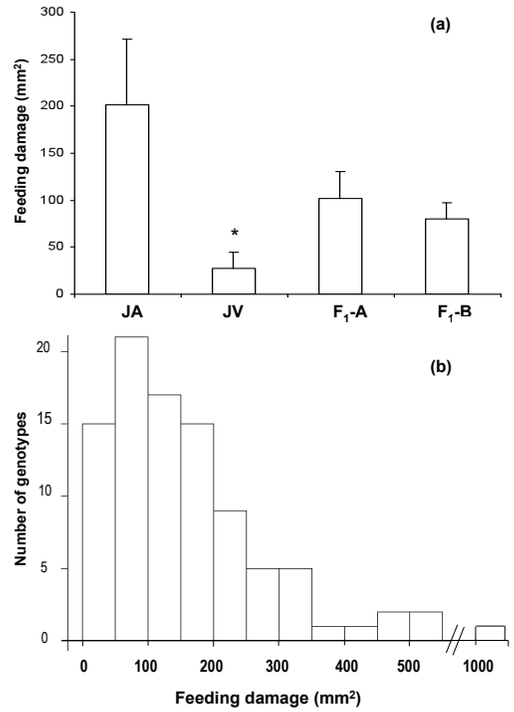

Fig.1 Variation of western flower thrips (WFT) feeding damage (mm^2) in *Jacobaea aquatica, Jacobaea vulgaris*, 2 F_1 and 94 F_2 hybrids. (a) Mean feeding damage for one *J. aquatica* genotype (JA), one *J. vulgaris* genotype (JV), and 2 F_1 (F_1-A and F_1 -B) genotypes. Error bars are standard errors, N = 12. *J. vulgaris* was significantly different from the other genotypes at * P < 0.05. (b) Distribution frequency for genotypic mean WFT feeding damage of 94 F_2 hybrids. N = 3-6 for each genotype. In total, 587 plants were used in WFT bioassay.

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* = -0.23, *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 low/high concentrations of these PAs; Table S2). A plot of PC1 versus PC2 (Fig.3) shows that F_2 hybrids can roughly be divided into different groups. Feeding damage, indicated by the size of the dots, is not clearly clustered either on the plot of PC1 versus PC2 or the plot of PC_1 versus PC_3 (Fig.3).

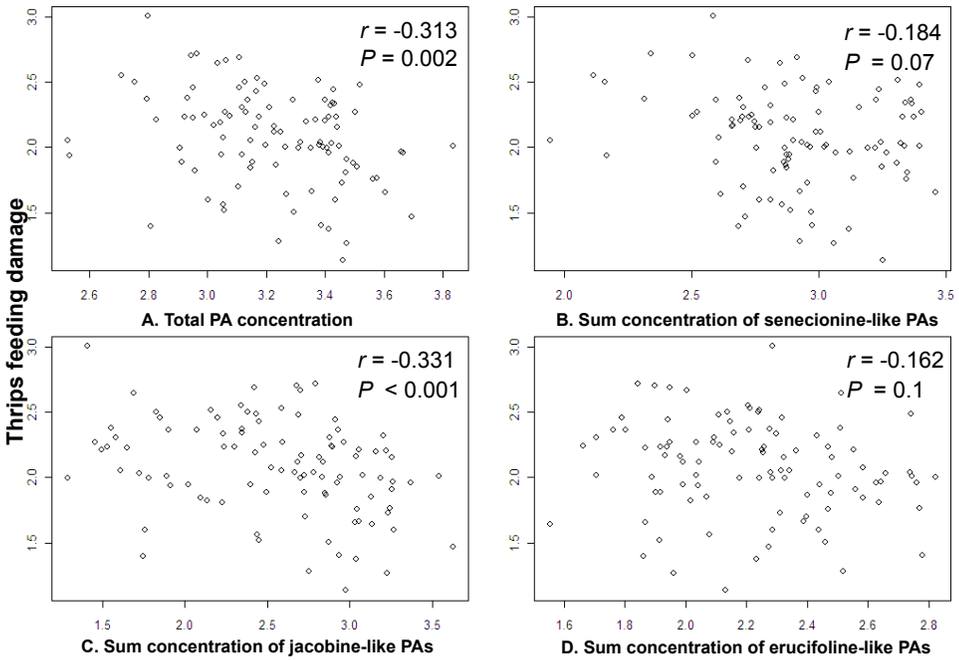

Fig 2. Relationship between feeding damage by western flower thrips (WFT) (mm²) and the concentration of total pyrrolizidine alkaloid (PA), senecionine-like, jacobine-like and erucifoline-like PAs (µg/g dw) of F_2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris*. The data for WFT feeding damage 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.

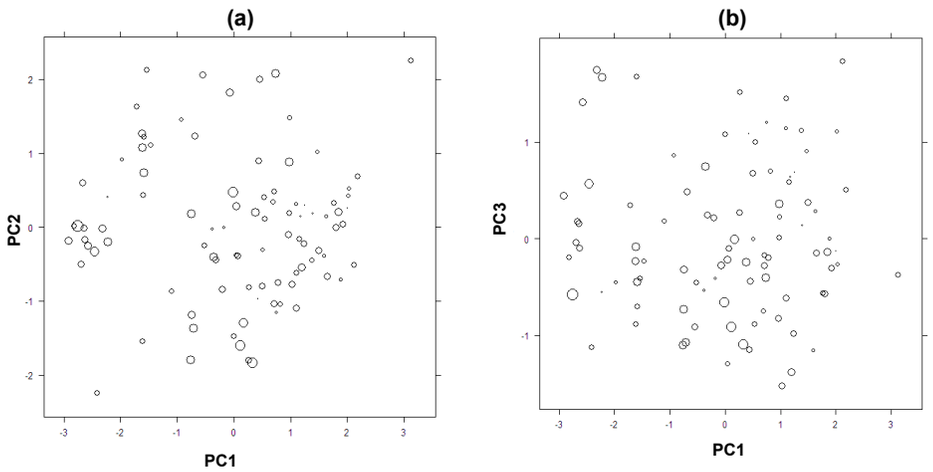

Fig.3 Principle component analysis (PCA) of the pyrrolizidine alkaloid (PA) profiles of F_2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris*. PCA was performed on the log-transformed genotypic mean concentrations of all individual PAs excluding six minor PAs that did not have normally distributed concentrations (see Table 2). One dot represents one of 94 F_2 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 F_2 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 [a]	Estimate	*t* value
PA groups	snt	-0.22	-1.50
	jbt	-0.22	-2.83**
	ert	-0.10	-0.72
	onet	0.06	0.78
Two-way interactions	snt:jbt	-0.43	-1.45
	snt:ert	-0.26	-0.49
	jbt:ert	-0.27	-0.95
	snt:onet	0.05	0.21
	jbt:onet	0.15	0.93
	ert:onet	-0.03	-0.10
Three-way interactions	snt:jbt:ert	1.79	1.89 +
	snt:jbt:onet	0.43	1.07
	snt:ert:onet	-1.19	-1.29
	jbt:ert:onet	1.13	1.63
Four-way interaction	snt:jbt:ert:onet	-3.02	-1.71+

[a] snt, jbt, ert, onet: the sum concentration of senecionine-, jacobine- erucifoline- and otosenine- type PAs, separately.

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 F_2 hybrids of *J. vulgaris* and *J. aquatica*. Although most F_2 hybrids were as susceptible as or even more susceptible than *J. aquatica* (73% and 11% among all F_2 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 F_2 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, retrorsine, senkirkine, heliotrine and monocrotaline, or mixtures of senecionine, seneciphylline, and retrorsine. 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, mm^2, for the models: $df = 97, 489$; $F = 5.30$; $P < 0.001$)

Genotype	*Jacobaea aquatica* as reference				*Jacobaea vulgaris* as reference				Thrips
	Estimate [b]	Std. Error	*t*	*P*	Estimate	Std. Error	*t*	*P*	Resistance [a]
intercept	2.10	0.13	16.49	<0.001	0.91	0.12	7.50	<0.001	--
The other parent	-1.18	0.18	-6.73	<0.001	1.18	0.18	6.73	<0.001	--
F1-A [c]	-0.25	0.17	-1.46	0.146	0.93	0.17	5.52	<0.001	Ds
F1-B	-0.31	0.18	-1.77	0.078	0.87	0.17	5.07	<0.001	Ds
60127	-0.83	0.21	-3.88	<0.001	0.35	0.21	1.68	0.093	Dr
60129	-0.93	0.21	-4.35	<0.001	0.25	0.21	1.20	0.232	Dr
60152	-0.86	0.27	-3.12	0.002	0.33	0.27	1.20	0.231	Dr
60161	-1.13	0.21	-5.26	<0.001	0.06	0.21	0.27	0.784	Dr
60223	-0.92	0.21	-4.28	<0.001	0.27	0.21	1.28	0.202	Dr
60260	-0.91	0.21	-4.23	<0.001	0.28	0.21	1.32	0.187	Dr
60270	-0.95	0.21	-4.45	<0.001	0.23	0.21	1.10	0.270	Dr
70120	-0.87	0.21	-4.06	<0.001	0.32	0.21	1.50	0.135	Dr
70159	-0.76	0.27	-2.77	0.006	0.42	0.27	1.56	0.120	Dr
60135	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	0.033	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	3.07	0.002	2.03	0.27	7.45	<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	0.736	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	1.53	0.21	7.24	<0.001	Ds
60110	-0.42	0.21	-1.94	0.053	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	0.10	0.21	0.47	0.636	1.29	0.21	6.10	<0.001	Ds
60137	0.25	0.21	1.18	0.237	1.44	0.21	6.82	<0.001	Ds
60140	0.12	0.21	0.55	0.582	1.30	0.21	6.18	<0.001	Ds
60141	0.03	0.21	0.14	0.885	1.22	0.21	5.76	<0.001	Ds
60146	-0.39	0.21	-1.83	0.068	0.79	0.21	3.76	<0.001	Ds
60157	0.26	0.21	1.20	0.233	1.44	0.21	6.83	<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	-1.50	0.134	0.86	0.21	4.09	<0.001	Ds
60183	0.39	0.21	1.83	0.068	1.58	0.21	7.47	<0.001	Ds
60184	0.12	0.21	0.54	0.586	1.30	0.21	6.17	<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	0.95	0.21	4.50	<0.001	Ds
60215	-0.27	0.21	-1.25	0.211	0.92	0.21	4.35	<0.001	Ds
60217	0.26	0.21	1.20	0.232	1.44	0.21	6.83	<0.001	Ds
60220	0.32	0.21	1.50	0.134	1.51	0.21	7.14	<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	-1.12	0.264	0.88	0.27	3.22	0.001	Ds

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Genotype	*Jacobaea aquatica* as reference				*Jacobaea vulgaris* as reference				Thrips Resistance
	Estimate [b]	Std. Error	*t*	*P*	Estimate	Std. Error	*t*	*P*	
60232	-0.20	0.21	-0.94	0.349	0.98	0.21	4.66	<0.001	Ds
60248	-0.37	0.21	-1.73	0.084	0.81	0.21	3.86	<0.001	Ds
60249	-0.42	0.27	-1.54	0.124	0.76	0.27	2.80	0.005	Ds
60256	0.08	0.21	0.38	0.707	1.26	0.21	6.00	<0.001	Ds
60259	-0.13	0.21	-0.62	0.533	1.05	0.21	4.98	<0.001	Ds
60261	-0.04	0.21	-0.19	0.849	1.14	0.21	5.42	<0.001	Ds
60262	0.07	0.21	0.31	0.758	1.25	0.21	5.93	<0.001	Ds
60264	0.33	0.21	1.56	0.120	1.52	0.21	7.20	<0.001	Ds
60265	0.38	0.21	1.79	0.073	1.57	0.21	7.44	<0.001	Ds
60267	-0.13	0.21	-0.60	0.548	1.06	0.21	5.01	<0.001	Ds
60269	0.12	0.21	0.56	0.574	1.30	0.21	6.19	<0.001	Ds
60276	0.06	0.21	0.26	0.792	1.24	0.21	5.88	<0.001	Ds
70101	-0.18	0.21	-0.84	0.402	1.00	0.21	4.76	<0.001	Ds
70103	-0.26	0.21	-1.19	0.234	0.93	0.21	4.41	<0.001	Ds
70106	0.20	0.21	0.95	0.344	1.39	0.21	6.58	<0.001	Ds
70107	-0.39	0.21	-1.82	0.069	0.79	0.21	3.77	<0.001	Ds
70108	-0.01	0.21	-0.06	0.951	1.17	0.21	5.55	<0.001	Ds
70109	-0.31	0.21	-1.47	0.143	0.87	0.21	4.13	<0.001	Ds
70110	0.18	0.21	0.84	0.403	1.36	0.21	6.47	<0.001	Ds
70116	0.15	0.21	0.68	0.495	1.33	0.21	6.31	<0.001	Ds
70125	-0.29	0.23	-1.28	0.200	0.89	0.22	3.98	<0.001	Ds
70132	-0.04	0.21	-0.17	0.864	1.15	0.21	5.44	<0.001	Ds
70135	0.11	0.21	0.52	0.602	1.30	0.21	6.15	<0.001	Ds
70138	-0.16	0.21	-0.74	0.462	1.03	0.21	4.87	<0.001	Ds
70140	-0.10	0.21	-0.46	0.646	1.09	0.21	5.15	<0.001	Ds
70143	-0.05	0.21	-0.24	0.811	1.13	0.21	5.37	<0.001	Ds
70146	-0.32	0.21	-1.51	0.131	0.86	0.21	4.08	<0.001	Ds
70149	0.04	0.21	0.21	0.836	1.23	0.21	5.83	<0.001	Ds
70154	0.08	0.23	0.34	0.731	1.26	0.22	5.62	<0.001	Ds
70160	-0.11	0.21	-0.52	0.605	1.07	0.21	5.09	<0.001	Ds
70201	0.10	0.21	0.46	0.649	1.28	0.21	6.08	<0.001	Ds
70203	0.18	0.21	0.83	0.409	1.36	0.21	6.46	<0.001	Ds
70206	-0.20	0.21	-0.95	0.342	0.98	0.21	4.65	<0.001	Ds
70207	-0.22	0.21	-1.02	0.310	0.97	0.21	4.59	<0.001	Ds
70209	-0.16	0.21	-0.76	0.446	1.02	0.21	4.84	<0.001	Ds
70218	0.33	0.21	1.55	0.123	1.52	0.21	7.19	<0.001	Ds
70220	-0.16	0.21	-0.74	0.460	1.03	0.21	4.87	<0.001	Ds
70222	0.00	0.23	-0.01	0.993	1.18	0.22	5.27	<0.001	Ds
70223	-0.04	0.21	-0.21	0.835	1.14	0.21	5.40	<0.001	Ds
70224	-0.34	0.21	-1.57	0.118	0.85	0.21	4.03	<0.001	Ds
70226	0.08	0.21	0.36	0.716	1.26	0.21	5.99	<0.001	Ds
70229	-0.27	0.21	-1.26	0.210	0.92	0.21	4.34	<0.001	Ds
70231	-0.01	0.20	-0.05	0.960	1.17	0.20	5.85	<0.001	Ds
70235	-0.33	0.27	-1.20	0.231	0.85	0.27	3.14	0.002	Ds
70238	-0.24	0.21	-1.14	0.256	0.94	0.21	4.46	<0.001	Ds
70239	0.23	0.21	1.05	0.292	1.41	0.21	6.69	<0.001	Ds
60101	-0.56	0.23	-2.44	0.015	0.63	0.22	2.80	0.005	S
60118	-0.57	0.21	-2.68	0.008	0.61	0.21	2.89	0.004	S
60209	-0.59	0.23	-2.58	0.010	0.60	0.22	2.66	0.008	S
60221	-0.60	0.27	-2.20	0.028	0.58	0.27	2.13	0.033	S
60118	-0.57	0.21	-2.68	0.008	0.61	0.21	2.89	0.004	S
60209	-0.59	0.23	-2.58	0.010	0.60	0.22	2.66	0.008	S
60221	-0.60	0.27	-2.20	0.028	0.58	0.27	2.13	0.033	S
60245	-0.58	0.21	-2.72	0.007	0.60	0.21	2.86	0.004	S
60257	-0.52	0.21	-2.42	0.016	0.67	0.21	3.16	0.002	S
70111	-0.47	0.21	-2.17	0.030	0.72	0.21	3.41	0.001	S
70117	-0.67	0.21	-3.11	0.002	0.52	0.21	2.46	0.014	S
70151	-0.44	0.21	-2.04	0.042	0.75	0.21	3.54	<0.001	S
70158	-0.62	0.21	-2.88	0.004	0.57	0.21	2.69	0.007	S

[a] Resistance patterns: Dr - dominant to resistant parent (9 F_2 genotypes, 9.57% among all F_2 hybrids); A - additive (resistance was intermediate to parents, 6 F_2 genotypes, 6.38%); Ds - dominant to susceptible parent (71 F_2 genotypes, 73.40%); S - more susceptible than both of the parents (10 F_2 genotypes, 10.62%),
[b] The estimated coefficient of a genotype indicates whether it suffered more or less damage than the reference (one of the parents).
[c] F_1-A and F_1-B represent F_1 hybrids; other genotypes represent F_2 hybrids.

- **Table S2** Statistics results of correlation tests between the first three PCs and individual Pyrrolizidine alkaloids (PAs) from PCA in the shoots of 94 F_2 hybrid genotypes of *Jacobaea aquatica, Jacobaea vulgaris* and the hybrids

Group	PAs [a]	*R* [b]			*P* [c]		
		PC1	PC 2	PC 3	PC 1	PC 2	PC 3
Senecionine-like PAs	senecionine	0.03	0.82	0.17	1	<0.001	1
	senecionine *N*-oxide	0.05	0.86	0.25	1	<0.001	1
	integerrimine	0.24	0.73	0.14	1	<0.001	1
	integerrimine *N*-oxide	0.30	0.75	0.26	1	<0.001	1
	retrorsine	0.54	0.14	-0.04	<0.001	1	1
	retrorsine *N*-oxide	-0.06	0.49	0.20	1	<0.001	1
	usaramine	0.39	0.10	-0.12	0.17	1	1
	riddelliine *N*-oxide	0.13	0.09	0.61	1	1	<0.001
	seneciphylline	0.16	0.55	0.60	1	<0.001	<0.001
	seneciphylline *N*-oxide	0.16	0.58	0.70	1	<0.001	<0.001
	spartioidine	0.25	0.00	0.68	1	1	<0.001
	spartioidine *N*-oxide	0.24	-0.03	0.69	1	1	<0.001
	acetylseneciphylline	0.08	0.53	0.01	1	<0.001	1
	acetylseneciphylline *N*-oxide	0.04	0.57	0.24	1	<0.001	1
	senecivernine	0.26	0.48	0.46	1	0.002	0.004
Jacobine-like PAs	jacobine	0.96	-0.10	-0.11	<0.001	1	1
	jacobine *N*-oxide	0.96	-0.09	0.08	<0.001	1	1
	jacoline	0.97	-0.12	-0.10	<0.001	1	1
	jacoline *N*-oxide	0.96	-0.11	0.05	<0.001	1	1
	jaconine	0.96	-0.13	-0.10	<0.001	1	1
	jaconine *N*-oxide	0.96	-0.11	0.08	<0.001	1	1
	jacozine	0.54	0.14	0.15	<0.001	1	1
	jacozine *N*-oxide	-0.03	0.02	0.79	1	1	<0.001
	dehydrojaconine	0.55	0.13	0.19	<0.001	1	1
Erucifoline--like PAs	erucifoline	0.02	0.15	0.26	1	1	1
	erucifoline *N*-oxide	0.00	0.16	0.56	1	1	<0.001
	acetylerucifoline	0.14	-0.04	0.14	1	1	1
	acetylerucifoline *N*-oxide	0.20	0.00	0.30	1	1	1
Otosenine--like PAs	otosenine	0.26	0.74	-0.46	1	0	0.004
	onetine	0.31	0.75	-0.45	1	0	0.008
	desacetyldoronine	0.27	0.73	-0.47	1	0	0.003

[a] excluding six minor PAs that did not show normally distributed concentrations (see details of six minor PAs in Table 2).
[b] The *R* values are the correlation coefficients from the Pearson correlations tests.
[c] The *P*-values were adjusted using the Bonferroni method.