Evidence for two genetically and chemically defined host races of *Tyria jacobaeae* (Arctiidae, Lepidoptera)

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Summary. A widely distributed host race of *Tyria jacobaeae* lives on *Senecio jacobaeae* and related species and accumulates pyrrolizidine alkaloids ("PA race"), another race, which is restricted to the Alps and found on *Petasites paradoxus*, sequesters sesquiterpenes, such as petasol and isopetasol. Nucleotide sequences of the mitochondrial 16S rDNA gene show 1\% sequence divergence, indicating that genetic differences exist between the PA exploiting and the terpene-sequestering host races of *T. jacobaeae*. This finding suggests that both host races of *T. jacobaeae* must have been separated for some time already, possibly since the Pleistocene.

Key words. *Tyria jacobaeae* – host races – mitochondrial DNA – pyrrolizidine alkaloids, sesquiterpenes – *Senecio* – *Petasites*

Introduction

Many insects are strictly monophagous and live on a single host plant species, whereas oligophagous species can exploit a wider number of hosts (Bernays & Chapman 1994). For a number of oligo- and even polyphagous insects evidence has been obtained that specific host races exist, i.e. lineages of insects within a species, which are confined to a single host plant. A number of genetic studies have already shown that some host races can be recognised by distinct mitochondrial haplotypes (Brown et al. 1996; Dobler & Farrell 1999; Shufan et al. 2000; Sperling et al. 1999). This finding indicates that the specific insect-plant relationship must have evolved some time ago because the occurrence of nucleotide substitutions, which characterise the haplotypes, is time dependent. But also the alternative has been documented: Boulding (1998) found no evidence for phylogenetically distinct host races in the pea aphid (*Acyrthosiphon pismum*) indicating a more recent or no consistent host plant selection. It is a matter of debate whether the genetically defined host races should be considered as an indication of genetic polymorphism within a single species without clear subspecific divisions, or as evidence for sibling or multiple cryptic species (Sperling et al. 1999; Adams & Funk 1997).

The Cinnabar moth, *Tyria jacobaeae* L. (syn. *Hippocrita jacobaeae* L.; *Thyria jacobaeae* L.; *Callimorpha jacobaeae*), is an apomastically coloured arcticid moth, which has been studied thoroughly by many groups. A remarkable trait of the Cinnabar moth is the acquisition of pyrrolizidine alkaloids (PAs) from *Senecio* host plants. PAs are taken up, are partly converted into new insect-specific PAs, such as callimorphine, and stored in the integument. Adults are capable of ejecting noxious secretions from their thoracic glands. These alkaloids obviously serve as chemical defence compounds, because this moth is unpalatable and almost invariably rejected by vertebrate predators (Aplin & Rothschild 1992; Edgar et al. 1980; Ehmke et al. 1990; Hartmann 1995; Lindigk et al. 1997; Von Nickisch-Rosenegk & Wink 1993; Vrielings & De Boer 1999; Van Zeele & Van der Meijden 1991; Watson & Whalley 1983).

Most authors consider *Senecio jacobaeae* and related *Senecio* species (*S. vulgaris*, *S. erucifolius*) as the main host plant of this moth (Carter & Hargreaves 1987; Watson & Whalley 1975). This is true for most parts of Central Europe and the English Islands, but not for Alps. Here, *Tyria jacobaeae* lives mostly on *Petasites* (maybe also on *Tussilago* and *Adenostyles*) (Koch 1991) but not on PA rich *Senecio* species (Von Nickisch-Rosenegk & Wink [1993] and unpublished observations) even if PA-rich *Senecio* plants are growing in the direct vicinity. The *Petasites* species which were selected as host plants were not PA producing species, such as *Petasites hybridus*, but mainly PA-free *Petasites paradoxus* and *P. albus* which are rich in sesquiterpenes, such a petasine.

In this communication, we have analysed the potential PA and terpene sequestration of *Tyria jacobaeae* from an alpine population by GLC and GLC-MS. In addition, we have determined the nucleotide sequences of two mitochondrial genes, i.e. 16S rDNA and ND1 from the alpine population and from PA sequestering lowland populations in order to understand whether these populations might represent genetically differentiated host races. Nucleotide sequences from 16S rDNA,
ND1 and other mitochondrial genes have been employed already to solve phylogenetic and phylogeographic questions in Lepidoptera and other insects (Aubert et al. 1999; Brown et al. 1996; Dobler & Farrell 1999; Schneider et al. 1999; Shufra et al. 2000; Simon et al. 1994; Sperling et al. 1999; Von Nickisch-Rosenegk & Wink 1993).

Material and methods

Samples of the PA-acumulating population came from different lowlands locations in France (mainly from northern France). Larvae (adult moths were not found) of the high-elevated alpine population were collected in the “Großes Walsertal” (Austria) in August 1998, feeding on Petasites paradoxus (Table 1).

DNA extraction

DNA was extracted from part of the abdomen (typically the anterior or middle section) of adults or larvae from which the midguts had been removed. In adults, the posterior part (genitalia) was avoided because of the presence of sclerified parts, and, in mated females, of a spermatophore of male origin. Extraction was performed by incubating ground samples for 3 hours at 37°C in 300 µl of 0.1 M Tris-HCl pH 8.0, 10 mM Na2EDTA, 100 mM NaCl, 0.1% sodium dodecyl sulfate, 50 mM dithiothreitol and 1.5 mg proteinase K (Kocher et al. 1989). Homogenates were then extracted twice with phenol/chloroform. Then DNA was precipitated with ethanol after addition of 6 µl of a 0.25% solution of linear acrylamide. The latter step was included in order to improve DNA recovery from dried specimens. DNA pellets were then dissolved in 50 µl TE.

Amplification and sequencing of DNA

A partial section of the 16S rRNA gene with 486 nucleotides (nt) was amplified by Polymerase Chain Reaction (PCR) using the oligonucleotide primers: 5'-CGC CTG TTT ATC AAA AAC AT-3' and 5'-CCG GTT TGA ACT CAG ATC A-3'. PCR amplification was carried out in a volume of 50 µl Taq-polymerase buffer containing 200 µM of each dNTP, 1 mM of each primer and approximately 1 µg total DNA. About 10 to 20 µg/ml bovine serum albumin were added to improve the efficiency of amplification from dried material. PCR was initiated by adding 5 units of Taq polymerase (Amersham Pharmacia Biotech) to samples that had been boiled for 2 min at 92°C. The cycling program included 5 cycles consisting of 10 s at 92°C, 45 s at 42°C and 2.5 min at 65°C (a controlled rate of heating of 7°C/min was used between 42 to 65°C), followed by 40 cycles consisting of 10 s at 92°C, 45 s at a more stringent reassociation temperature (50°C) and 2.5 min at 65°C; a relatively low polymerisation temperature was chosen because of the A + T richness of moth mitochondrial DNA.

Sequencing was carried out by “Cycle sequencing” using two internal overlapping fluorescent primers: 5'-GTC CAA AGG TAG CAT AAT C-3' and 5'-TAA AAC TCT ATA GGG TCT TCT CG-3'. Thermo Sequenase labelled primer kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) was employed, according to a protocol provided by the manufacturer. Sequencing was performed on an ALFExpress II DNA sequencer, AMV 3.0 (Amersham Pharmacia Biotech) with the following running conditions-800 V; 60 mA; 25 W and 55°C. Sequencing was carried out using 5.5% acrylamide gels (7.5%) Long ranger solution [FMC Corp.], 7M urea, 1X TBE. Sequences were further treated on the ALF WIN V 1.0 PC interface. Those which are illustrated in Figs. 1 and 2 will be deposited at the EMBL sequence data library.

For ND1, the following PCR primers were used for PCR amplification: 597: 5'-CGT AAA GTC GTA TAA TAT CAG ATT CGG-3' and 3264: 5'-ATC GAA AGG AGC TGG ATT GTT-3'. Sequencing primers were: 6631: 5'-TAA TCT AAC TTC ATA TGA AAT CTT TTG-3' and ND1-1: 5'-ATT CCT TAT TAT TTT AA-3' or ND1-2: 5'-CAR CWA TTT CWT ATA RGT-3'. Other PCR conditions were identical to those used for the analysis of 16S rDNA.

Secondary structure constraints of 16S rRNA were deduced from data described in Maidaik et al. (1996), and according to the rules of nucleotide linkages as in Michel and Costa (1998), and the reference sequence and secondary structure of Papilio machaon (Lepidoptera) given in Aubert et al. (1999) and for Arctiidae in Schneider et al. (1999).

Phylogenetic analysis was performed using the phylogeny programs PAUP* 4.0b8 (Swofford 1998) and MEGA (Kumar et al. 1993). All heuristic searches for optimal trees were carried out by TBR (Tree-bisection-reconnection) branch swapping with option MULPARS in effect. Parsimony-based analyses (MP). Starting trees were obtained by stepwise addition. We checked that random addition of taxa did not lead to alternative, more parsimonious trees. Distance- (minimum evolution) based analyses (NJ) used Jukes Cantor distance algorithm. Analyses based on Maximum Likelihood (ML): Starting trees were obtained by heuristic search; a new optimal tree was sought and the process was repeated until a stable topology was achieved (different starting trees led to the same final topology, as it was the case without any starting tree). Rates for variable sites were always assumed to follow a Gamma distribution, and both the shape of this distribution and the fraction of invariable sites were estimated. Using Ulteiseta as an outgroup and Panaxia as a related ingroup (Wink et al. 1998) MP, NJ and ML produced identical trees.

Chemical analysis

Alkaloid and terpene extraction

Frozen plant material, larvae, pupae, imagines or collected faeces were extracted twice with 0.5 N HCl after homogenisation by pestle and mortar and left to stand for one hour each. The extract was
Table 2: Profile of secondary metabolites in *Tyria jacobaeae* living on *Petasites parodoxus* host plants. *tr* = traces, = not detected.

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Presence (% of total terpenes)**</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>larvae</td>
<td>faeces</td>
</tr>
<tr>
<td>1. Petasitol</td>
<td>51.4</td>
<td>55.2</td>
</tr>
<tr>
<td>2. Isopetasol</td>
<td>40.5</td>
<td>35.3</td>
</tr>
<tr>
<td>3. Petarine</td>
<td>8.5</td>
<td>5.8</td>
</tr>
<tr>
<td>4. Isopetamine</td>
<td>tr</td>
<td>3.6</td>
</tr>
<tr>
<td>5. PAs</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

* Because of material shortage, we had to extract PAs and sesquiterpenes from the same homogenate. Using HCl/zinc procedure which is optimal for PA analysis, the sesquiterpenes profile could have been altered by the acidic procedure in that furanoeremophilanes become decomposed (Siegenthaler & Neuschwander 1996). We also observed a shift from petasine and isopetamine to petasol and isopetasol under these conditions.

** In order to have enough material for a chemical analysis about 10 larvae or pupae (coming from the same population) were pooled.

brought to 2N HCl and reduced with Zn dust with stirring over night to convert PA N-oxides to the corresponding free bases. The aqueous solution was made alkaline with NH4OH, and extracted with methylene chloride on a Chemelut column, and evaporated to obtain the total alkaloids (tertiary PAs and PA N-oxides) in form of tertiary PAs. Aliquots were extracted without reduction to determine the free tertiary PAs or terpenes. Extracts were dissolved in methanol and analysed by GLC and GLC-MS.

GLC-MS analysis: A Carlo Erba HRGC 4160 gas chromatograph equipped with OV1 capillary column was coupled to a quadrupole mass spectrometer Finnigan MAT 4500 was used. EI mass spectra were recorded at 40 eV. Conditions: injector 250°C; temperature program 150–300°C, 6°C/min.; split ratio 1:20; carrier gas He 50 kPa.

GLC: A Carlo Erba ICU 600 gas chromatograph was employed equipped with FID, Spectra Physics integrator, and a DB1-30W (J & W Scientific) fused silica capillary column (30 m × 0.317 mm). GLC conditions: carrier gas He (2 ml/min.); detector temp. 300°C; injector temp. 250°C; oven temp. program; initial temp. 170°C, 5 min. isothermal, 170–300°C, 10°C/min., 300°C, 15 min. isothermal. Senecionine and seneciphylline were used as external standards.

Results and discussion

Chemical analysis

Extracts were prepared from *T. jacobaeae* larvae, their faeces and from pupae or imagines and analysed by capillary GLC and GLC-MS. PAs and sesquiterpenes were identified according to mass spectral data reported in Witte *et al.* (1993), or Bickel *et al.* (1994) respectively (Table 2).

As several studies have shown, larvae of *T. jacobaeae*, reared on *Senecio jacobaeae* take up the plant alkaloids and partially convert them into callimorphine and other PA derivates (Aplin & Rothschild 1972; Edgar *et al.* 1980; Ehmke *et al.* 1990; Hartmann 1995; Lindigk et al. 1997; Von Nickisch-Roseneck & Wink 1993). Two reference samples (Bu1 and Bu2) collected from low elevation areas show substantial amounts of pyrrolizidine alkaloids (110 and 124 ng PAs per mg insect, respectively). Two thirds of these alkaloids could be attributed to senecionine and the other third to seneciphylline. Only traces of callimorphine were detected. The alkaloid profile provides a clear indication that the insects had been feeding on *Senecio jacobaeae* and are in agreement with previous studies. Note that all samples from Brittany (Br 1–4) and samples from Burgundy (Bu1–5) derive from larvae which had actually been feeding on *S. jacobaeae* host-plant in the field.

![Fig. 1 Separation of sesquiterpenes extracted from Tyria jacobaeae by capillary GLC. A. Faeces, B. starved larvae, C. pupae. 1 = petasol, 2 = isopetasol, 3 = petasine, 4 = isopetamine](image-url)
The analysed alpine samples did not reveal any trace of PAs; this is plausible, because also the host plant, *Petasites paradoxus* had no PAs. But *P. paradoxus* leaves are a rich source of sesquiterpenes. In *T. jacobaeae* larvae and to a minor degree in pupae we could detect petasol, isopetasol, petasine and isopetasine by GLC-MS indicating that the larvae were able to sequester the terpenes of its food plant. However, most terpenes were discarded with the faeces (Table 1, Fig. 1). Although our extraction procedure might produce a bias into the quantitative sesquiterpene profile, the basic finding, that *Tyria* larvae sequester these compounds is still valid. We need a larger sampling of larvae to follow fate and distribution of sesquiterpenes in more detail. Because of material shortage such an investigation was not possible in the present study.

In all alpine populations studied, we found several PA plants in the direct vicinity of *Petasites* host plants,
such as *Senecio caccallae*, *S. fuchsi*, *S. alpinus*, and *Adenostyles glabra*. But not a single *Tyria* larva could be seen on these plants; similar observations have been made earlier (M.W.) in the area of “Großer Ahornboden” and in the Rauris valley, which are 150 km and 240 km away from the Walsertal. Although these observations were not quantified at that time, the finding is based on more than 20 local populations.*

* However, in 1990 (M.W.) had analysed samples of *T. jacobaeae*, which were collected from *Adenostyles glabra* host plants, in the German Alps near Garmisch-Partenkirchen by E. Priesner. Our GC-MS analysis revealed that these larvae contained seneciphylline as major PA. Thus, it is likely that even a third host race could exist. Unfortunately, no samples were preserved for a genetic study and since Priesner had died on a mountain excursion shortly after, we have no locality information to find the original population and to repeat the study.
Fig. 2 Nucleotide sequences of the 16S rRNA from Tyria jacobaeae (A; partial sequences covering position 700 to 1170 corresponding to the Drosophila melanogaster sequence, Maidak et al. 1996) and ND1 gene (B; from position 123 to 585 according to the reference sequence of Papilio machaon, Aubert et al. 1999)

In 1988 and 1989 we had transferred larvae, which were collected from Petasites plants, experimentally to containers with PA-rich Senecio or Adenostyles leaves. The larvae started feeding on the plant material and PA sequestration could be confirmed by GLC measurements (Von Nickisch-Rosenegk & Wink 1993). This indicates, that although the alpine larvae naturally prefer PA-free Petasites plants they have maintained the capacity to tolerate PA plants and to accumulate PAs.

The chemical studies imply that the two host races of T. jacobaeae exist, which differ significantly in their defence strategy. Whereas the effectiveness of PA defences have been documented (Hartmann & Witte 1995), a sesquiterpene-mediated defence is likely but needs to be confirmed experimentally for insects. Some
Experimental evidence has already been obtained that these sesquiterpenes are physiological active compounds that ward off herbivorous snails (Hägele et al. 1998).

**DNA analysis**

Two main genetic lineages can be recognised among populations sampled (Fig. 2, 3). Only 16S rDNA revealed substantial but consistent differences between animals from high elevation and low elevation localities (Fig. 2). Five positions in the 16S rRNA (=1.02% nucleotide substitution) were found to be variable between these two lineages (Positions: 147, 148, 226, 297, 298, Fig. 4). All nucleotide substitutions were transitions between adenine and guanine (Fig. 2, 4). Such kind of changes do not play a significant role in terms of secondary structure of ribosomal RNA because A–U pairs have approximately the same thermodynamic stability as G–U pairs (Michel & Costa 1998).

Using previously collected *Tyria* moths we can assess the variation of the 16S rDNA over a wider range of lowland populations (Fig. 2). Except for a single point mutation at position 148, which separates the populations from western France (Brittany) and central/eastern France, the lowland populations appear to differ substantially from the alpine populations. Genetic distances account for 0.2% within the lowland populations and 1.03% between alpine and lowland populations. Because the variation between both mayor lines exceeds substantially the genetic distance between various lowland populations we are certain that our data provide evidence for the existence of PA- and terpene host races.

In butterflies and insects the ND1 gene is generally more variable than 16S rRNA (Aubert et al. 1999; Simon et al. 1994). Interestingly, the overall variability of this gene is not higher in *Tyria* than that of 16S RNA. Among the 4 variable positions found, two are autapomorphies (positions 166 and 295), a third one (position 85) represents a transition (C–T) which appears to be randomly distributed among both host races and populations (Fig. 2). Only a point mutation shows some phylogeographic information as it separates populations from French
Brittany from all the others (Fig. 3). All substitutions are synonymous and thus selection neutral. Alpine high elevation populations share a haplotype with some populations from central/eastern France. This findings indicates that the alpine population probably derived from the PA-host race and that sesquiterpenes sequestration is a new trait.

A Maximum Likelihood and a strict Maximum Parsimony tree are shown in Fig. 3 (using the combined data set of 16S rDNA and ND1 sequences; Fig. 2) clearly illustrates the split between alpine and lowland populations.

A genetic distance of 1% in the 16S rRNA is comparable to that found between two closely related species of Papilionidae (Papilio machaon and Papilio hospiton; Aubert et al. 1999), indicating that both main “races” of T. jacobaeae must have been genetically separated for some thousands of years. A divergence during the glacial periods (Pleistocene or Holocene), which has been described for several organisms, would be a plausible phylogeographic scenario. The alpine population lives in an environment in which the vegetation period is much shorter and where plant life becomes abundant at a much later date during the season as compared to the lowland populations. This has also consequences for the biology of T. jacobaeae: Larvae of the alpine population have been observed in August and September, whereas larvae of the lowland population emerge at a much earlier date in June. This implicates that adults from both population hardly ever have the chance to meet and to reproduce together because they are separated in time and space. This separation will prevent gene flow between both populations and thus enhance the observed specialisation and genetic differentiation.

Conclusion

Although our data are still preliminary, as we have not sampled more alpine populations and a restricted set of continental ones, they nevertheless indicate that genetic differences obviously exist between the PA-exploiting and the terpene-sequestering host races of T. jacobaeae. A closer study of the morphology of these moths might reveal even more differences. If this would be the case, we might even be dealing with distinct sibling species instead of host races.

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