DNA sequence variation at the mitochondrial cytochrome oxidase I subunit among pheromotypes of the sibling taxa Diachrysia chrysitis and D. tutti (Lepidoptera: Noctuidae)

Article *in* Zoologica Scripta · January 2005

DOI: 10.1111/j.1463-6409.2005.00171.x

CITATIONS
13

READS
768

3 authors, including:

Sven Erlacher
Natural History Museum of Chemnitz
38 PUBLICATIONS **364** CITATIONS

SEE PROFILE

DNA sequence variation at the mitochondrial cytochrome oxidase I subunit among pheromotypes of the sibling taxa *Diachrysia chrysitis* and *D. tutti* (Lepidoptera: Noctuidae)

Axel Hille, Michael A. Miller & Sven Erlacher

Accepted: 19 April 2004

Hille A., Miller M. A. & Erlacher S. (2005). DNA sequence variation at the mitochondrial cytochrome oxidase I subunit among pheromotypes of the sibling taxa *Diachrysia chrysitis* and *D. tutti* (Lepidoptera: Noctuidae). — *Zoologica Scripta*, *34*, 49–56.

We surveyed variation in the mtDNA cytochrome oxidase subunit I (COI) gene in the noctuid sibling species *Diachrysia chrysitis* and *D. tutti*, whose taxonomic status has been queried. Taxonomically, these taxa are separated on the basis of wing pattern and time of flight period. Samples were field-collected from different geographical sites where pheromone traps were baited to attract males containing different mixtures of two blends of pheromone components: (Z)-5-decenyl acetate and (Z)-7-decenyl acetate. Most specimens were sequenced over a 709-bp segment of the COI gene. Single specimens each of *D. chrysitis* and *D. tutti* were sequenced over a region of 1.5 kilobases. mtDNA variation within and among *D. chrysitis* and *D. tutti* is most simply interpreted as DNA polymorphism within a complex of closely related, but well-differentiated pheromotypes. Maximal nucleotide difference per site among haplotypes was 0.28%, which is at the lower end of the range for interspecific mtDNA nucleotide diversity in Lepidoptera. Coefficient of differentiation G_{ST} was *c.* 76.3% ± 11.7%, a typical value at the intraspecific level. Sequences revealed stable diagnostic differences between pheromotypes irrespective of geographical origin. Identification of pheromone-trapped males based on morphology remained vague and uncorrelated to mtDNA haplotypes. The survey illustrated the potential utility of direct DNA sequencing in assessing lineage structures or taxon limits among moths that have been previously found to be different using the pheromone mate recognition system, but which have not been subjected to DNA analysis. The results of mtDNA analyses presented here support recognition of *chrysitis* and *tutti* lineages as presented in previous allozyme studies.

Axel Hille, Sven Erlacher, Landesinstitut für den Öffentlichen Gesundheitsdienst des Landes Nordrhein-Westfalen (lögd), Dezernat Gesundheitsberichterstattung, Westerfeldstr. 35-37, D-33611 Bielefeld, Germany. E-mail: axel.hille@loegd.nrw.de

Axel Hille, Michael A. Miller & Sven Erlacher, Zoologische Staatssammlung München, AG DNA-Tax, Münchhausenstr. 21, D-81247 München, Germany. E-mail: dnatax@zsm.mwn.de

Introduction

The Common Burnished Brass, *Diachrysia chrysitis* (Linnaeus, 1758) (Lepidoptera, Noctuidae, Plusiinae) is a common and widespread noctuid moth inhabiting temperate climates in the Palearctic region. Kostrowicki's (1961) taxonomic revision of the palearctic Plusiinae (Noctuidae, Lepidoptera) included a description of the two forms, *D. chrysitis* (Linnaeus 1758) and *D. tutti* (Kostrowicki 1961), distinguished on the basis of the metallic bands of the forewings and minute differences of male genitalia.

In the subsequent 40 years numerous authors (Järvinen & Vespäläinen 1979; Rezbanyai-Reser 1985; Bruun 1987; Lempke 1965; Urbahn 1967) have investigated the problem of whether *D. tutti* can be unambigously distinguished from the nominate taxon using a variety of morphological techniques. Defining biological species based on indicators for reproductive isolation was first realized by allozyme analysis of pheromone-trapped male moths performed by Svensson *et al*. (1989). Measures of genetic divergence suggested that *D. tutti* is a separate species occurring sympatrically with *D. chrysitis* in the Swedish study area. Samples were very similar with respect to allele frequencies and lacked diagnostic alleles for each taxon, suggesting absence of full reproductive isolation.

Differences in flight period and habitat preference derived from large samples from a variety of trapping sites (Sweden, southern Germany, Hungary) have also been noted (Löfstedt *et al.*

1994; Reichholf 1985), indicating ecological differentiation and ongoing niche separation. In a recent monograph, *D. tutti* has been synonymized with the Pacific *D. stenochrysis* as the western Palearctic representative form, based on genital morphology (Goater *et al*. 2003). The authors state that while these differences are not conclusive, they can be used for identification in the majority of problematic cases. We believe, however, that the underlying species concept is typological and does not validate the synonomization; we have therefore decided to continue to use the pre-existing names *D. chrysitis* and *D. tutti*.

Sex pheromones play a crucial role in the process of mate recognition in almost all species of moths (Roelofs *et al*. 1987; Baker 2002). A general overview of insect pheromones is given by Linn & Roelofs (1995), and H. Arn has compiled a pheromone list available on http://www.nysaes.cornell.edu/fst/faculty/acree/pheronet/phlist/Diachrysia.html.

Changes in pheromone synthesis system (Roelofs *et al*. 1987; Willet 2000) and the ability of conspecifics to detect the pheromone have often been found to be involved in the speciation process. Exemplary pheromone studies have been performed by Newcomb & Gleeson (1998) and Sperling & Hickey (1995) for Tortricidae, and by Sperling *et al*. (1996) for Noctuidae. Priesner (1985) reported that two distinct *Diachrysia* populations were attracted to two different mixtures of the sex attractants (Z)-5-decenyl acetate (Z5-10:OAc) and (Z)-7-decenyl acetate (Z7-10:OAc), here referred to as components of the 'specific mate recognition system' (SMRS) of *Diachrysia*. Ratios of pheromone components of 3 : 100 were assignable to *D. tutti* and of 100 : 10 to *D. chrysitis*.

A series of pheromone field tests was conducted, showing that both forms are sympatric over a very large part of their geographical distribution (Hungary: Tóth & Szöcs 1988; Switzerland: Rezbanyai-Reser 1985; southern Germany: Priesner 1985; northern Germany, Lower Saxony: Robenz 1988; North Rhine-Westphalia: Schulze 1988; Fennoscandia: Svensson *et al*. 1989). On a more local scale, both taxa were found to co-occur at no less than 90% of all collecting sites, except in some xerotherm localities, higher mountain regions (Bavaria), and some areas of northern Germany (for example, on the border between Lower Saxony and North Rhine-Westphalia, *D. chrysitis* was the only form trapped).

In this study we report the results of a survey of mitochondrial DNA (mtDNA) sequence lineage distributions between the two pheromotypes, *D. chrysitis* and *D. tutti*. Our analysis stresses the diagnostic power of DNA-based identification of morphologically problematic species (Tautz *et al*. 2003). Non-recombinant mtDNA was examined because it is more likely to display drift effects due to lineage sorting (Avise 1994) and hence is particularly useful for showing differences between reproductively isolated species (Moore 1995). The specificity of the SMRS of *D. chrysitis* and *D. tutti* reveals that members of different pheromotypes are genetically distinguishable from each other and actually experience very low mating rates. This is an independent test of the expectation that these pheromotypes actually represent species *in statu nascendi*, or full sibling species.

Materials and methods

Methods for collecting male Diachrysia *specimens*

The specimens used in this study were provided by our collaborators Priesner, Naumann and Müller-Tappe. Trapping with synthetic pheromones was conducted in accordance with field work standards as described by Priesner (1985) at sites in Seewiesen (See) in Bavaria, and Bielefeld (Bi) and Bielefeld-Lämershagen (BiL) in North Rhine-Westphalia.

The bioactive chemical components of the pheromones were identified from prepared individual female *Diachrysia* ssp. gland extracts using a gas chromatograph. These were found to be stimulating when tested by male electroantennographic and flight tunnel bioresponse. Trapped specimens were frozen at –70 °C and stored in 95–100% EtOH for a minimum of 2 weeks prior to DNA analysis. Samples of the *Diachrysia* species complex comprised two forms which were pheromone-trapped by using two different bioactive blends which differentially attracted males in previous field tests. A summary relating to the individuals that were examined genetically is provided in the web-based SYSTAX taxonomy database (http://www.biologie.uni-ulm.de/systax). Specimens came from two geographically disparate sites (straight-line distance among sites of over 600 km) in northern (Bielefeld, North Rhine-Westphalia, represented by two locations *c.* 10 km apart) and southern (Seewiesen, Starnberg, Bavaria) Germany. We sampled a minimum of five specimens from each site to determine the extent of sequence divergence within geographical populations.

DNA survey

Total genomic DNA was extracted from thoracic muscles of whole individuals by means of the DNeasy Tissue Kit (QIAGEN) following protocols supplied by the manufacturer. Prior to extraction, specimens were identified as far as possible by phenotype, specifically forewing pattern, with pheromone samples grouped under either '*chrysitis*' or '*tutti*'. The abdomen and, where possible, wings from each pheromone-trapped specimen were preserved in a vial for confirmation of identification, and these vouchers together with the extracted DNA were deposited in the 'DNA-Tax' collection of the Zoologische Staatssammlung, Munich (ZSM). The male genitalia of four individuals were slide-mounted and deposited at ZSM (*D. chrysitis*: DNATAX-00426, ZSM N3795, DNATAX-00428, ZSM N3796; *D. tutti*: DNATAX-00247, ZSM N3797, DNATAX-00249, ZSM N3798). All other specimens were determined by preparation and investigation of male genitalia by L. Rezer (Luzern, CH).

The mitochondrial cytochrome oxidase subunit I (COI) was chosen in accordance with a larger scale insect DNA taxonomy project ('DNA-Tax') underway at ZSM aimed at identifying species from selected genes. All subsequent specimen numbers beginning '00' are DNATAX numbers. Five specimens of pheromotype '*chrysitis*' (00241, 00260, 00426, 00427, 00430) and two of '*tutti*' (00249, 00425) were sequenced over a region of 1.5 kilobases. This region encompassed a leading tRNA tyrosine gene, the COI gene, and parts of a trailing tRNA leucine gene. No other specimens were sequenced over this full region, since shorter sequences of 709 base pairs positioned between bases nos. 751 and 1458 in the respective alignment of *Feltia herilis* (EMBL acc. no U60991) revealed that its mtDNA contained considerable diagnostic and taxonomic information in this stretch of synonymous mtDNA protein coding sequences for both pheromotypes.

Universal primers for PCR amplification and sequencing were selected from Simon *et al*. (1994), corresponding to primers TY-J-1460 (forward), C1-J-2183 (forward), C1-N-2329 (reverse) and TL2-N-3014 (reverse) (Table 1). PCR amplification of the complete COI gene was carried out using combinations of these primers. A PTC 220 DYAD thermocycler (MJ Research) with a 25 µL reaction volume was employed, together with the Expand PCR system (Roche Diagnostics), including 25 pmol of each primer, 20 pmol of dNTPs, 12.5 pmol $MgCl_2$ and 0.88 units of *Taq* polymerase. PCR parameters were 94 °C for 4 min, 45 cycles with 94 °C for 1.5 min, 48 °C for 1 min, 72 °C for 1.5 min, and finally 72 °C for 3 min. Results were visualized using ethidium bromide stained agarose gels under UV light and products cleaned using MinElute PCR purification kit (QIAGEN) following instructions given in the accompanying manual.

Products of dsDNA of each specimen were used as templates for cycle-sequencing reaction (Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit ('Big Dye'; Applied Biosystems) with each of the PCR primers. Cycle parameters were as follows: 94 °C, 2 min; 25 cycles of 94 °C, 20 s; 52 °C, 10 s; 60 °C, 4 min; with final cooling to 4 °C. The sequenced product was filtered through Sephadex-G50 fine (Fluka) packed spin columns (Amersham) to remove unincorporated dye terminators, primers and salts, and finally dried in a speed-vac. These products were resuspended and electrophoresed on an ABI 377 automated sequencer. All fragments were sequenced in both directions. The sequences contained in the ABI files were checked manually and aligned to the noctuid reference sequences of *Feltia herilis* and *F. jaculifera* (EMBL nos U60991, U60990) using BioEdit 5.0.9 (Hall 1999) and ClustalX 1.81 (Thompson *et al*. 1997). Sequences were submitted to the EMBL Nucleotide Sequence Database under accession numbers AJ420352–366 and AJ420375 for *D. chrysitis* and numbers AJ420367–373 for *D. tutti*.

Substitution, transversion/transition ratios, the aggregated genetic distances and their bootstrap values (Nei 1978, 1987) were calculated using MEGA2 (Kumar *et al*. 2001). Pairwise distances between the taxa were calculated as uncorrected percent sequence divergence (p-distance). In order to complement the data presented in the study of Hebert *et al*. (2003), we also calculated percent nucleotide sequence diversity by computation of Kimura's 2-parameter distance where necessary.

Results

Reliable sequences were obtained from 23 individual specimens, including both forms of *D. chrysitis* and *D. tutti*, from two geographically disparate localities (data available at http://www.biologie.uni-ulm.de/systax/daten/index.html).

With an average composition of 30.6% A, 43.1% T, 13.7% C and 12.6% G, the sequenced fragment shows the typical AT bias of insect mitochondrial DNA and cytochrome oxidase genes in particular (Clary & Wolstenholme 1985). Of the 709 nucleotide sites used for all comparisons, nine were variable, and sequences obtained exhibited only one unique base substitution. The *D. chrysitis* and the *D. tutti* lineages have five parsimony-informative nucleotide substitutions at alignment position 157 (C vs. T), 169 (T vs. C), 245 (A vs. T), 295 (G vs. A), and position 698 (T vs. C).

Sequence variation between the *chrysitis* and *tutti* pheromotype results in one conservative amino acid replacement (threonine to serine at amino acid position 82/236) in the serine codon at nucleotide position 245/709 (Table 2). The Ts: Tv ratio range is on average 2.4; at the first codon position it is on average 3.8 (Table 2).

In total, 16 *chrysitis* and 7 *tutti* sequences of 709 bp were derived. Of all haplotypes described, three *chrysitis* haplotypes (00260, 00263, 00264, all See) were unique to single specimens. The most common haplotype, that of *chrysitis*, was

Table 1 Primers used for PCR amplification and mtDNA sequencing (Simon *et al*. 1994), their designations, primer sequences, and alignment positions within the sequence of *Bombyx mori*.

Gene	Primer designation	Primer sequence (5′–3′)	Primer position (and location)
COI	TY-J-1460 (forward)	TACAATTTATCGCCTAAACTTCAGCC	11 782–11 807 (tRNA-Tyrosine)
COI	C1-J-2183 (forward)	CCACATTTATTTTGATTTTTTGG	12 515–12 537 (COI)
COI	C1-N-2329 (reverse)	ACTGTAAATATATGATGAGCTCA	12 683–12 705 (COI)
COI	TL2-N-3014 (reverse)	TCCAATGCACTAATCTGCCATATTA	13 364–13 389 (tRNA-Leucine)

found in 13 specimens from both disparate northern (Bi: 00254, 00257, 00428; BiL: 00429, 00430) and southern (See: 00237, 00241, 00259, 00274, 00275, 00276, 00426, 00427) localities. The other most frequent haplotype was *tutti*-specific and only found in the south (See: 00247, 00424, 00267, 00269, 00271, 00425). Molecular data revealed a maximum difference of two base pairs (2/709 = 0.282% nucleotide diversity) within the two pheromotypes (*chrysitis*: 00263, 00264, 2 bp difference from other *chrysitis* haplotypes; 00260, 0.141% difference; *tutti*: 00249, 0.282% difference from other *tutti* haplotypes 00247, 00424).

Pairwise comparisons of percent nucleotide differences (uncorrected p-distances) between 23 individuals of the genus *Diachrysia* and two members of the outgroup *Feltia* (including the 709 nucleotides comparable throughout) are shown in Table 3.

Diachrysia specimens fell into two groups of haplotypes. Group 1 corresponds to the *chrysitis* pheromotype and shows average nucleotide differences of 0.068% ± 0.040% between sequences. Group 2 corresponds to the *tutti* pheromotype and shows average nucleotide differences of 0.134% ± 0.095%. The mean p-distance between groups is 0.864% ± 0.324%. The two noctuid species of the genus *Feltia* which serve as outgroup taxa are on average differentiated by 5.219% ± 0.839%. Their average distance to *D. chrysitis* is 10.869% ± 1.048%; to *D. tutti* it is 10.588% ± 1.061%.

Another perspective of the problem of genetic differentiation is the population-genetic approach of measuring the coefficient of nucleotide diversity within and among haplotype populations in a hierarchical manner analogous to G_{ST} (Nei 1987) or F_{ST} (Wright 1978). Sequence diversity was compared within each of the *chrysitis* and the *tutti* pheromone population samples and related to that of the entire *Diachrysia* sample by estimating the following indices and their dispersions (500 bootstrap replications; Nei & Kumar 2000).

The mean diversity within groups (*chrysitis* and *tutti* averaged) was estimated to result in a value of $\pi = 0.101\% \pm 0.052\%$, that for the entire pheromotype population (*Diachrysia* averaged) was $\pi = 0.426\% \pm 0.147\%$, which left a component of mean interpopulational diversity (among *chrysitis* and *tutti* pheromotype samples) of $\pi = 0.325\% \pm 0.128\%$. Thus the hierarchical decomposition of variance components of nucleotide diversity resulted in the coefficient of differentiation of $G_{ST} = 76.228\% \pm 11.721\%$ for the entire sample.

Table 2 Variable sites (sequence positions vertically arranged, in brackets) among *D. chrysitis* and *D. tutti* COI sequences

DNATAX ID	Pheromone type	Morphological determination (by L. Reser)	Locality	Var. Pos. 112223566 564494319 791550868
00237	*chrysitis*	*tutti*	See	CTCAGTATT
00241	*chrysitis*	*tutti*	See	....
00254	*chrysitis*	*chrysitis*	Bi	....
00257	*chrysitis*	*chrysitis*	Bi	....
00259	*chrysitis*	*chrysitis*	See	....
00260	*chrysitis*	*chrysitis*	See	C
00263	*chrysitis*	*tutti*	See	......G..
00264	*chrysitis*	*tutti*	See	......GG.
00274	*chrysitis*	*tutti*	See	....
00275	*chrysitis*	*tutti*	See	....
00276	*chrysitis*	*tutti*	See	....
00426	*chrysitis*	*tutti*	See	....
00427	*chrysitis*	*tutti*	See	....
00428	*chrysitis*	*tutti*	Bi	....
00429	*chrysitis*	*chrysitis*	BiL	....
00430	*chrysitis*	*chrysitis*	BiL	....
00247	*tutti*	*tutti*	See	TCTTA...C
00249	*tutti*	*tutti*	See	TC.TAG..C
00267	*tutti*	*chrysitis*	See	TC.TAG..C
00269	*tutti*	*chrysitis*	See	TC.TAG..C
00271	*tutti*	*tutti*	See	TC.TAG..C
00424	*tutti*	*chrysitis*	See	TCTTA...C
00425	*tutti*	*chrysitis*	See	TC.TAG..C

The molecular tree generated (based on simple p-distances) graphically expresses the clear separation between the two groups (Fig. 1). Specimens belonging to the same pheromotype irrespective of geographical origin are grouped in well supported, separate mtDNA lineage clades which are represented by homogeneous clusters of the trees. By contrast, there was no concordance in patterns of molecular haplotypes and genitalic characters (Table 2).

Discussion

Mitochondrial DNA has proved useful for elucidating genetic variation and species limits of insect species complexes (Bogdanovic *et al.* 1993; Frey & Frey 1995; Sperling & Hickey 1995; Brower 1999; Landry *et al.* 1999; Kruse & Sperling 2001). The COI gene was recently shown to have

	Nucleotide p-distance ± SE (within taxon average)	Nucleotide p-distance ± SE (between taxon average)	
		outgroup	*chrysitis*
outgroup (*n* = 2)	5.219% ± 0.839%		
chrysitis (*n* = 16)	0.068% ± 0.040%	10.869% ± 1.048%	
tutti (*n* = 7)	0.134% ± 0.095%	10.588% ± 1.061%	0.864% ± 0.315%

Table 3 Nucleotide distances (percentage) and standard errors (SE) estimated from 500 bootstrap replications for chrysitis and tutti COI sequences. Distances measured as within- and between-taxon distances.

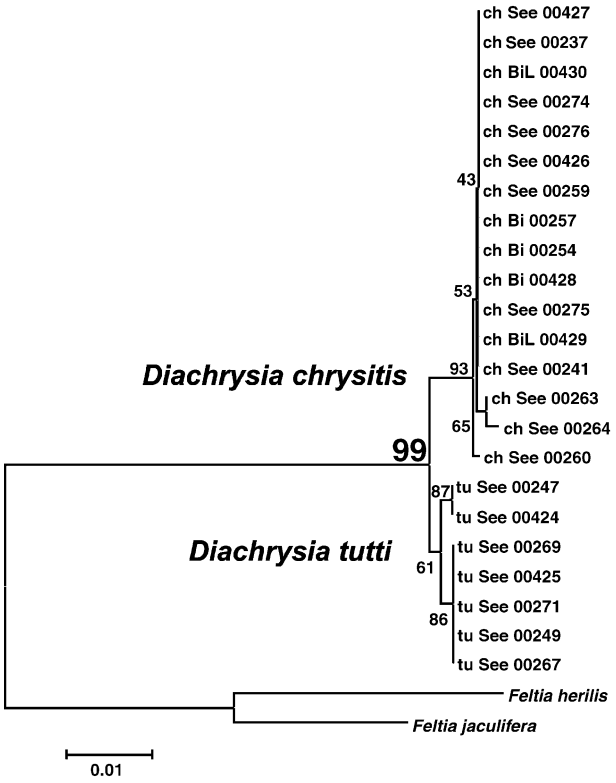

Fig. 1 Genetic relationships between COI haplotypes of *D. chrysitis* and *D. tutti*, with *Feltia* taxa as outgroups, inferred by Neighbour-joining clustering of percent nucleotide pairwise distances. Robustness was estimated by standard errors derived by the bootstrap method (Number of replications = 500; Nei & Kumar 2000). Values are presented as percentages on the tree.

the potential of a general taxonomic tool assigning newly analysed taxa to the appropriate phylum, order or species. In particular, Hebert *et al.* (2003) demonstrated that the comprehensive COI profiles they termed taxon 'barcodes' are especially useful for discrimination at the level of species. For example, a model COI profile, based upon the analysis of a single individual from each of 200 closely allied species of lepidopterans, was 100% successful in correctly identifying subsequent specimens. The threshold adopted for the rate of molecular evolution of the COI gene is *c.* 3% divergence per Myr.

mtDNA of the *D. chrysitis* complex provides another illustration of the utility of this approach where morphological differences are extremely subtle, as has been found in related entomological work (e.g. Sperling *et al.* 1996). The results of the analyses presented here support the recognition of *chrysitis* and *tutti* as presented in previous work (Priesner 1985; Reichholf 1985; Svensson *et al.* 1989; Löfstedt *et al.* 1994). Initially, rigid morphological investigation was invaluable for delimiting *D. tutti* as a separate taxon of the species complex. Later, pheromone and allozyme differentiation were instrumental in addressing the *chrysitis/tutti* problem at greater depth.

Measures of genetic distance, such as simple percent sequence divergence, are highly variable among closely related lepidopterans and are not necessarily a good predictor of biological species (Landry *et al.* 1999; Ferguson 2002). For example, among three species of ermine moths (Yponomeutidae), 1% divergence occurred across the entire 2.3 kb COI/II region (Sperling *et al.* 1995), and sequence divergence between haplotypes from different populations of *Greya obscura* (Lep., Prodoxidae) ranged from 1% to as much as 5.7% across a 765 bp region of COI/II (Brown *et al.* 1994). Hebert *et al.* (2003) reported an average level of Poisson corrected percent divergences of 5.4% in 24 families analysed in the insect order of Lepidoptera. Average percentages of nucleotide sequence divergence (Kimura 2-parameter distances) at COI between 42 species of the family Noctuidae ranged from higher within-genus distances of 5.8% (n = 12 genera) to low within-species values of 0.17%; the Kimura-2 distance between a 617 bp stretch of *D. chrysitis* (00260) and *D. aereoides* (AF549732.1 in Hebert *et al.* 2003) was 7.2%, indicating a level of within-genus molecular differentiation.

Molecular evidence for the two pheromotypes revealed a maximum per site difference of 0.282% nucleotides within both haplotypes. Thus, the range of genetic differentiation is rather small (p-distance below 1%), although it is an order of magnitude greater in intergeneric comparisons to the noctuid taxa of *Feltia* (> 10% Gooding *et al.* 1992; in Hebert *et al.*'s 2003 meta-analysis the respective mean within-family distance is 10.4%). All in all, the value of divergence in the nominal species *D. chrysitis* is indicative of an amount of differentiation that is settled at the genus/species level, and often found between subspecies or geographical subpopulations and species at *statu nascendi*.

Kostrowicki (1961) suggested that *D. chrysitis* and *D. tutti* are not variants of a single species, but rather are morphologically similar — sibling species. We found that population/species and male genitalia divergence is decoupled in the species complex. Genitalic evolution does not definitively reflect the pattern of incipient speciation that we expected based on patterns of molecular divergence. It involves molecular phylogenetic patterns consistent with recent population/species divergence (e.g. relatively short branches, variability in lineages, bifurcating nodes and incipient lineage sorting), concomitant with a variable pattern of divergent genitalia and putative premating isolation.

An alternative explanation may be that male genitalic morphology is relatively unimportant and thus evolving neutrally in an unlinked manner in this sibling species complex. Because we know very little about the details of the mating behaviour, there may be many other premating and copulatory

cues, including those furnished by the chemical communication system. The phylogenetic patterns of incomplete genitalic/molecular divergence can, nonetheless, be considered to be concordant with rapid, neutrally divergent evolution of male genitalia in an imperfect specific mate recognition system.

Much of the information provided by DNA about the amount and grade of differentiation is consistent with, but not conclusive proof of, the proposition that the sympatric pheromotypes are reproductively isolated. Their pheromonal systems are not completely distinct, and cross-attraction of males to lure females or synthetic attractants has been reported (Löfstedt *et al*. 1994). It is therefore possible that interbreeding between the pheromotypes may occur at a level sufficient to preclude major genetic differentiation. Although sample sizes are low and have limited statistical resolving power, the results obtained in the present study indicate that there is genetic substructuring within the nominal species *D. chrysitis*.

The levels of genetic distance among the pheromotypes were found to be low, indicating differentiation well below that characteristic of sibling species. On the other hand, based on the DNA divergence, the present results neither contradict nor confirm conclusively that *chrysitis* and *tutti* are siblings. Parts of the mitochondrial COI gene can be used as a diagnostic marker to differentiate these two forms from their DNA 'barcode'. It was also found that COI sequence differences present in one pheromotype are not present in the other (reciprocal monophyly). If such phenomena are not due to simple genetic drift in fluctuating populations, they could be interpreted as reproductive isolation. Insects collected from different locations, habitats and seasons could form separate clusters. Therefore, it is necessary to sample the species over a wider geographical range before conclusive taxonomic decisions can be made.

D. chrysitis and *D. tutti* are reported as being distributed sympatrically over large parts of their area, but show differences in flight periods and habitat preferences. Since morphological characters for reliable diagnosis have yet to be found, we believe that further ecological study of *D. chrysitis* populations — including research on foodplant relationships and pheromone chemistry — is needed to justify the species status of both forms.

Molecular analysis in this study supported the status of the two pheromotypes as diagnosable biological taxa by the aid of molecular typing. While neutral molecular markers are useful characters for determining the patterns of phylogenetic relationships, understanding of the process of pheromone evolution will require the isolation of the genes required for pheromone production in females (e.g. desaturases and reductases; cf. Baker 2002) and the complementary detection systems in males (e.g. carrier proteins and receptors, Löfstedt 1993). Indeed, differences in these genes may be responsible for speciation (Linn & Roelofs 1995) and also provide the most relevant characters to investigate speciation within the *D. chrysitis* species complex, and to track pheromone evolution.

In conclusion, our results are consistent with those of previous studies using allozymes that indicated that there are two genetically differentiated forms (Svensson *et al*. 1989) although we were unable to correlate these with male genitalic structures. We found diagnostic mitochondrial COI gene sequences that distinguish the two groups which will prove useful for future analysis of the population structure. It is also clear from their low mtDNA sequence divergence that the two forms are very closely related. Like any single marker, however, mtDNA may not always mirror species boundaries. This may occur because of gene introgression between species, rapid speciation, or random sorting of retained ancestral polymorphism (Avise 1994). It remains particularly important for future studies of *D. chrysitis* and *D. tutti* to view variation of mtDNA or nuclear genes in the context of divergent patterns of pheromone response behaviour.

Acknowledgements

This paper is dedicated to Clas M. Naumann, who died prematurely earlier this year, and to Ernst Priesner, who died in 1994, commemorating their many contributions to the study of lepidoteran chemical ecology and biodiversity. They laid the groundwork for the project and provided most of the specimens.

L. Reser (Luzern, CH) determined specimens by genital preparation and inspection. His help is highly appreciated. We also thank J. Reichholf for helpful insights and K. Witt for technical assistance. We thank M. Spies and two anonymous reviewers who improved the content and style of the manuscript.

This sequencing project is integral to the DNA-TAX project aimed at establishing insect sequence databases as part of the Entomological Data Information System (EDIS) initiative under the direction of BIOLOG, founded by the German Federal Ministry for Education and Research (BMBF project ID 01LC99004/2 granted to Gerd Haszprunar, ZSM, Munich).

References

Avise, J.C. (1994). *Molecular Markers, Natural History and Evolution*. New York: Chapman & Hall.

Baker, T. C. (2002). Mechanism for saltational shifts in pheromone communication systems. *Proceedings of the National Academy of Sciences of the USA*, *99* (*21*), 13368–13370.

Bogdanovic, S. M., Wallner, W. E., Bell, J., Odell, T. M. & Harrison, R. G. (1993). Asian Gypsy moths (Lepidoptera: Lymantriidae) in North America. Evidence from molecular data. *Annals of the Entomological Society of America*, *86* (*6*), 710–715.

Brower, A. W. (1999). Delimitation of phylogenetic species with DNA sequences: a critique of Davis and Nixon's Population Aggregation Analysis. *Evolution*, *48*, 199–213.

Brown, J. M., Pellmyr, O., Thompson, J. M. & Harrison, R. G. (1994). Phylogeny of *Greya* (Lepidoptera: Prodoxidae), based on

nucleotide sequence variation in mitochondrial cytochrome oxidase I and II: congruence with morphological data. *Molecular Biology and Evolution*, *11*, 128–141.

Bruun, H. (1987). Longitudinal ridge density of hind-wing scales of *Diachrysis chrysitis* (L.) and *D. tutti* (Kostr.) captured with pheromones (Lepidoptera: Noctuidae). *Notulae Entomologicae*, *67*, 125–127.

Clary, D. O. & Wolstenholme, D. R. (1985). The mitochondrial DNA molecule of *Drosophila yakuba*: Nucleotide sequence, gene organization, and genetic code. *Journal of Molecular Evolution*, *22*, 252–271.

Ferguson, J. W. H. (2002). On the use of genetic divergence for identifying species. *Biological Journal of the Linnean Society*, *75*, 509–516.

Frey, J. E. & Frey, B. (1995). Molecular identification of six species of scale insects (Quadraspidiotus sp.) by RAPD-PCR: Assessing the field-specificity of pheromone traps. *Molecular Ecology*, *4*, 777–780.

Goater, B., Ronkay, L. & Fibiger, M. (2003). Catocalinae & Plusiinae. In M. Honey & M. Fibiger (Eds) *Noctuidae Europaeae*, Vol. 10 (pp. 191–193). Sorø, Denmark: Entomological Press.

Gooding, R. H., Rolseth, B. M., Byers, J. R. & Herle, C. E. (1992). Electrophoretic comparisons of pheromotypes of the dingy cutworm, *Feltia jaculifera* (Gn.) (Lepidoptera: Noctuidae). *Canadian Journal of Zoology*, *70*, 79–86.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT [Computer software and manual]. *Nucleic Acids Research*, *Symposium Series*, *41*, 95–98.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society*, *Series B*, *London*, *270* (*1512*), 313–321.

Järvinen, O. & K. Vespäläinen (1979). Morphological variation in *Diachrysis chrysitis* (Lepidoptera, Noctuidae): a statistical analysis of the wing pattern. *Notulae Entomologicae*, *59*, 19–26.

Kostrowicki, A. S. (1961). Studies on the Palaearctic species of the subfamily Plusiinae (Lepidoptera, Phalaenidae). *Acta Zoologica Cracoviensia*, *6*, 376–472.

Kruse, J. J. & F. A. H. Sperling (2001). Molecular phylogeny within and between species of the *Archips argyrospila* complex (Lepidoptera: Tortricidae). *Annals of the Entomological Society of America*, *94* (*2*), 166–173.

Kumar, S., Tamura, K., Jakobsen, I. & Nei, M. (2001). *MEGA2: Molecular Evolutionary Genetics Analysis*, Version 2.1 [Computer Software and Manual]. University Park, PA and Tempe, AZ: Pennsylvania and Arizona State Universities.

Landry, B., Powell, J. A. & Sperling, F. A. (1999). Systematics of the *Argyrotaenia franciscana* (Lepidoptera: Tortricidae) species group: Evidence from mitochondrial DNA. *Annals of the Entomological Society of America*, *92* (*1*), 40–46.

Lempke, B. L. (1965). *Plusia tutti* Kostrowicki and *Plusia chrysitis* L. *Entomologische Berichten*, *Amsterdam*, *25*, 73–76.

Linn, C. E. & Roelofs, W. L. (1995). Pheromone communication in moths and its role in the speciation process. In D. M. Lambert & H. G. Spencer (Eds) *Speciation and the Recognition Process* (pp. 263–300). Baltimore, MD: Johns Hopkins University Press.

Löftstedt, C. (1993). Moth pheromone genetics and evolution. *Philosophical Transactious of the Royal Society London*, *B*, *340*, 167–177.

Löfstedt, C., Hansson, B. S., Tóth, M., Szöcs, G., Buda, V., Bengtsson, M., Ryrholm, N., Svensson, M. & Priesner, E. (1994). Pheromone differences between sibling taxa *Diachrysia chrysitis* (Linnaeus, 1758) and *D. tutti* (Kostrowicki, 1961) (Lepidoptera: Noctuidae). *Journal of Chemical Ecology*, *20* (*1*), 91–109.

Moore, W. S. (1995). Inferring phylogenies from mtDNA variation: Mitochondrial gene trees versus nuclear trees. *Evolution*, *49*, 718–726.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, *83*, 583–590.

Nei, M. (1987). *Molecular Evolutionary Genetics*. New York: Columbia University Press.

Nei, M. & Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

Newcomb, R. D. & Gleeson, D. M. (1998). Pheromone evolution within the genera *Ctenopseustis* and *Planotortrix* (Lepidoptera: Tortricidae) inferred from a phylogeny based cytochrome oxidase I gene variation. *Biochemical Systematics and Ecology*, *26*, 473–484.

Priesner, E. (1985). Artspezifische Sexuallockstoffe für Männchen von *Diachrysis chrysitis* (L.) und *D. tutti* (Kostr.) (Lepidoptera, Noctuidae: Plusiinae). *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, *58*, 373–391.

Reichholf, J. H. (1985). Speciation dynamics in the noctuid moth *Plusia chrysitis* L. (Lepidoptera, Noctuidae). *Spixiana*, *8* (*1*), 75–81.

Rezbanyai-Reser, L. (1985). *Diachrysia chrysitis* (Linnaeus, 1758) and *D. tutti* (Kostrowicki, 1961) in der Schweiz. *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, *58*, 345–372.

Robenz, W. (1988). Pheromonfallenfänge von *Diachrysia chrysitis* (L.) und *D. tutti* (Kostr.) an einem Standort im niedersächsischen Wendland (Lep., Noctuidae). *Mitteilungen der Arbeitsgemeinschaft Ostwestfälisch-Lippischer Entomologen*, *4* (*43*), 120–122.

Roelofs, W., Glover, T., Tang, X.-H., Sreng, I., Robbins, P., Eckenrode, C., Löfstedt, C., Hansson, B. & Bengtsson, B. O. (1987). Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proceedings of the National Academy of Sciences of the USA*, *84*, 7585–7589.

Schulze, W. (1988). Bericht über Untersuchungen zum Vorkommen von *Diachrysia chrysitis* (L.) und *D. tutti* (Kostr.) in Westfalen und Niedersachsen (Lep., Noctuidae, Plusiinae). *Mitteilungen der Arbeitsgemeinschaft Ostwestfälisch-Lippischer Entomologen*, *4* (*43*), 113–120.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994). Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, *87*, 651–702.

Sperling, F., Byers, R. & Hickey, D. (1996). Mitochondrial DNA sequence variation among pheromotypes of the dingy cutworm, *Feltia jaculifera* (Gn.) (Lepidoptera: Noctuidae). *Canadian Journal of Zoology*, *74*, 2109–2117.

Sperling, F. & Hickey, D. (1995). Amplified mitochondrial DNA as a diagnostic markers for species of conifer-feeding Choristoneura (Lepidoptera: Tortricidae). *The Canadian Entomologist*, *127* (*3*), 277–288.

Sperling, F. A. H., Landry, J. F. & Hickey, D. (1995). DNA-based identification of introduced Ermine moth species in North America (Lepidoptera: Yponomeutidae). *Annals of the Entomological Society of America*, *88* (*2*), 155–162.

Svensson, I., Douwes, P. & Stille, B. (1989). Are *Diachrysis chrysitis* (L.) and *D. tutti* (Kostrowicki) different species? (Lepidoptera: Noctuidae). *Entomologica Scandinavica*, *20*, 15–22.

Tautz, D., Arctander, P., Minelli, A., Thomas, R. H. & Vogler, A. P. (2003). A plea for DNA taxonomy. *Trends in Ecology and Evolution*, *18* (*2*), 70–74.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & D. G. Higgins (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools [Computer software and manual]. *Nucleic Acids Research*, *24*, 4876–4882.

Tóth, M. & Szöcs, G. (1988). Field tests with sex attractants of *Diachrysis chrysitis* (L.) und *D. tutti* (Kostr.) (Lepidoptera, Noctuidae) at several sites in Hungary. *Zeitschrift für Naturforschung*, *43*, 463–466.

Urbahn, E. (1967). Zur Klärung der *Plusia chrysitis-tutti*-Frage durch Eizuchtuntersuchungen (Lepidoptera, Noctuidae). *Reichenbachia*, *8*, 133–137.

Willett, C. S. (2000). Do pheromone binding proteins converge in amino acid sequence when pheromones converge? *Journal of Molecular Evolution*, *50*, 175–183.

Wright, S. (1978). *Variability Within and Among Natural Populations*. Chicago, IL: University of Chicago Press.