*Systematic Entomology* (2003) **28**, 257–273

# Phylogenetic relationships of *Phyciodes* butterfly species (Lepidoptera: Nymphalidae): complex mtDNA variation and species delimitations

NIKLAS WAHLBERG*, RITA OLIVEIRA* and JAMES A. SCOTT†
*Metapopulation Research Group, Department of Ecology and Systematics, University of Helsinki, Finland, and †Lakewood, Colorado, U.S.A.

**Abstract.** Mitochondrial DNA variation was studied in the butterfly genus *Phyciodes* (Lepidoptera: Nymphalidae) by sequencing 1450 bp of the COI gene from 140 individuals of all eleven currently recognized species. The study focused on four species in particular that have been taxonomically difficult for the past century, *P. tharos*, *P. cocyta*, *P. batesii* and *P. pulchella*. A cladistic analysis of ninety-eight unique haplotypes showed that *Phyciodes* forms a monophyletic group with *P. graphica* as the most basal species. Of the three informal species groups described for *Phyciodes*, only one (the *mylitta*-group) is unambiguously monophyletic. Within the *tharos*-group, seven well supported clades were found that correspond to three taxa, *P. tharos*, *P. pulchella* and a grade consisting of *P. cocyta* and *P. batesii* haplotypes interdigitated with each other. None of the clades is formed exclusively by one species. The patterns of haplotype variation are the result of both retained ancient polymorphism and introgression. Introgression appears to be most common between *P. cocyta* and *P. batesii*; however, these two species occur sympatrically and are morphologically and ecologically distinct, suggesting that the level of current introgression does not seem to be enough to threaten their genetic integrity. The results indicate that mitochondrial DNA sequences must be used with great caution in delimiting species, especially when infraspecific samples are few, or introgression seems to be rampant.

## Introduction

The wealth of information on butterflies is unrivaled among invertebrates. The superfamily Papilionoidea is taxonomically very well known, with the majority of species probably already described. Butterflies have been used as model systems for a wide range of evolutionary and ecological studies (Boggs *et al*., 2003), yet the study of speciation in butterflies has focused on few groups such as *Heliconius* (Jiggins *et al*., 1996, 2001; Jiggins & Davies, 1998) and *Papilio* (Sperling, 1987; Sperling & Harrison, 1994). These studies have generally looked at pairs of closely related species to elucidate the factors leading to their divergence. However, like other groups of organisms, butterflies include numerous examples of species complexes in which there are several to many entities that may or may not represent species. With the increasing ease of generating molecular datasets suitable for studies of speciation, we can now assess whether these species complexes represent one or more species.

The rapid evolution of mitochondrial DNA (mtDNA) sequences has often been used to investigate the relationships of populations within species (Avise, 2000) and the relationships of closely related species (Sperling, 2003). More recently, the use of mtDNA sequences to identify (Kruse & Sperling, 2001) and delimit (Brower, 1999; Templeton, 2001; Wiens & Penkrot, 2002) species has been advocated. Mitochondrial DNA sequences are thought to better recover species-level relationships because a smaller effective population size for this essentially non-recombining, maternally inherited genome leads to shorter coalescence times (Moore, 1995; Sperling, 2003). A shorter coalescence time means that a gene tree is more likely to reflect a species tree (Avise, 2000).

Correspondence: Niklas Wahlberg, Department of Zoology, Stockholm University, S-106 91 Stockholm, Sweden. E-mail: niklas.wahlberg@zoologi.su.se

We investigated the mitochondrial haplotype diversity of the cytochrome oxidase subunit I (COI) gene in the butterfly genus *Phyciodes*, which is widespread in the Nearctic Region. Ten of the eleven species belonging to *Phyciodes* form a well defined group. One species, *P. graphica*, has been placed either within *Phyciodes* (Higgins, 1981) or in the Neotropical genus *Eresia* (Scott, 1994). Nine species included in a cladistic analyses of mtDNA sequences of tribe Melitaeini (to which *Phyciodes* belongs) formed a strongly supported monophyletic group (Wahlberg & Zimmermann, 2000).

The ten species other than *P. graphica* have been informally divided into three species groups (Scott, 1994, 1998), the *mylitta*-, *tharos*- and *phaon*-groups (Table 1). The only available phylogenetic hypothesis for *Phyciodes*, using DNA sequences, suggests that the first two species groups form monophyletic groups, whereas the latter species group is polyphyletic (Wahlberg & Zimmermann, 2000). The potential monophyly of the *phaon*-group is nevertheless supported by three distinct genitalic characters (Scott, 1994).

The *tharos*-group of four species has greatly troubled taxonomists because some of the species are similar in wing pattern and other traits, and several species have been distinguished only recently. New characters of the genitalia, antennae, larvae, pupae, hostplants and ecology (Scott, 1994, 1998) have proven to be of some help, but most characters vary considerably even within populations, and many taxa can only be identified through unique combinations of character states, even if some character states are apparently plesiomorphic. There is some parallel variation in *Phyciodes*, including possible mimicry. For instance, the higher-altitude subspecies *P. orseis herlani* and *P. pulchella montana* in the Sierra Nevadas are much more orange than all other populations of the species, and perhaps are Batesian mimics of the orange *Euphydryas chalcedona sierra* (Scott, 1986a). Also, genitalic structures of *P. tharos* are identical to those of *P. mylitta*, whereas they differ in other species of the genus, although this similarity could be due to common ancestry (e.g. retention of the plesiomorphic condition).

Currently, the *tharos*-group is thought to consist of four *bona fide* species: *P. tharos, P. pulchella, P. cocyta* and *P. batesii*. *Phyciodes cocyta* and *P. batesii* are mainly found in northern North America and they are sympatric over most of their range (Fig. 1). *Phyciodes tharos* and *P. pulchella* have mainly parapatric ranges both with each other and with *P. cocyta* and *P. batesii*, though there are areas of sympatry with the latter two (Fig. 1). *Phyciodes tharos* and *P. cocyta* have been considered conspecific until recently (Scott, 1994), yet they occur sympatrically over large areas without apparently interbreeding. *Phyciodes batesii* and *P. cocyta* are also considered to clearly be different species as they have widely differing ecologies, population structures and phenotypes over much of their range (Scott, 1994, 1998). However, in the Rocky Mountains of Colorado and Utah, *P. cocyta* and *P. batesii* are often more difficult to identify, and the two species may actually interbreed in this area (Scott, 1994, 1998).

For such morphologically difficult groups, it is thought that molecular characters, particularly mtDNA, can help in assessing the species boundaries (Brower, 1999; Templeton, 2001; Wiens & Penkrot, 2002). With appropriate sampling for molecular data, it is also possible to discover something

**Table 1.** The species and subspecies belonging to the three species groups of *Phyciodes* according to Scott (1994, 1998), Gatrelle (1998) and Austin (1998a,b). Taxa marked with an asterisk were included in this study.

*P. mylitta*-group

*mylitta*
- *mylitta** (Edwards, 1861)
- *arizonensis** Bauer, 1975
- *mexicana* Hall, 1928
- *arida** (Skinner, 1917)
- *thebais* Godman & Salvin, 1878

*pallida*
- *pallida** (Edwards, 1864)
- *barnesi** Skinner, 1897

*orseis*
- *orseis** Edwards, 1871
- *herlani** Bauer, 1975

Placed in *Eresia*

*graphica*[1]* (Felder, 1869)

*P. tharos*-group

*tharos*
- *tharos** (Drury, 1773)
- *riocolorado** Scott, 1992
- *orantain** Scott, 1998

*cocyta*[2]
- *cocyta* (Cramer, 1777)
- *diminutor** Scott, 1998
- *selenis** (Kirby, 1873)
- *arenacolor* Austin, 1998

*batesii*
- *batesii** (Reakirt, 1865)
- *lakota** Scott, 1994
- *apsaalooke** Scott, 1994
- *anasazi** Scott, 1994
- *maconensis** Gatrelle, 1998

*P. tharos*-group

*pulchella*[3]
- *pulchella** (Bdv., 1852)
- *deltarufa* Scott, 1998
- *montana** (Behr, 1863)
- *owimba** Scott, 1998
- *tutchone** Scott, 1994
- *camillus** Edwards, 1871
- *shoshoni* Scott, 1994
- *vallis* Austin, 1998
- *inornatus* Austin, 1998

*P. phaon*-group
- *pallescens** (Felder, 1869)

*picta*
- *picta** (Edwards, 1865)
- *canace** (Edwards, 1871)

*phaon*
- *phaon** (Edwards, 1864)
- *jalapeno** Scott, 1998

[1]*Phyciodes graphica* is a senior subjective synonym of *P. vesta* (see Opler & Warren, 2002).
[2]*Phyciodes cocyta* is also known as *P.* '*tharos* type B', *P. pascoensis* and *P. morpheus*. The latter three names were synonymized with *P. cocyta* by Scott (1994).
[3]*Phyciodes pulchella* is also known as *P. campestris* and *P. pratensis*. The latter two names were synonymized with *P. pulchella* by Scott (1994).

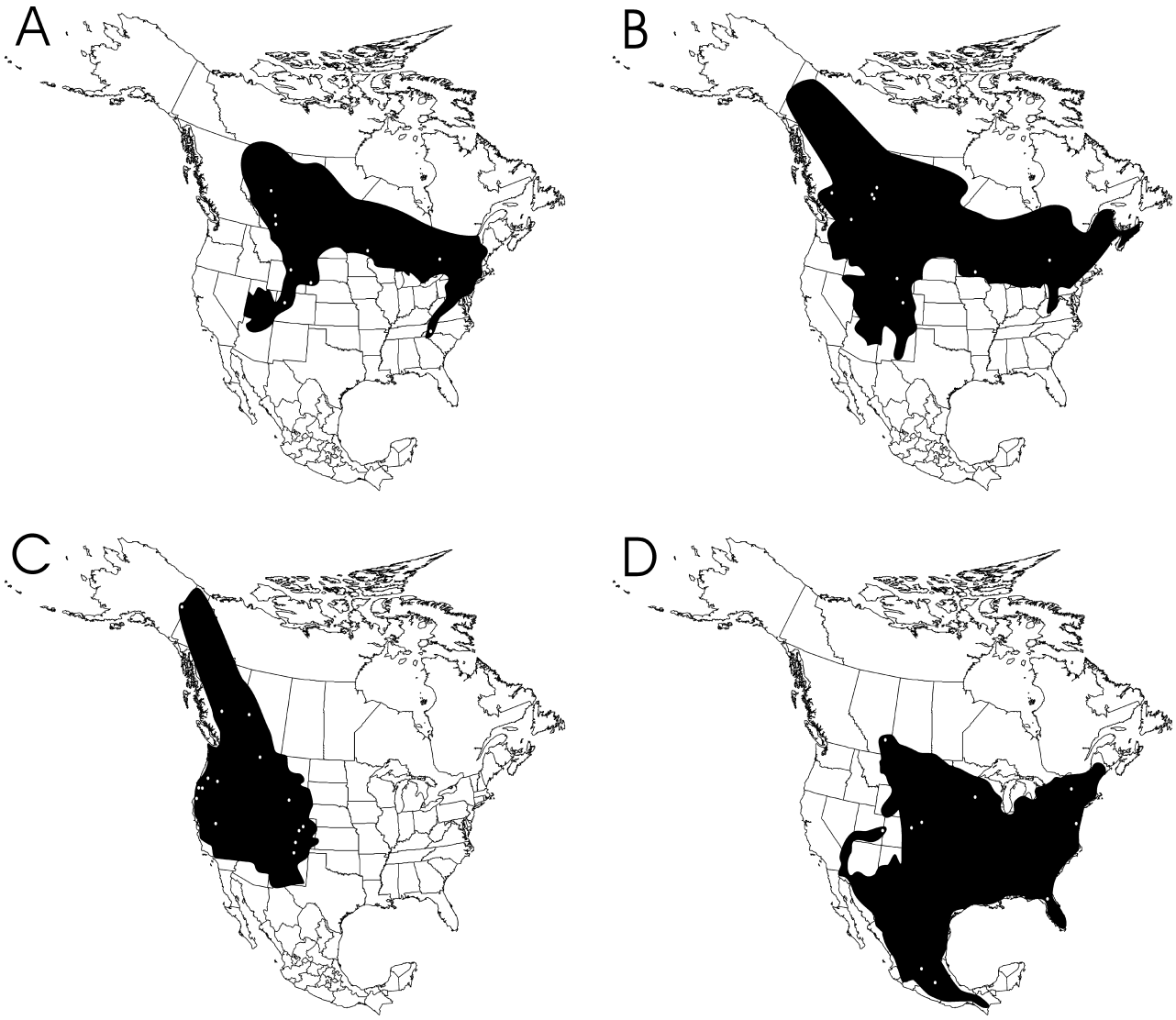


**Fig. 1.** Distributions of the four currently recognized *Phyciodes* species in the *tharos*-group. The distributions are modified from Layberry *et al*. (1998) for Canada and Opler *et al*. (1995) for the U.S.A and northern Mexico. A, *Phyciodes batesii*; B, *Phyciodes cocyta*; C, *Phyciodes pulchella*; D, *Phyciodes tharos*. The white dots show the collection localities of specimens used in this study.

about the processes behind the patterns. In this paper we investigate the phylogenetic relationships of *Phyciodes* species based on mtDNA sequences, including elucidation of the generic placement of *P. graphica*. We specifically aim to gain new insights into the taxonomic status and evolutionary history of the entities presently recognized as four species in the taxonomically difficult *tharos*-group.

## Materials and methods

### *Taxon sampling and sequenced gene*

We attempted to sample as many individuals per taxon from as many different populations as possible (Appendix 1, Fig. 1). Butterflies were collected by J.A.S. and colleagues (see Acknowledgements). Samples were stored under dry conditions at room temperature and sent by airmail to N.W. and R.O. for further processing. Two to forty individuals (total of 140) were sampled for all species (Appendix 1). Each individual was identified to subspecies by one of us (J.A.S.) prior to sequencing.

A 1450-bp segment of the COI gene was sequenced for all individuals. The sequence corresponds to positions 1539–2989 in the *Drosophila yakuba* Burla mtDNA sequence (Clary & Wolstenholme, 1985). The COI gene has been proposed as a standard for studies of the molecular phylogenetics of insects (Caterino *et al*., 2000; Sperling, 2003).

To investigate the monophyly of *Phyciodes*, we analysed the dataset with the COI dataset of Wahlberg & Zimmermann

(2000), excluding species for which only half of COI was successfully sequenced, using parsimony jackknifing (Farris *et al*., 1996). The outgroup dataset thus included sixty-three species, of which eight species in three genera (*Anthanassa*, *Castilia* and *Tegosa*) belong to subtribe Phyciodina and are part of the putative sister group of *Phyciodes*. Trees were rooted with *Asterocampa leilia* (Nymphalidae: Apaturinae) as in Wahlberg & Zimmermann (2000). Owing to the cumbersome number of trees found for the entire dataset (see Results), we used two outgroup species, *Chlosyne acastus* (GenBank Accession number AF187735) and *Anthanassa ardys* (GenBank Accession number AF187743) for more detailed analyses of *Phyciodes* sequences.

### *Molecular techniques*

Genomic DNA was extracted from the legs of dried specimens that had not been relaxed for spreading. Relaxing specimens may result in invasion of tissues by fungi or bacteria that break down cells, and thus DNA. DNA was extracted either using a standard phenol/chloroform protocol (for details see Zimmermann *et al*., 2000) or QIAgen's DNEasy extraction kit. All specimens can be viewed at http://www.zoologi.su.se/research/wahlberg/phyciodes.htm. Most voucher specimens are stored in the Department of Zoology, Stockholm University, but some remain in the private collections of the collectors.

Two primer pairs were used to amplify the COI gene, LCO1490-J-1514 (5′GGTCAACAAATCATAAAGATATTGG) and HCO2198-N-2175 (5′TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al*., 1994), and C1-J-2183 (5′CAACAYTTATTTTGATTTTTTGG) and TL2-N-3014 (5′ATCCATTACATATAATCTGCCATA) (Simon *et al*., 1994). All fragments were amplified with PCR in a total volume of 20 µl. The following thermal cycling protocol was used: 5 min at 95 °C, thirty-five cycles of 30 s at 94 °C, 30 s at 47 °C and 1 min 30 s at 72 °C, and a final extension of 10 min at 72 °C. PCR fragments were then sequenced using either an ABI 377 Automated Sequencer (Espoo, Finland) or a Beckman-Coulter CEQ2000 capillary sequencer (Bromma, Sweden) and a dye terminator cycle sequencing kit. Sequences were aligned by eye as the length of this protein-coding gene is highly conserved and insertions or deletions have not been observed in melitaeines (Wahlberg & Zimmermann, 2000; Zimmermann *et al*., 2000). Each unique haplotype sequence is available from GenBank (Accession Nos. AF187747, AF187785, AF187789, AF187798, AF187800, AF187783, AF187807, AY156595-AY156686).

### *Phylogenetic analyses*

The patterns of nucleotide substitutions among sequences were analysed using the program MEGA2 (Kumar *et al*., 2001). The number of transitions and transversions were plotted against sequence divergence values to investigate the degree of transition saturation apparent in the dataset using the program DAMBE (Xia & Xie, 2001).

Most parsimonious cladograms were searched for from the equally weighted and unordered data matrix (*Phyciodes* plus the two outgroups mentioned above) using a heuristic search algorithm of NONA 2.0 (Goloboff, 1998). The heuristic searches were conducted with 10–1000 random addition replicates using tree bisection-reconnection (TBR) branch swapping with up to twenty trees held during each step. Trees were drawn with the aid of WINCLADA (Nixon, 2002).

The robustness of the clades in the resulting cladograms was evaluated using jackknife (Farris *et al*., 1996) and Bremer support (Bremer, 1988, 1994) analyses. Jackknife values were calculated for the entire data matrix (i.e. including the sixty-three outgroup species) from 10 000 replicates at a cutoff point of 50% using the program PARSIMONY JACKKNIFER (Farris, 1995). Bremer support was calculated using the dataset with only two outgroup species with the program TREEROT (Sorensen, 1999), in conjunction with PAUP* 4.0b10 (Swofford, 1998).

## Results

Ninety-eight unique mitochondrial haplotypes were found in the 140 individuals of *Phyciodes* sequenced. In these ninety-eight haplotypes, there were 315 variable sites in the 1450 bp of the COI gene, of which 245 were parsimony informative (Table 2). The uncorrected pairwise sequence divergences ranged from close to 0% to about 5%, excluding the *P. graphica* sequences, and about 7% between the *P. graphica* sequences and all the other ingroup sequences (Fig. 2). Saturation of substitutions was not obvious in the data as the number of transitions and transversions in

**Table 2.** Basic statistics of the *Phyciodes* sequences.

| | | | | Empirical base frequencies (%) | | | |
|---|---|---|---|---|---|---|---|
| Position | No. of sites | No. variable | No. informative | A | G | C | T |
| All positions | 1450 | 321 | 246 | 31.3 | 13.7 | 14.4 | 40.6 |
| First position | 484 | 52 | 31 | 31.9 | 24.2 | 14.2 | 29.7 |
| Second position | 483 | 14 | 4 | 18.7 | 15.6 | 23.5 | 42.1 |
| Third position | 483 | 255 | 211 | 43.3 | 1.1 | 5.7 | 49.9 |

pairwise comparisons increased with sequence divergence, with the former increasing faster than the latter (Fig. 2). The number of transitions still exceeded the number of transversions at the highest level of divergence, although the transition/transversion ratio approached 1. Interestingly, the inferred types of changes are highly asymmetric when plotted on to the most parsimonious cladogram described below (Table 3). For instance, the transition from T to C is much more common than the opposite transition (from C to T) and, likewise, the transversion from T to A is more than twice as common as the opposite transversion (from A to T). Any modelling of these sequences would have to take these assymmetries into account. However, a more detailed investigation of the molecular evolution of COI sequences in *Phyciodes* is beyond the scope of this paper.

In the jackknife analysis, *Phyciodes* (including *P. graphica*) formed a well supported clade (Fig. 3). The sister group of *Phyciodes* was unresolved in the jackknife tree, although the eight other Phyciodina species form a monophyletic sister group to *Phyciodes* with relatively good Bremer support in the strict consensus of the 37 056 most parsimonious trees found for the entire dataset (Fig. 3). *Phyciodes graphica* is clearly a species of *Phyciodes*, albeit a basal and highly divergent (relatively speaking) one.

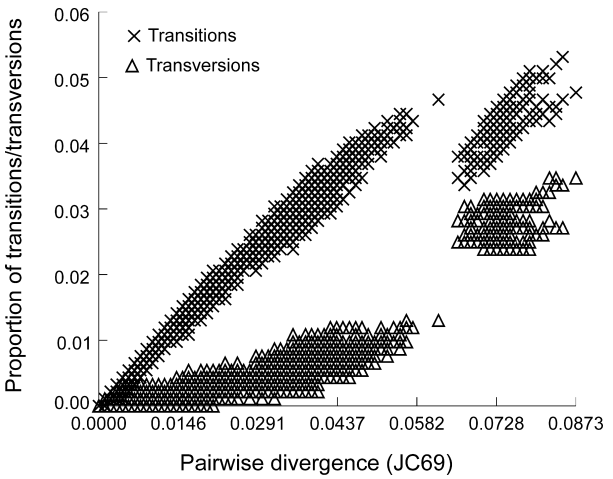


**Fig. 2.** The number of transitions and transversions plotted against the uncorrected pairwise sequence divergences. The comparisons between *Phyciodes graphica* and the other *Phyciodes* species can be seen as an independent cluster on the right side of the graph.

**Table 3.** The inferred directions and numbers of changes in the *Phyciodes* sequences (excluding outgroup taxa).

| | | To | | | |
|---|---|---|---|---|---|
| | | A | C | G | T |
| F | A | – | 8 | 66 | 19 |
| r | C | 3 | – | 0 | 43 |
| o | G | 20 | 1 | – | 0 |
| m | T | 41 | 205 | 7 | – |

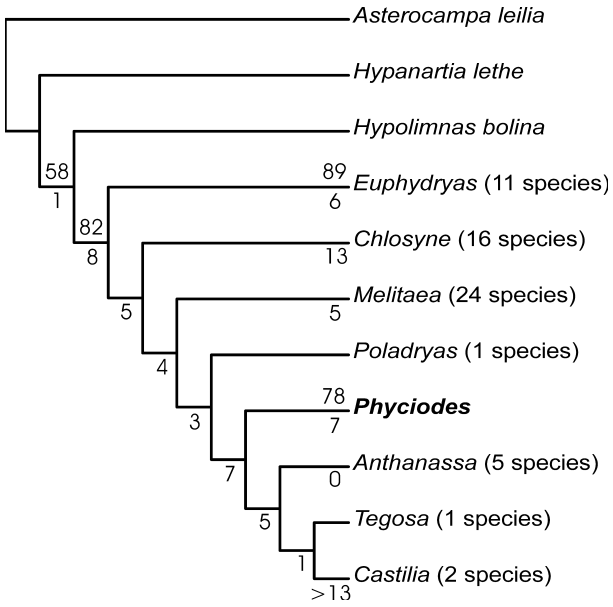


**Fig. 3.** The strict consensus of 37 056 equally parsimonious trees (length 3582 steps, CI = 0.22, RI = 0.72, the latter two indexes calculated with uninformative characters removed) with jackknife percentages/Bremer support values to the left of each node. The consensus tree is simplified to show only genera. For the internal relationships of genera other than *Phyciodes*, see Wahlberg & Zimmermann (2000).

Whether there are still more species in Phyciodina that could be considered to be part of genus *Phyciodes* needs to be investigated with a larger sampling of the about 130 currently recognized Neotropical species.

Parsimony analysis of the dataset with only two outgroup species produced 1728 equally parsimonious trees, for which the strict consensus is shown in Fig. 4. Within *Phyciodes*, the species other than *P. graphica* form a strongly supported monophyletic group, although only one of the three traditional species groups (the *mylitta*-group) is unambiguously monophyletic. The *mylitta*-group of three species is very strongly supported, with *P. orseis* basal to *P. pallida* and *P. mylitta* (Fig. 4). The *P. mylitta* haplotypes form a monophyletic clade with relatively good support, but the sister relationship of the two *P. pallida* haplotypes in the parsimony analyses has no support. In fact, prior to the addition of *P. mylitta arida* (67-10) to the data matrix, the *P. pallida barnesi* haplotype was sister to the *P. mylitta* clade, with the *P. pallida pallida* haplotype basal. For mtDNA, as for wing pattern, *P. orseis herlani* seems to be just a subset of the variation that occurs within *P. orseis orseis*.

The *phaon*-species group is paraphyletic as observed by Wahlberg & Zimmermann (2000). *Phyciodes picta* and *P. pallescens* form a very strong monophyletic group that is basal to the other *Phyciodes* species, excluding *P. graphica*. The sister relationship of *P. picta* and *P. pallescens* is not surprising as they are sometimes considered to be the same species (e.g. Higgins, 1981), although the wing pattern of

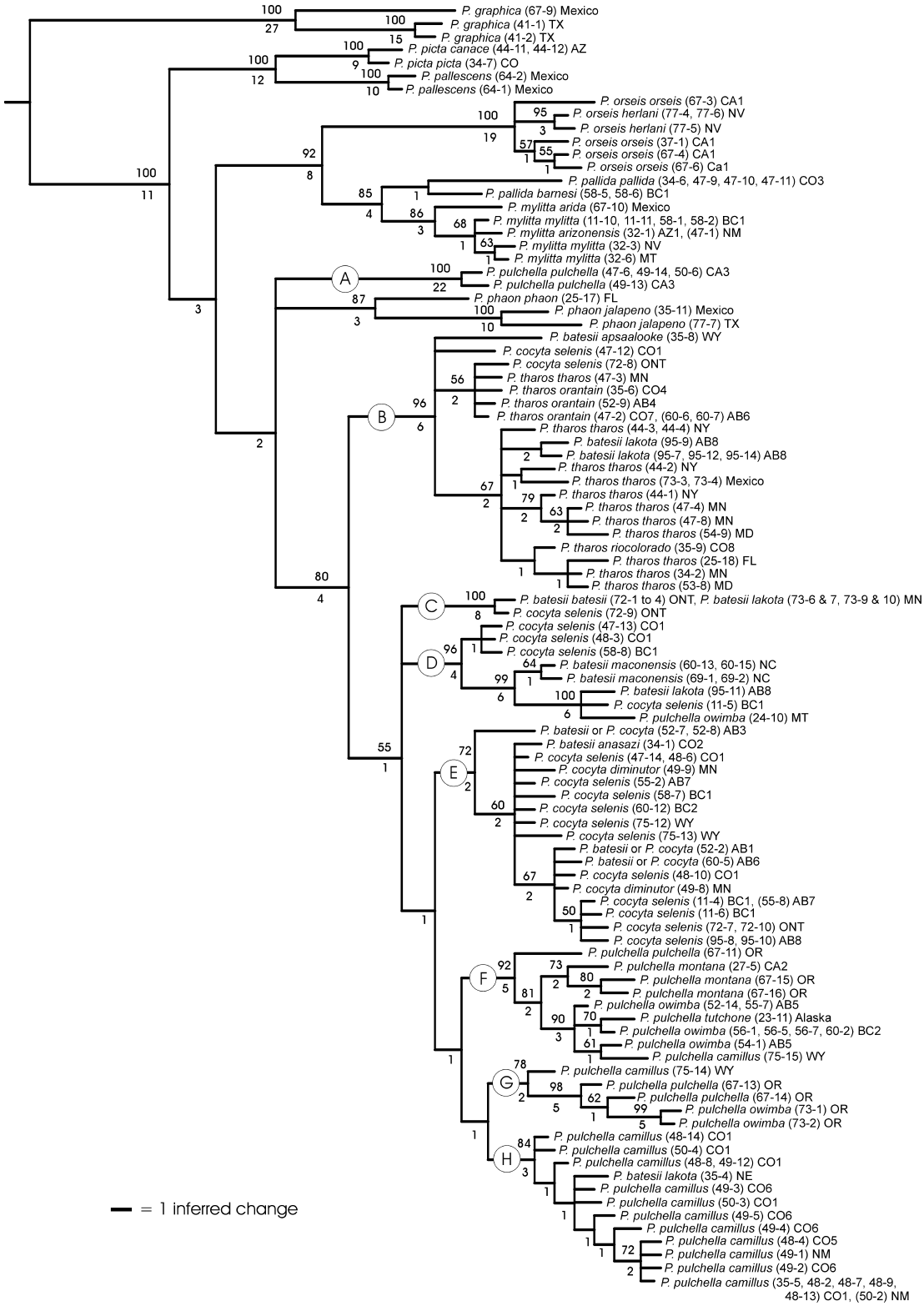


**Fig. 4.** The strict consensus of 1728 equally parsimonious trees (length 841 steps, CI = 0.48, RI = 0.84, the latter two indexes calculated with uninformative characters removed) found for the dataset with only two outgroup species (see text for details). Jackknife percentages/Bremer support values are given to the left of each node. Numbers in parentheses after the species names identify individuals from which DNA was extracted (see Appendix 1). Clades discussed in the text are marked with capital letters in circles.

*P. picta* more closely resembles that of *P. phaon*. The position of *P. phaon* is not entirely clear, but it appears to be basally related to the *tharos*-group. Its position is confounded by two anomalous *P. pulchella* haplotypes (see below).

The *tharos*-group of four species, with 107 individuals sequenced, forms a well supported monophyletic group with the exception of two *P. pulchella* haplotypes (labelled clade A in Fig. 4), which form a basal polytomy with *P. phaon* and the rest of the *tharos*-group. There are seven well supported major clades in the *tharos*-group (labelled clades B–H in Fig. 4). Clade B mainly consists of *P. tharos* haplotypes with one *P. cocyta* haplotype and two *P. batesii* haplotypes among the *P. tharos* haplotypes, and one *P. cocyta* and one *P. batesii* haplotype basal to the *P. tharos* haplotypes. Clades C to E mainly consist of *P. cocyta* haplotypes, although almost all *P. batesii* haplotypes fall within these clades as well. There is also one *P. pulchella* haplotype in clade D. It is interesting to note that *P. cocyta* haplotypes from individuals collected on the same day from the same population (in Jefferson County, Colorado) fall into three clades (clades B, D and E). Likewise, *P. cocyta* haplotypes from one British Columbian population fall into two clades (D and E), and *P. cocyta* haplotypes from one population in Ontario are found in three clades (B, C and E). The remaining clades (F to H) are consist almost exclusively of *P. pulchella* haplotypes, with the exception of one *P. batesii* haplotype in clade H. The relationships of the seven clades are not well supported. The most parsimonious solution (Fig. 4) suggests that the *P. tharos* clade (B) is basal to the other clades. *Phyciodes cocyta* (C to E) forms a paraphyletic assemblage with respect to *P. pulchella* (F to H), with clade E being sister to the *P. pulchella* clades. The three *P. pulchella* clades (F to H) appear to form a monophyletic assemblage.

In summary, the main results for the *tharos*-group are that *P. tharos* forms a distinct clade basal to most of the other *tharos*-group species; *P. cocyta* is not closely related to *P. tharos* (as has always been assumed from morphological–ecological traits), rather the majority of its mtDNA haplotypes are closer to *P. pulchella*; the haplotypes of *P. cocyta* and *P. batesii* do not form monophyletic groups, but are paraphyletic with regard to *P. pulchella*; the haplotypes of *P. pulchella* appear to form a monophyletic group, with the exception of two anomalous haplotypes that may even be outside of the *tharos*-group clade; and, finally, *P. batesii* and *P. cocyta* haplotypes are interdigitated with each other for the most part.

## Discussion

### *mtDNA, closely related species and sampling strategy*

Theoretical models suggest that during divergence a species pair may go through stages of polyphyly to paraphyly before reaching monophyly (Neigel & Avise, 1986; Pamilo & Nei, 1988; Avise & Ball, 1990; Avise, 2000). Thus, assuming that species limits have been accurately assessed and introgressive hybridization is not a factor, the presence of non-monophyletic lineages in different species can be attributed to the inheritance of allelic polymorphisms from ancestral populations and their persistence in descendent species (Neigel & Avise, 1986; Pamilo & Nei, 1988; Avise & Ball, 1990). The appearance of non-monophyletic lineages may also be due to interspecific hybridization leading to genomic introgression (Boyce *et al*., 1994; Odorico & Miller, 1997; Sota & Vogler, 2001). These two explanations are more fully discussed below.

Species are often assumed to be monophyletic entities in ecological and evolutionary studies (Harrison, 1998). This assumption is also implicit in the many molecular systematic studies concerned with species-level phylogenies based on only one individual per species. Such an assumption is valid if each species has gone through a severe population bottleneck during speciation or lineage sorting has gone to completion (Pamilo & Nei, 1988), something which may or may not be the case and certainly should not be assumed. The potential presence of poly- and paraphyletic lineages must especially be taken into account in recently diverged species, as has been shown both theoretically (Neigel & Avise, 1986; Avise & Ball, 1990) and empirically (Avise *et al*., 1983; Brown *et al*., 1994; Parker & Kornfield, 1997; Funk, 1999; Sota & Vogler, 2001).

The COI gene in the mitochondrial genome proved to be an excellent source of information for the set of very closely related species belonging to the genus *Phyciodes*. The hierarchical information content of the dataset was not obscured by saturation of substitutions and the relationships of the different mtDNA lineages were well supported in general. However, species defined using non-DNA characters were not well defined using mtDNA characters, and indeed there were a large number of poly- and paraphyletic mtDNA lineages in different taxa of *Phyciodes*.

Several previous studies advocated the use of mtDNA sequences as aids in identifying closely related species (Sperling & Hickey, 1994; Sperling *et al*., 1995; Cognato *et al*., 1999; Kruse & Sperling, 2001), and many studies explicitly stated the appropriateness of mtDNA in resolving the relationships of closely related species (e.g. in butterflies, Brunton & Hurst, 1998; Caterino & Sperling, 1999; Rand *et al*., 2000; Monteiro & Pierce, 2001). Our results show that both of these assumptions need to be investigated in more detail. We did not observe haplotypes being shared between species of *Phyciodes*, although some haplotypes were sufficiently similar (only one or two base pairs out of 1450 bp differed) that they may be found to be shared with broader sampling. The results also show that a broad sampling strategy is imperative for closely related species, as has been emphasized by Funk (1999). If we had sampled only one specimen per species, the resulting relationships would have been well supported, but dependent on the haplotypes sampled by chance.

The quality of the dataset is not only due to the large number of individuals sampled, but also the length of the sequence analysed (1450 bp). Many studies looking at

closely related species sequence only 400–600 bp per specimen and find few informative characters between species (e.g. Pedersen, 1996; Walton *et al*., 1997; Brunton & Hurst, 1998; Brunton, 1998; Crespi *et al*., 1998; Wetterer *et al*., 1998; Brown *et al*., 1999; Nice & Shapiro, 1999, 2001). Our relatively long sequence shows that there can be much information in mtDNA despite low pairwise divergence values. This added information may be able to resolve polytomies evident in studies using shorter sequences, and indeed even longer sequences than we analysed may be able to resolve the polytomies found in our study (Fig. 4).

### *Species concepts and* Phyciodes

Taxonomists have defined eleven species as belonging to *Phyciodes* as delimited in this study. These species were described mainly on the basis of morphological and eco-ethological criteria and are the products of the typological species concept. Such a concept has its utility when attempting to establish some order for the perceived chaos nature confronts us with, but in the long run will not help to understand how and why the entities in nature that we call species have come to be there. The multitude of species concepts that have been developed attempt to partition the continuum of variation in natural populations into discrete boxes (species) based on different criteria. In many cases it is possible to do so, but in some cases different sources of evidence can be in serious conflict, as is the case with the *tharos*-group of species.

It is obvious that taxa that do not interbreed naturally and produce inviable offspring in the laboratory can be considered to be separate species. Problems arise when a group of populations are in the process of speciation and have not yet become fully reproductively isolated. It is at this interface that the traditional biological species concept does not satisfactorily define what is one species contra what are several species (Harrison, 1998). Here it is the four species in the *tharos*-group that need to be scrutinized.

A series of interspecific hybridization experiments on *Phyciodes* indicate that some populations are reproductively isolated from each other, at both the prezygotic and the postzygotic stage (Oliver, 1972, 1978, 1979, 1980, 1982). The clearest results from these experiments are the much reduced egg hatching rates observed in crosses between *P. tharos* and *P. pulchella montana* (Oliver, 1978), *P. tharos* and *P. phaon* (Oliver, 1982), *P. tharos* and *P. batesii* (Oliver, 1979) and *P. cocyta* and *P. batesii* (Oliver, 1979; whose Syracuse 'tharos type B' were *P. cocyta*). Crosses between *P. tharos* and *P. cocyta* gave ambiguous results and viable offspring (Oliver, 1972, 1980; Scott, 1986b), but some of the parents in Oliver's (Oliver, 1972, 1980) hybridization studies may not have been identified as *P. tharos* or *P. cocyta* properly, as the taxon *P. cocyta diminutor* was only recently described from the northeastern United States (Scott, 1998). A recent enzyme electrophoretic study which showed that *P. tharos* and *P. cocyta* are conspecific in Ohio and Michigan (Porter & Mueller, 1998) actually used specimens that Scott has since reidentified as *P. cocyta selenis* and *P. cocyta diminutor*. This unfortunate misidentification invalidates the conclusion of Porter & Mueller (1998), but does show that there is no reproductive barrier between the two subspecies of *P. cocyta*.

From Oliver's (1972, 1978, 1979, 1980, 1982) experiments it is clear that *P. tharos* is not conspecific with *P. pulchella* or *P. batesii*. Also, *P. cocyta* and *P. batesii* show strong postzygotic isolation in the northeastern part of their range. However, the degree of reproductive isolation between *P. cocyta* and the *P. tharos* remains ambiguous. Reproductive isolation between *P. cocyta* and the other three *tharos*-group species seems likely in nature based on the distinct phenotypes observed in sympatric populations, although in the western parts of the ranges of *P. cocyta* and *P. batesii* the subspecies *P. batesii anasazi* appears to be *P. batesii apsaalooke* that has introgressed with *P. cocyta* (Scott, 1998).

Based on Oliver's observations and the long-term familiarity with these species in the field of J.A.S., we prefer to interpret our results in the framework of the traditionally held concepts of species in the *tharos*-group. Our results can be interpreted to be in strong conflict with the traditionally defined species in the *tharos*-group, especially if one would redefine species based on mtDNA. Since mtDNA disagrees so strongly with what any good field biologist can observe in nature regarding *Phyciodes*, we will question the traditional species concepts, but will defer any actual changes to them until further investigations have taken place.

### *Phylogenetic history of* Phyciodes

Different species have had different histories of population division and isolation, and thus each species requires an individual explanation of how it came to be (Hewitt, 1996). In the present study, we found the most complex relationships within the *tharos*-group, where there are seven well supported haplotype clades (Fig. 4). Since we sampled the *tharos*-group most intensively, we will discuss the patterns found in that group in the most detail, but first we will briefly discuss the implications of the results for the other seven species.

The most basal species of *Phyciodes*, according to the results, is *P. graphica*, which has sometimes been placed in the putative sister group to *Phyciodes*, the Neotropical Phyciodina (Scott, 1994). The three individuals sequenced for this study differed from all the other *Phyciodes* by about 7% (uncorrected pairwise divergences). There is potentially much more variation within *P. graphica*, as the Texan individuals differed from the Mexican individual by about 3%.

The next clade up includes *P. picta* and *P. pallescens*, which differ from each other by about 2%. These two species are sometimes considered to be conspecific (Higgins, 1981). The two haplotypes found within each differ little from one another. The difference between the two species is within the range of difference between *P. graphica* and *P. phaon*, which do not seem to belong to any well defined

species group. On the other hand, the difference between *P. picta* and *P. pallescens* is also within the range of differences between species in the *mylitta*- and *tharos*-groups. Thus we are unable to make any strong statements about the specific status of *P. pallescens* with our limited sampling. In wing pattern, *P. pallescens* differs greatly from *P. picta* and resembles *P. pulchella camillus*.

*Phyciodes phaon* seems to be more closely related to the *tharos*-group than to *P. picta* and *P. pallescens* (Fig. 4) based on mtDNA. There is, however, a possibility that the *phaon*-group forms a paraphyletic grade with regard to the *mylitta*- and *tharos*-groups since the support for the basal nodes is very weak and new information (e.g. nuclear gene sequences) may change the position of *P. phaon*. The three *P. phaon* individuals sequenced in this study form a strong monophyletic group even though the Floridian individual shows almost 3% difference to the Texan and Mexican individuals, and the latter two are over 1% different. Once again there is potentially much more variation in *P. phaon* that would be uncovered by more intensive sampling of this species.

*Phyciodes orseis* is quite clearly the basal member of the *mylitta*-group. The six haplotypes found for this species differ by over 3% from the haplotypes of *P. pallida* and *P. mylitta*, and they form a very strong monophyletic clade. Also, the five haplotypes found in *P. mylitta* form a well supported monophyletic clade, with very little variation within the species (despite our very wide range of samples of *P. mylitta* analysed). Individuals sampled from British Columbia, Nevada, Montana, Arizona and New Mexico differed by at most two base pairs in the 1450-bp sequence studied. The Mexican individual differed by almost 1% from the others, suggesting that there might be more variation in the southern parts of the species' range. *Phyciodes pallida* presents an interesting situation. The two haplotypes that we found differ from each other by 1.7%, but the *P. pallida barnesi* haplotype differs from the *P. mylitta* haplotypes by about 1.1%, whereas the *P. pallida pallida* haplotype differs by 2.1%. Our analyses place the two *P. pallida* haplotypes in a sister relationship, but the support for this clade is weak. Obviously more sampling of the widespread *P. pallida* is needed to survey the extent of variation in haplotypes.

The complex patterns of mtDNA variation found in the *tharos*-group may be the result of two non-exclusive mechanisms. One mechanism is gene flow through hybridization and the second is incomplete lineage sorting, i.e. the presence of ancestral lineages, in two or more species, that are more closely related to each other than to other lineages within a species. Both of these mechanisms may have contributed to the complex mtDNA variation present in the *tharos*-group, as explained below.

Disregarding clade A (Fig. 4) for the moment, the four putative species in the *tharos*-group form a well supported monophyletic clade. The present distributions of the four species may provide some insight into the patterns we have uncovered (Fig. 1). It is interesting to note that *P. tharos* and *P. pulchella* have a largely parapatric distribution and their haplotypes fall into exclusive clades. *Phyciodes cocyta* and *P. batesii* are found sympatrically over much of their range, and both are found sympatrically with the other two species as well. The haplotypes of *P. cocyta* and *P. batesii* are interdigitated and together they form a paraphyletic assemblage with regard to the western species *P. pulchella*. Also *P. cocyta* and *P. batesii* haplotypes are found within the *P. tharos* and *P. pulchella* clades.

Within the major *tharos*-group clade, all *P. tharos* haplotypes can be found in clade B, a monophyletic clade with good support. Clade B does not, however, include *P. tharos* haplotypes exclusively. One *P. cocyta* haplotype and two *P. batesii* haplotypes are found well within the *P. tharos* haplotypes (Fig. 4). These haplotypes are sufficiently similar to the *P. tharos* haplotypes that we hypothesize their origins in *P. cocyta* and *P. batesii* to be the result of introgressive hybridization. However, the individuals carrying the *P. tharos* haplotype do not exhibit any morphological characters of *P. tharos* and, thus, the hybridization event would have had to happen more than one generation prior to collecting the individuals. *Phyciodes tharos* occurs sympatrically with *P. cocyta* in the area where *P. cocyta* individual 72-8 was collected (R. Layberry, personal communication), but *P. tharos* does not occur in the area where the *P. batesii* individuals were collected (C. Schmidt, personal communication). There are two haplotypes basal to clade B, one from *P. batesii* and one from *P. cocyta*, that show a large number of autapomorphic changes, leading us to hypothesize that they represent ancestral lineages that have not yet been sorted out of their respective species. The *P. cocyta* haplotype is found in a population that does not occur sympatrically with *P. tharos*, but *P. batesii apsaalooke* is sympatric with *P. tharos*.

Similarly, clades F–H do not contain *P. pulchella* haplotypes exclusively. A *P. batesii* haplotype is found within clade H. The *P. batesii* haplotype is very similar to the *P. pulchella* haplotypes, so its origin in *P. batesii* possibly could be due to past introgressive hybridization; however, *P. pulchella* is rare in the area where the *P. batesii* individual was collected. One *P. pulchella* haplotype found outside clades F–H is in a predominantly *P. cocyta* clade (clade D), and may represent an ancient polymorphism that has been eliminated from most *P. pulchella* populations, but is still present in at least one population in Montana. Unfortunately, the individual was the only representative of the *tharos*-group from its region available for study. Further sampling is clearly needed.

The two distinctive haplotypes of *P. pulchella* in clade A are also problematic. They may represent a cryptic species, as they are so divergent from the rest of the *tharos*-group haplotypes and are only found in one of the *P. pulchella* populations sampled (from the northern Californian coastal area). Shapiro *et al.* (1979) noted that in northern California there are two allopatric populations of *P. pulchella* that do not interbreed, *P. pulchella pulchella* along the coast, and *P. pulchella montana* at higher altitudes inland. However, Shapiro (personal communication) later found that they interbreed completely in the Feather River region of California.

These populations appear to continue northwards and meet in southern Oregon, but as individual variants in variable populations. Oregon *P. pulchella* haplotypes from individuals that can phenotypically be identified as *P. pulchella pulchella* occur in clades F and G, as do all of the *P. pulchella montana* haplotypes. Whether clade A represents an ancient polymorphism that is retained in the Californian *P. pulchella* population, or the Californian population is a cryptic species, warrants further investigation.

*Phyciodes cocyta* and *P. batesii* must be considered together because their haplotypes are interdigitated. Our sample sizes of both taxa are nearly equal (twenty-one individuals of *P. batesii* vs twenty-three individuals of *P. cocyta*, plus four females that could be either species), yet we found no clades made up exclusively of one taxon or the other. The haplotype diversity of *P. cocyta* (twenty haplotypes) was much higher than that of *P. batesii* (nine haplotypes). From our rather limited sampling it would appear that clade C is a largely *P. batesii* clade, whereas clades D and E are largely *P. cocyta* clades. The four females that could not be definitely assigned to species are in clade E, suggesting that they may in fact be *P. cocyta* females. Both species also appear in clade B. Our rather scattered sampling over the distributions of the two taxa precludes a more detailed analysis of the co-occurrence of their haplotypes using, for example, Templeton's methods (Templeton *et al.*, 1995; Templeton, 2001). The patterns shown in Fig. 4 could result from extensive gene flow between *P. cocyta* and *P. batesii*, or ancient polymorphisms retained in both species today. Both mechanisms appear to be at work, since some of the haplotypes in the two species are very similar to each other (implying introgression), whereas others are relatively autapomorphic (implying ancient polymorphisms).

Morphological data suggest that *P. cocyta* and *P. batesii* do not interbreed over most of their ranges. Introgression appears to be happening today in the Rocky Mountains of Colorado and Utah. In these areas, *P. cocyta* and *P. batesii* are often more difficult to identify where the two approach each other altitudinally. In the eastern parts of their ranges, the males are easily separated in the field (Scott, 1994, 1998). Females sometimes are not identifiable in northern areas. *Phyciodes* females in general are more difficult to identify than males because they show fewer distinguishing features. However, even very rare events of introgression may allow the mtDNA genome of one species to enter the gene pool of another species, unless processes such as Haldane's rule are affecting the direction of gene flow (see Sperling, 1994).

The genetic and morphological evidence presented above suggests that *P. cocyta* and *P. batesii* have diverged allopatrically more recently than they diverged from *P. pulchella*, and were in contact before complete reproductive barriers (either prezygotic or postzygotic) developed throughout their ranges. To get a fuller picture of what is happening in *P. cocyta* and *P. batesii*, one would have to sample both taxa much more systematically over their ranges than we have done to discover the extent of their mtDNA haplotype variation.

By comparing Fig. 4 with the distribution maps (Fig. 1), we can deduce that the ancestral *tharos*-group species was distributed throughout North America; clade B haplotypes prevailed in southeastern U.S.A. and Mexico, later evolving into *P. tharos*; clades F to H were mostly in western North America, later evolving into *P. pulchella*; and clades C, D and E were mostly in the north, later evolving into *P. cocyta* and *P. batesii*. The boreal northern populations surely evolved into *P. cocyta*, whereas populations a little further south in more savannah-like habitats apparently evolved into *P. batesii*. Glaciation in the Rocky Mountains may have isolated the western populations more than the others, with the result that *P. pulchella* is more distinctive now than the other three members of the *tharos*-group. The correspondence of ranges with clades seems to add support to the conclusion that much of the mtDNA variation is ancient, and was passed down to the modern butterflies relatively intact.

### *Ancient polymorphisms or introgression?*

A recent method advocating the use of mtDNA to delimit species (Wiens & Penkrot, 2002) would force us to conclude that *P. cocyta* and *P. batesii* are conspecific, whereas morphological evidence points to these two being separate species. We must thus investigate in more detail the mechanisms that might lead to the patterns of haplotype variation observed in these two species and their close relatives.

We know that *Phyciodes* are quite variable in morphological traits. Wing patterns in the *tharos*-group vary greatly, the genitalia vary, larval and pupal colour pattern varies, the amount of silk spun by larvae varies, etc. From morphological traits, it seems clear that these butterflies have descended from variable ancestors, and today's descendants have retained this large variation mostly intact. The reproductive isolation mechanisms that permitted speciation evidently spread through ancestral populations without greatly affecting the remaining genome.

The morphological evidence suggests that hybridization presently only occurs between *P. cocyta selenis* and *P. batesii anasazi* where they approach each other altitudinally in western Colorado. Elsewhere, *P. batesii apsaalooke* is quite distinct from nearby *P. cocyta* and, going further away, *P. batesii lakota* is even more distinct, and *P. batesii batesii* and *P. batesii maconensis* are very distinct from *P. cocyta*. So far, wild hybrids between these pairs have not been identified, and past confusion in identifying '*P. batesii*' generally involved misidentified specimens of *P. cocyta* with *P. tharos*. Mating does not occur between *P. batesii batesii* and *P. cocyta* even in the laboratory (C. G. Oliver, personal communication).

Butterfly taxonomists previously thought that hybridization was frequent between *P. cocyta* and *P. tharos*. Oliver (1972, 1980) found laboratory hybridization to occur easily between them, and Scott (1986b) even crossed them in nature by releasing females of *P. cocyta* in front of wild

*P. tharos* males to produce F1 hybrids and backcrosses. But our mtDNA results (Figs 3, 4) show a single clade for *P. tharos*, suggesting that it is a distinct, non-hybridizing species. *Phyciodes tharos* and *P. cocyta* were considered to be one species until recently, then it was thought that they were barely distinct hybridizing species, but our DNA results suggest that actually they hybridize rarely.

Also, *P. pulchella* does not hybridize with the other members of the *tharos*-group. Nearly all *P. pulchella* differ in genital structure from the other *tharos*-group species: they can all be separated from the others by the colour of a ventral spot on the front margin of the forewing, and nearly all of their larvae are separable by head pattern. Oliver (1978) found that they would not mate even in the laboratory. Furthermore, *P. pulchella* always has blacker wings than the other *tharos*-group species everywhere they are sympatric. Hybrids would be easily detected by wing pattern if they occurred, yet J.A.S. has never seen an individual that looked like it could be a hybrid. Only in California–Oregon–western Nevada do oranger *P. pulchella* occur, but the other three *tharos*-group species do not occur there.

Thus, based on morphology, we are left with only one apparent case where hybridization may be happening today, in western Colorado between *P. cocyta* and *P. batesii anasazi*, where the latter seems to resemble *P. cocyta* the more closely it approaches it in altitude. Therefore, we conclude that most of the more autapomorphic haplotypes of *P. batesii*, *P. cocyta* and *P. pulchella* that are included in clades made up of largely heterospecific haplotypes are the result of retained ancient polymorphisms. Some haplotypes (specifically haplotypes 72-8, 95-7 and 95-9 in clade B; haplotype 72-9 in clade C; haplotype 34-1 in clade E; and haplotype 35-4 in clade H) are too similar (only 1 or 2 bp difference in 1450 bp of sequence) to the related heterospecific haplotypes to be explained by retained ancient polymorphisms, unless one invokes parallel evolution, which is highly unlikely in selectively neutral third positions of the codon triplet. These haplotypes suggest that there is some gene flow between *P. cocyta* and *P. tharos*, *P. batesii* and *P. pulchella*, *P. batesii* and *P. tharos* and especially *P. cocyta* and *P. batesii*. However, as the morphological evidence indicates, this gene flow is not enough to break down the phenotypic integrity of any of the four species, even *P. cocyta* and *P. batesii*.

Finally, since we have been able to sample individuals of a large majority of the subspecies described (Table 1), a word or two must be said about mtDNA and subspecies. To put it briefly, the confusing patterns we see at the species level are multiplied at the subspecies level, i.e. none of the subspecies form exclusive, monophyletic groups. This is reassuring since by definition a subspecies is part of a species. Subspecies are usually described based on morphological characters that differ between allopatric populations. These characters are likely to be subject to selection, which can lead to their clinal variation over space. mtDNA is not subject to these same selection pressures, and thus acts like a neutral marker that can indicate gene flow or lack of gene flow between different taxonomic entities.

## Conclusions

This study has shown that the utility of mtDNA to resolve the relationships of sets of closely related species is questionable unless a large number of individuals are sampled for each taxonomic entity. Analysing just one individual per species in species-level systematics work may give misleading, though strongly supported, results (Funk, 1999). On the other hand, analysing a large number of individuals for each taxonomic entity can be very informative about the history of the species group, as shown by our work on the *tharos*-group of genus *Phyciodes*. However, the utility of mtDNA on its own in assessing the boundaries of traditionally recognized species (e.g. Wiens & Penkrot, 2002) is suspect. One must combine all possible knowledge, including morphological, ecological and molecular, to understand the species boundaries of groups of very closely related species. Our study has raised more questions than it has answered and will certainly help focus future research on the process of speciation in the *tharos*-group of species in *Phyciodes*.

In this study, we showed that the morphologically difficult *tharos*-group is made up of three distinct genetic entities (four if the enigmatic clade A is taken into account). The most basal entity in the major *tharos*-group clade corresponds to the species *P. tharos*. The *P. cocyta* and *P. batesii* clades can be interpreted as plesiospecies (Olmstead, 1995) or ferespecies (Graybeal, 1995) with respect to the genetic entity, which corresponds to *P. pulchella*. Indeed, given that a *P. batesii* and a *P. cocyta* haplotype are basal to the *P. tharos* clade (B in Fig. 4), the variation present today in *P. batesii* and *P. cocyta* may be representative of the variation present in the common ancestor to all four species. Gene flow appears to happen between the *P. cocyta* and *P. batesii* populations and *P. tharos*, and the *P. cocyta* and *P. batesii* populations and *P. pulchella*, yet the *P. tharos* and *P. pulchella* species maintain their genetic integrity in sympatry with *P. cocyta* and *P. batesii*. There is no evidence for gene flow between *P. tharos* and *P. pulchella* in our dataset. Gene flow appears to be more frequent between *P. cocyta* and *P. batesii*, and evidently is happening today in the southern Rocky Mountains. Yet these two species remain morphologically distinct over most of their sympatric ranges, suggesting that they too are maintaining their genetic integrity.

The northeastern populations of *P. batesii* and *P. cocyta* need to be sampled much more intensively to see whether they exhibit any modern gene flow. Crosses between sympatric populations of *P. cocyta* and *P. batesii* from various parts of their ranges may give valuable additional information regarding their present taxonomic status. One might predict that resulting offspring will have phenotypes that lie within the variation observed in nature if hybridization were to have a major effect on the genetic make up of these two species.

## Acknowledgements

We are very grateful to the following people for sending specimens to us: G. R. Ballmer, B. Beck, R. Dana, T. Feldman,

R. Gatrelle, C. Guppy, W. Harrison, S. Kohler, N. Kondla, R. Layberry, P. Opler, G. F. Pratt, D. Ross, J. Salazar, C. Schmidt, T. Tammaru, M. Walker, J. B. Walsh, A. Warren, E. Williams and M. Zimmermann. We thank J. Iisalo and T. Marjomaa for help in the laboratory. We thank R. Gatrelle, C. Guppy, S. Nylin, two anonymous referees and especially N. Kondla and A. Warren for useful comments and discussions. Thanks to S. Nylin for allowing us to use the facilities at Stockholm University to complete the project. This study was funded by the Academy of Finland (grant nos. 38604 and 44887 to Ilkka Hanski).

## References

Austin, G.T. (1998a) New subspecies of Nymphalidae (Lepidoptera) from Nevada and Arizona. *Systematics of Western North American Butterflies* (ed. by T. C. Emmel), pp. 573–586. Mariposa Press, Gainesville, Florida.

Austin, G.T. (1998b) Variation of *Phyciodes pulchellus* (Boisduval) (Lepidoptera: Nymphalidae) in the Great Basin of Nevada. *Systematics of Western North American Butterflies* (ed. by T. C. Emmel), pp. 737–748. Mariposa Press, Gainesville, Florida.

Avise, J.C. (2000) *Phylogeographyraphy: the History and Formation of Species*. Harvard University Press, Cambridge, Massachussetts.

Avise, J.C. & Ball, R.M. Jr (1990) Principles of genealogical concordance in species concepts and biological taxonomy. *Oxford Surveys in Evolutionary Biology*, **7**, 45–67.

Avise, J.C., Shapira, J.F., Daniel, S.W., Aquadro, C.F. & Lansman, R.A. (1983) Mitochondrial DNA differentiation during the speciation process in *Peromyscus*. *Molecular Biology and Evolution*, **1**, 38–56.

Boggs, C.L., Watt, W.B. & Ehrlich, P.R. (eds) (2003) *Butterflies: Evolution and Ecology Taking Flight*. University of Chicago Press, Chicago.

Boyce, T.M., Zwick, M.E. & Aquadro, C.F. (1994) Mitochondrial DNA in the bark weevils: phylogeny and evolution in the *Pissodes strobi* species group (Coleoptera: Curculionidae). *Molecular Biology and Evolution*, **11**, 183–194.

Bremer, K. (1988) The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution*, **42**, 795–803.

Bremer, K. (1994) Branch support and tree stability. *Cladistics*, **10**, 295–304.

Brower, A.V.Z. (1999) Delimitation of phylogenetic species with DNA sequences: a critique of Davis and Nixon's population aggregation analysis. *Systematic Biology*, **48**, 199–213.

Brown, B., Emberson, R.M. & Paterson, A.M. (1999) Phylogeny of "*Oxycanus*" lineages of hepialid moths from New Zealand inferred from sequence variation in the mtDNA COI and II gene regions. *Molecular Phylogenetics and Evolution*, **13**, 463–473.

Brown, J.M., Pellmyr, O., Thompson, J.N. & 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.

Brunton, C.F.A. (1998) The evolution of ultraviolet patterns in European *Colias* butterflies (Lepidoptera, Pieridae): a phylogeny using mitochondrial DNA. *Heredity*, **80**, 611–616.

Brunton, C.F.A. & Hurst, G.D.D. (1998) Mitochondrial DNA phylogeny of brimstone butterflies (genus *Gonepteryx*) from the Canary Islands and Madeira. *Biological Journal of the Linnean Society*, **63**, 69–79.

Caterino, M.S., Cho, S. & Sperling, F.A.H. (2000) The current state of insect molecular systematics: a thriving Tower of Babel. *Annual Review of Entomology*, **45**, 1–54.

Caterino, M.S. & Sperling, F.A.H. (1999) *Papilio* phylogeny based on mitochondrial cytochrome oxidase I and II genes. *Molecular Phylogenetics and Evolution*, **11**, 122–137.

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.

Cognato, A.I., Seybold, S.J. & Sperling, F.A.H. (1999) Incomplete barriers to mitochondrial gene flow between pheromone races of the North American pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *Proceedings of the Royal Society of London Series B. Biology Sciences*, **266**, 1843–1850.

Crespi, B.J., Carmean, D.A., Mound, L.A., Worobey, M. & Morris, D. (1998) Phylogenetics of social behavior in Australian gall-forming thrips: evidence from mitochondrial DNA sequence, adult morphology and behavior, and gall morphology. *Molecular Phylogenetics and Evolution*, **9**, 163–180.

Farris, J.S. (1995) *Parsimony Jackknifer, Version 4.22*. Published by the author.

Farris, J.S., Albert, V.A., Källersjö, M., Lipscomb, D. & Kluge, A.G. (1996) Parsimony jackknifing outperforms neighbor-joining. *Cladistics*, **12**, 99–124.

Folmer, O., Black, M.B., Hoch, W., Lutz, R.A. & Vrijehock, R.C. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294–299.

Funk, D.J. (1999) Molecular systematics of cytochrome oxidase I and 16S from *Neochlamisus* leaf beetles and the importance of sampling. *Molecular Biology and Evolution*, **16**, 67–82.

Gatrelle, R.R. (1998) Two new Nymphalidae from western North Carolina: new subspecies of *Speyeria aphrodite* and *Phyciodes batesii*. *Taxonomic Report*, **1**(3), 1–8.

Goloboff, P.A. (1998) *NONA, Version 2.0*. Published by the author.

Graybeal, A. (1995) Naming species. *Systematic Biology*, **44**, 237–250.

Harrison, R.G. (1998) Linking evolutionary pattern and process: the relevance of species concepts for the study of speciation. *Endless Forms: Species and Speciation* (ed. by D. J. Howard & S. H. Berlocher), pp. 19–31. Oxford University Press, Oxford.

Hewitt, G.M. (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, **58**, 247–276.

Higgins, L.G. (1981) A revision of *Phyciodes* Hübner and related genera, with a review of the classification of the Melitaeinae (Lepidoptera: Nymphalidae). *Bulletin of the British Museum of Natural History*, **43**, 77–243.

Jiggins, C.D. & Davies, N. (1998) Genetic evidence for a sibling species of *Heliconius charithonia* (Lepidoptera: Nymphalidae). *Biological Journal of the Linnean Society*, **64**, 57–67.

Jiggins, C.D., McMillan, W.O., Neukirchen, W. & Mallet, J. (1996) What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae). *Biological Journal of the Linnean Society*, **59**, 221–242.

Jiggins, C.D., Naisbit, R.E., Coe, R.L. & Mallet, J. (2001) Reproductive isolation caused by colour pattern mimicry. *Nature*, **411**, 302–305.

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

Kumar, S., Tamura, K., Jakobsen, I.B. & Nei, M. (2001) MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics*, **17**, 1244–1245.

Layberry, R.A., Hall, P.W. & Lafontaine, J.D. (1998) *The Butterflies of Canada*. University of Toronto Press, Toronto.

Monteiro, A. & Pierce, N.E. (2001) Phylogeny of *Bicyclus* (Lepidoptera: Nymphalidae) inferred from COI, COII, and EF-1alpha gene sequences. *Molecular Phylogenetics and Evolution*, **18**, 264–281.

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

Neigel, J.E. & Avise, J.C. (1986) Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. *Evolutionary Processes and Theory* (ed. by E. Nevo and S. Karlin), pp. 515–534. Academic Press, London.

Nice, C.C. & Shapiro, A.M. (1999) Molecular and morphological divergence in the butterfly genus *Lycaeides* (Lepidoptera: Lycaenidae) in North America: evidence of recent speciation. *Journal of Evolutionary Biology*, **12**, 936–950.

Nice, C.C. & Shapiro, A.M. (2001) Population genetic evidence of restricted gene flow between host races in the butterfly genus *Mitoura* (Lepidoptera: Lycaenidae). *Annals of the Entomological Society of America*, **94**, 257–267.

Nixon, K.C. (2002) *Winclada, Version 1.00.08*. Published by the author.

Odorico, D.M. & Miller, D.J. (1997) Variation in the ribosomal internal transcribed spacers and 5.8S rDNA among five species of *Acropora* (Cnidaria; Scleractinia): patterns of variation consistent with reticulate evolution. *Molecular Biology and Evolution*, **14**, 465–473.

Oliver, C.G. (1972) Genetic and phenotypic differentiation and geographic distance in four species of Lepidoptera. *Evolution*, **26**, 221–241.

Oliver, C.G. (1978) Experimental hybridization between the nymphalid butterflies *Phyciodes tharos* and *P. campestris montana*. *Evolution*, **32**, 594–601.

Oliver, C.G. (1979) Experimental hybridization between *Phyciodes tharos* and *P. batesii* (Nymphalidae). *Journal of the Lepidopterists' Society*, **33**, 6–20.

Oliver, C.G. (1980) Phenotypic differentiation and hybrid breakdown within *Phyciodes 'tharos'* (Lepidoptera: Nymphalidae) in northeastern United States. *Annals of the Entomological Society of America*, **73**, 715–721.

Oliver, C.G. (1982) Experimental hybridization between *Phyciodes tharos* and *P. phaon* (Nymphalidae). *Journal of the Lepidopterists' Society*, **36**, 121–131.

Olmstead, R.G. (1995) Species concepts and plesiomorphic species. *Systematic Botany*, **20**, 623–630.

Opler, P.A., Pavulaan, H. & Stanford, R.E. (1995) *Butterflies of North America*. Northern Prairie Wildlife. Research Center Home Page. http://www.npwrc.usgs.gov/resource/distr/lepid/bflyusa/bflyusa.htm.

Opler, P.A. & Warren, A.D. (2002) *Butterflies of North America. 2. Scientific Names List for Butterfly Species of North America, North of Mexico*. Gillette Museum Publications, Fort Collins, Colorado.

Pamilo, P. & Nei, M. (1988) Relationships between gene trees and species trees. *Molecular Biology and Evolution*, **5**, 568–583.

Parker, A. & Kornfield, I. (1997) Evolution of the mitochondrial DNA control region in the *mbuna* (Cichlidae) species flock of Lake Malawi, East Africa. *Journal of Molecular Evolution*, **45**, 70–83.

Pedersen, B.V. (1996) A phylogenetic analysis of cuckoo bumblebees (*Psithyrus*, Lepeletier) and bumblebees (*Bombus*, Latreille) inferred from sequences of the mitochondrial gene cytochrome oxidase I. *Molecular Phylogenetics and Evolution*, **5**, 289–297.

Porter, A.M. & Mueller, J.C. (1998) Partial genetic isolation between *Phyciodes tharos* and *P. cocyta* (Nymphalidae). *Journal of the Lepidopterists' Society*, **52**, 182–205.

Rand, D.B., Heath, A., Suderman, T. & Pierce, N.E. (2000) Phylogeny and life history evolution of the genus *Chrysotis* within Aphnaeini (Lepidoptera: Lycaenidae), inferred from mitochondrial *cytochrome oxidase I* sequences. *Molecular Phylogenetics and Evolution*, **17**, 85–96.

Scott, J.A. (1986a) *The Butterflies of North America*. Stanford University Press, Stanford.

Scott, J.A. (1986b) The courtship of *Phyciodes*, and the relationship between *P. tharos tharos* and *P. tharos morpheus* (=*pascoensis*) in Colorado. *Papilio (New Series)*, **5**, 1–8 (privately published, available from author).

Scott, J.A. (1994) Biology and systematics of *Phyciodes* (*Phyciodes*). *Papilio (New Series)*, **7**, 1–120 (privately published, available from author).

Scott, J.A. (1998) *Phyciodes* (*Phyciodes*): new discoveries, new subspecies, and convergence. *Papilio (New Series)*, **10**, 1–42 (privately published, available from author).

Shapiro, A.M., Palm, C.A. & Wcislo, K.L. (1979) The ecology and biogeography of the butterflies of the Trinity Alps and Mount Eddy, northern California. *Journal of Research on the Lepidoptera*, **18**, 69–152.

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**, 651–701.

Sorensen, M.D. (1999) *Treerot, Version 2.0*. Boston University, Massachussetts.

Sota, T. & Vogler, A.P. (2001) Incongruence of mitochondrial and nuclear gene trees in the carabid beetles *Ohomopterus*. *Systematic Biology*, **50**, 39–59.

Sperling, F.A.H. (1987) Evolution of the *Papilio machaon* species group in western Canada (Lepidoptera: Papilionidae). *Quaestiones Entomologicae*, **23**, 198–315.

Sperling, F.A.H. (1994) Sex-linked genes and species differences in Lepidoptera. *Canadian Entomologist*, **126**, 807–818.

Sperling, F. (2003) Butterfly species and molecular phylogenies. *Butterflies: Evolution and Ecology Taking Flight* (ed. by C. Boggs W. Watt & P. Ehrlich), pp. 431–458. University of Chicago Press, Chicago.

Sperling, F.A.H. & Harrison, R.G. (1994) Mitochondrial DNA variation within and between species of the *Papilio machaon* group of swallowtail butterflies. *Evolution*, **48**, 408–422.

Sperling, F.A.H. & Hickey, D.A. (1994) Mitochondrial DNA sequence variation in the spruce budworm species complex (*Choristoneura*: Lepidoptera). *Molecular Biology and Evolution*, **11**, 656–665.

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

Swofford, D.L. (1998) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10*. Sinauer Associates, Massachussetts.

Templeton, A. (2001) Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology*, **10**, 779–791.

Templeton, A.R., Routman, E. & Phillips, C.A. (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767–782.

Wahlberg, N. & Zimmermann, M. (2000) Pattern of phylogenetic relationships among members of the tribe Melitaeini (Lepidoptera: Nymphalidae) inferred from mtDNA sequences. *Cladistics*, **16**, 347–363.

Walton, C., Butlin, R.K. & Monk, K.A. (1997) A phylogeny for grasshoppers of the genus *Chitaura* (Orthoptera: Acrididae) from Sulawesi, Indonesia, based on mitochondrial DNA sequence data. *Biological Journal of the Linnean Society*, **62**, 365–382.

Wetterer, J.K., Schultz, T.R. & Meier, R. (1998) Phylogeny of fungus-growing ants (tribe Attini) based on mtDNA sequence and morphology. *Molecular Phylogenetics and Evolution*, **9**, 42–47.

Wiens, J.J. & Penkrot, T.A. (2002) Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). *Systematic Biology*, **51**, 69–91.

Xia, X. & Xie, Z. (2001) DAMBE: Data analysis in molecular biology and evolution. *Journal of Heredity*, **92**, 371–373.

Zimmermann, M., Wahlberg, N. & Descimon, H. (2000) Phylogeny of *Euphydryas* checkerspot butterflies (Lepidoptera: Nymphalidae) based on mitochondrial DNA sequence data. *Annals of the Entomological Society of America*, **93**, 347–355.

Accepted 20 November 2002

**Appendix 1.** Localities of *Phyciodes* specimens sequenced in this study. An asterisk (*) indicates that specimens were reared in the lab and thus their 'collection' dates do not correspond to their natural flight periods.

| Species | Sample | Collection locality | Code | Collection or rearing date |
|---|---|---|---|---|
| *Phyciodes batesii anasazi* | 34-1 | U.S.A.: Colorado, Eagle Co. | CO2 | 24 Aug 1996 |
| *Phyciodes batesii apsaalooke* | 35-8 | U.S.A.: Wyoming, Bighorn Co. | WY | 15 Sep 1995 |
| *Phyciodes batesii batesii* | 72-1 | Canada: Ontario, Carleton Co. | ONT | 12 Jun 2001 |
| *Phyciodes batesii batesii* | 72-2 | Canada: Ontario, Carleton Co. | ONT | 12 Jun 2001 |
| *Phyciodes batesii batesii* | 72-3 | Canada: Ontario, Carleton Co. | ONT | 12 Jun 2001 |
| *Phyciodes batesii batesii* | 72-4 | Canada: Ontario, Carleton Co. | ONT | 12 Jun 2001 |
| *Phyciodes batesii batesii* | 72-9 | Canada: Ontario, Carleton Co. | ONT | 12 Jun 2001 |
| *Phyciodes batesii lakota* | 35-4 | U.S.A.: Nebraska, Sioux Co. | NE | 11 Aug 1994 |
| *Phyciodes batesii lakota* | 73-10 | U.S.A.: Minnesota, Lake Co. | MN | 01 Jul 2001 |
| *Phyciodes batesii lakota* | 73-6 | U.S.A.: Minnesota, Lake Co. | MN | 01 Jul 2001 |
| *Phyciodes batesii lakota* | 73-7 | U.S.A.: Minnesota, Lake Co. | MN | 01 Jul 2001 |
| *Phyciodes batesii lakota* | 73-9 | U.S.A.: Minnesota, Lake Co. | MN | 07 Jul 2001 |
| *Phyciodes batesii lakota* | 95-7 | Canada: Alberta, Fort Assiniboine | AB8 | 06 Jul 2002 |
| *Phyciodes batesii lakota* | 95-9 | Canada: Alberta, Lac La Biche | AB8 | 05 Jul 2002 |
| *Phyciodes batesii lakota* | 95-11 | Canada: Alberta, Fort Assiniboine | AB8 | 06 Jul 2002 |
| *Phyciodes batesii lakota* | 95-12 | Canada: Alberta, Lac La Biche | AB8 | 05 Jul 2002 |
| *Phyciodes batesii lakota* | 95-14 | Canada: Alberta, Lac La Biche | AB8 | 05 Jul 2002 |
| *Phyciodes batesii maconensis* | 60-13 | U.S.A.: North Carolina, Clay Co. | NC | 24 Jul 2000 |
| *Phyciodes batesii maconensis* | 60-15 | U.S.A.: North Carolina, Clay Co. | NC | 30 Jul 2000 |
| *Phyciodes batesii maconensis* | 69-1 | U.S.A.: North Carolina, Clay Co. | NC | 18 May 2001 |
| *Phyciodes batesii maconensis* | 69-2 | U.S.A.: North Carolina, Clay Co. | NC | 18 May 2001 |
| *Phyciodes batesii* or *cocyta* | 52-2 | Canada: Alberta, Elk Island | AB1 | 08 Jul 2000 |
| *Phyciodes batesii* or *cocyta* | 52-7 | Canada: Alberta, Strathcona Co. | AB3 | 03 Jul 2000 |
| *Phyciodes batesii* or *cocyta* | 52-8 | Canada: Alberta, Strathcona Co. | AB3 | 03 Jul 2000 |
| *Phyciodes batesii* or *cocyta* | 60-5 | Canada: Alberta, Dinosaur PP | AB6 | Jul 2000 |
| *Phyciodes cocyta diminutor* | 49-8 | U.S.A.: Minnesota, Freeborn Co. | MN | 21 Jul 1999 |
| *Phyciodes cocyta diminutor* | 49-9 | U.S.A.: Minnesota, Freeborn Co. | MN | 21 Jul 1999 |
| *Phyciodes cocyta selenis* | 11-4 | Canada: British Columbia,Trail | BC1 | 12 Jul 1997 |
| *Phyciodes cocyta selenis* | 11-5 | Canada: British Columbia,Trail | BC1 | 12 Jul 1997 |
| *Phyciodes cocyta selenis* | 11-6 | Canada: British Columbia,Trail | BC1 | 12 Jul 1997 |
| *Phyciodes cocyta selenis* | 47-12 | U.S.A.: Colorado, Jefferson Co. | CO1 | 29 Jun 1998 |
| *Phyciodes cocyta selenis* | 47-13 | U.S.A.: Colorado, Jefferson Co. | CO1 | 29 Jun 1998 |
| *Phyciodes cocyta selenis* | 47-14 | U.S.A.: Colorado, Jefferson Co. | CO1 | 29 Jun 1998 |
| *Phyciodes cocyta selenis* | 48-10 | U.S.A.: Colorado, Jefferson Co. | CO1 | 02 Jul 1998 |
| *Phyciodes cocyta selenis* | 48-3 | U.S.A.: Colorado, Jefferson Co. | CO1 | 11 Jun 1999 |
| *Phyciodes cocyta selenis* | 48-6 | U.S.A.: Colorado, Jefferson Co. | CO1 | 12 Jun 1998 |
| *Phyciodes cocyta selenis* | 55-2 | Canada: Alberta, Edmonton | AB7 | 24 Jul 2000 |
| *Phyciodes cocyta selenis* | 55-8 | Canada: Alberta, Edmonton | AB7 | 24 Jul 2000 |
| *Phyciodes cocyta selenis* | 58-7 | Canada: British Columbia, Pend-d'Oreille Valley | BC1 | 01 Jul 2000 |
| *Phyciodes cocyta selenis* | 58-8 | Canada: British Columbia, Pend-d'Oreille Valley | BC1 | 01 Jul 2000 |
| *Phyciodes cocyta selenis* | 60-12 | Canada: British Columbia, Nazko | BC2 | 12 Jul 2000 |
| *Phyciodes cocyta selenis* | 72-10 | Canada: Ontario, Carleton Co. | ONT | 12 Jun 2001 |
| *Phyciodes cocyta selenis* | 72-7 | Canada: Ontario, Carleton Co. | ONT | 12 Jun 2001 |
| *Phyciodes cocyta selenis* | 72-8 | Canada: Ontario, Carleton Co. | ONT | 12 Jun 2001 |
| *Phyciodes cocyta selenis* | 75-12 | U.S.A.: Wyoming, Sublette Co. | WY | 05 Aug 2001 |
| *Phyciodes cocyta selenis* | 75-13 | U.S.A.: Wyoming, Sublette Co. | WY | 05 Aug 2001 |
| *Phyciodes cocyta selenis* | 95-8 | Canada: Alberta, Lac La Biche | AB8 | 05 Jul 2002 |
| *Phyciodes cocyta selenis* | 95-10 | Canada: Alberta, Fort Assiniboine | AB8 | 06 Jul 2002 |
| *Phyciodes mylitta arida* | 67-10 | Mexico: Mexico State, Jilotepec | MX | 01 Apr 2001 |
| *Phyciodes mylitta arizonensis* | 32-1 | U.S.A.: Arizona, Chocise Co. | AZ1 | 20 May 1998 |
| *Phyciodes mylitta arizonensis* | 47-1 | U.S.A.: New Mexico, Taos Co. | NM | 09 Sep 1998 |
| *Phyciodes mylitta mylitta* | 11-10 | Canada: British Columbia,Trail | BC1 | 17 Aug 1997 |
| *Phyciodes mylitta mylitta* | 11-11 | Canada: British Columbia,Trail | BC1 | 17 Aug 1997 |
| *Phyciodes mylitta mylitta* | 32-3 | U.S.A.: Nevada, Washoe Co. | NV | 19 Aug 1994 |
| *Phyciodes mylitta mylitta* | 32-6 | U.S.A.: Montana, Missoula Co. | MT | 25 Aug 1992 |
| *Phyciodes mylitta mylitta* | 58-1 | Canada: British Columbia, Pend-d'Oreille Valley | BC1 | 05 Jul 2000 |
| *Phyciodes mylitta mylitta* | 58-2 | Canada: British Columbia, Rock Creek | BC1 | 25 May 2000 |

**Appendix 1.** Continued.

| Species | Sample | Collection locality | Code | Collection or rearing date |
|---|---|---|---|---|
| *Phyciodes orseis herlani* | 77-4 | U.S.A.: Nevada, Douglas Co. | NV | 18 Jun 2001 |
| *Phyciodes orseis herlani* | 77-5 | U.S.A.: Nevada, Douglas Co. | NV | 18 Jun 2001 |
| *Phyciodes orseis herlani* | 77-6 | U.S.A.: Nevada, Douglas Co. | NV | 18 Jun 2001 |
| *Phyciodes orseis orseis* | 37-1 | U.S.A.: California, Siskiyou Co. | CA1 | 08 Aug 1992 |
| *Phyciodes orseis orseis* | 67-3 | U.S.A.: California, Siskiyou Co. | CA1 | 24 Apr 2001 |
| *Phyciodes orseis orseis* | 67-4 | U.S.A.: California, Siskiyou Co. | CA1 | 24 Apr 2001 |
| *Phyciodes orseis orseis* | 67-6 | U.S.A.: California, Siskiyou Co. | CA1 | 24 Apr 2001 |
| *Phyciodes pallescens* | 64-1 | Mexico: Michoacán State, Ziracuaretiro | | 27 Aug 1997 |
| *Phyciodes pallescens* | 64-2 | Mexico: Michoacán State, Uruapan | | 26 Jul 1996 |
| *Phyciodes pallida barnesi* | 58-5 | Canada: British Columbia, Crater Mountain | BC1 | 27 Jun 2000 |
| *Phyciodes pallida barnesi* | 58-6 | Canada: British Columbia, Crater Mountain | BC1 | 27 Jun 2000 |
| *Phyciodes pallida pallida* | 34-6 | U.S.A.: Colorado, Boulder Co. | CO3 | 08 Jun 1994 |
| *Phyciodes pallida pallida* | 47-10 | U.S.A.: Colorado, Jefferson Co. | CO1 | 11 Jun 1999 |
| *Phyciodes pallida pallida* | 47-11 | U.S.A.: Colorado, Jefferson Co. | CO1 | 11 Jun 1999 |
| *Phyciodes pallida pallida* | 47-9 | U.S.A.: Colorado, Jefferson Co. | CO1 | 11 Jun 1999 |
| *Phyciodes phaon jalapeno* | 35-11 | Mexico: Mazatlan | Mex | 26 May 1991 |
| *Phyciodes phaon jalapeno* | 77-7 | U.S.A.: Texas, San Benito Co. | TX | 09 Sep 2001 |
| *Phyciodes phaon phaon* | 25-17 | U.S.A.: Florida, Alachua Co. | FL | 30 May 1998 |
| *Phyciodes picta canace* | 44-11 | U.S.A.: Arizona, Santa Cruz Co. | AZ2 | 25 Aug 1997 |
| *Phyciodes picta canace* | 44-12 | U.S.A.: Arizona, Santa Cruz Co. | AZ2 | 25 Aug 1998 |
| *Phyciodes picta picta* | 34-7 | U.S.A.: Colorado, Morgan Co. | CO4 | 28 Jul 1995 |
| *Phyciodes pulchella camillus* | 35-5 | U.S.A.: Colorado, Jefferson Co. | CO1 | 28 Sep 1998 |
| *Phyciodes pulchella camillus* | 48-13 | U.S.A.: Colorado, Jefferson Co. | CO1 | 28 May 1998 |
| *Phyciodes pulchella camillus* | 48-14 | U.S.A.: Colorado, Jefferson Co. | CO1 | 28 May 1998 |
| *Phyciodes pulchella camillus* | 48-2 | U.S.A.: Colorado, Jefferson Co. | CO1 | 18 Jun 1998 |
| *Phyciodes pulchella camillus* | 48-4 | U.S.A.: Colorado, Adams Co. | CO5 | 02 Sep 1998 |
| *Phyciodes pulchella camillus* | 48-7 | U.S.A.: Colorado, Jefferson Co. | CO1 | 10 Jun 1998 |
| *Phyciodes pulchella camillus* | 48-8 | U.S.A.: Colorado, Jefferson Co. | CO1 | 10 Jun 1998 |
| *Phyciodes pulchella camillus* | 48-9 | U.S.A.: Colorado, Jefferson Co. | CO1 | 10 Jun 1998 |
| *Phyciodes pulchella camillus* | 49-1 | U.S.A.: New Mexico, Taos Co. | NM | 10 Sep 1998 |
| *Phyciodes pulchella camillus* | 49-12 | U.S.A.: Colorado, Jefferson Co. | CO1 | 04 Sep 1998 |
| *Phyciodes pulchella camillus* | 49-2 | U.S.A.: Colorado, Costilla Co. | CO6 | 08 Sep 1998 |
| *Phyciodes pulchella camillus* | 49-3 | U.S.A.: Colorado, Costilla Co. | CO6 | 04 Sep 1998 |
| *Phyciodes pulchella camillus* | 49-4 | U.S.A.: Colorado, Costilla Co. | CO6 | 04 Sep 1998 |
| *Phyciodes pulchella camillus* | 49-5 | U.S.A.: Colorado, Costilla Co. | CO6 | 04 Sep 1998 |
| *Phyciodes pulchella camillus* | 50-2 | U.S.A.: New Mexico, Taos Co. | NM | 09 Sep 1998 |
| *Phyciodes pulchella camillus* | 50-3 | U.S.A.: Colorado, Jefferson Co. | CO1 | 18 May 1998 |
| *Phyciodes pulchella camillus* | 50-4 | U.S.A.: Colorado, Jefferson Co. | CO1 | 18 May 1998 |
| *Phyciodes pulchella camillus* | 75-14 | U.S.A.: Wyoming, Sublette Co. | WY | 05 Aug 2001 |
| *Phyciodes pulchella camillus* | 75-15 | U.S.A.: Wyoming, Sublette Co. | WY | 05 Aug 2001 |
| *Phyciodes pulchella montana* | 27-5 | U.S.A.: California, Mono Co. | CA2 | 09 Jul 1994 |
| *Phyciodes pulchella montana* | 67-15 | U.S.A.: Oregon, Deschutes Co. | OR | 21 Aug 2000 |
| *Phyciodes pulchella montana* | 67-16 | U.S.A.: Oregon, Deschutes Co. | OR | 21 Aug 2000 |
| *Phyciodes pulchella owimba* | 24-10 | U.S.A.: Montana, Missoula Co. | MT | 08 Jun 1992 |
| *Phyciodes pulchella owimba* | 52-14 | Canada: Alberta, Cardinal River Divide | AB5 | 22 Jul 2000 |
| *Phyciodes pulchella owimba* | 54-1 | Canada: Alberta, Cardinal River Divide | AB5 | 22 Jul 2000 |
| *Phyciodes pulchella owimba* | 55-7 | Canada: Alberta, Cardinal River Divide | AB5 | 22 Jul 2000 |
| *Phyciodes pulchella owimba* | 56-1 | Canada: British Columbia, Nazko | BC2 | 12 Jul 2000 |
| *Phyciodes pulchella owimba* | 56-5 | Canada: British Columbia, Nazko | BC2 | 12 Jul 2000 |
| *Phyciodes pulchella owimba* | 56-7 | Canada: British Columbia, Nazko | BC2 | 12 Jul 2000 |
| *Phyciodes pulchella owimba* | 60-2 | Canada: British Columbia, Nazko | BC2 | 12 Jul 2000 |
| *Phyciodes pulchella owimba* | 73-1 | U.S.A.: Oregon, Benton Co. | OR | 21 Jun 2001 |
| *Phyciodes pulchella owimba* | 73-2 | U.S.A.: Oregon, Benton Co. | OR | 21 Jun 2001 |
| *Phyciodes pulchella pulchella* | 47-6 | U.S.A.: California, Humboldt Co. | CA3 | 20 Oct 1998 |
| *Phyciodes pulchella pulchella* | 49-13 | U.S.A.: California, Humboldt Co. | CA3 | 21 Oct 1998 |
| *Phyciodes pulchella pulchella* | 49-14 | U.S.A.: California, Humboldt Co. | CA3 | 21 Oct 1998 |

**Appendix 1.** Continued.

| Species | Sample | Collection locality | Code | Collection or rearing date |
|---|---|---|---|---|
| *Phyciodes pulchella pulchella* | 50-6 | U.S.A.: California, Humboldt Co. | CA3 | 21 Oct 1998 |
| *Phyciodes pulchella pulchella* | 67-11 | U.S.A.: Oregon, Curry Co. | OR | 26 Jun 2000 |
| *Phyciodes pulchella pulchella* | 67-13 | U.S.A.: Oregon, Josephine Co. | OR | 25 Jun 2000 |
| *Phyciodes pulchella pulchella* | 67-14 | U.S.A.: Oregon, Josephine Co. | OR | 25 Jun 2000 |
| *Phyciodes pulchella tutchone* | 23-11 | U.S.A.: Alaska, Delta Junction | AK | 23 Jun 1996 |
| *Phyciodes tharos orantain* | 35-6 | U.S.A.: Colorado, Morgan Co. | CO4 | 28 Oct 1998 |
| *Phyciodes tharos orantain* | 47-2 | U.S.A.: Colorado, Sedgwick Co. | CO7 | 22 Jul 1998 |
| *Phyciodes tharos orantain* | 52-9 | Canada: Alberta, Spondin | AB4 | 05 Jun 2000 |
| *Phyciodes tharos orantain* | 60-6 | Canada: Alberta, Dinosaur PP | AB6 | Jul 2000 |
| *Phyciodes tharos orantain* | 60-7 | Canada: Alberta, Dinosaur PP | AB6 | Jul 2000 |
| *Phyciodes tharos riocolorado* | 35-9 | U.S.A.: Colorado, Montrose Co. | CO8 | 10 Sep 1993 |
| *Phyciodes tharos tharos* | 25-18 | U.S.A.: Florida, Alachua Co. | FL | 30 May 1998 |
| *Phyciodes tharos tharos* | 34-2 | U.S.A.: Minnesota, Freeborn Co. | MN | 25 Jun 1998 |
| *Phyciodes tharos tharos* | 44-1 | U.S.A.: New York, Oneida Co. | NY | 03 Sep 1998 |
| *Phyciodes tharos tharos* | 44-2 | U.S.A.: New York, Oneida Co. | NY | 03 Sep 1998 |
| *Phyciodes tharos tharos* | 44-3 | U.S.A.: New York, Oneida Co. | NY | 03 Sep 1998 |
| *Phyciodes tharos tharos* | 44-4 | U.S.A.: New York, Oneida Co. | NY | 03 Sep 1998 |
| *Phyciodes tharos tharos* | 47-3 | U.S.A.: Minnesota, Freeborn Co. | MN | 30 Jul 1999 |
| *Phyciodes tharos tharos* | 47-4 | U.S.A.: Minnesota, Freeborn Co. | MN | 30 Jul 1999 |
| *Phyciodes tharos tharos* | 47-8 | U.S.A.: Minnesota, Freeborn Co. | MN | 25 Jun 1998 |
| *Phyciodes tharos tharos* | 53-8 | U.S.A.: Maryland, College Park | MD | 23 Jun 2000 |
| *Phyciodes tharos tharos* | 54-9 | U.S.A.: Maryland, College Park | MD | 25 Jun 2000 |
| *Phyciodes tharos tharos* | 73-3 | Mexico: Mexico State, Jilotepec | MX | 01 Apr 2001 |
| *Phyciodes tharos tharos* | 73-4 | Mexico: Guanajuato State, Pénjamo | MX | 30 Mar 2001 |
| *Phyciodes graphica* | 41-1 | U.S.A.: Texas, Val Verde Co. | TX | 21 Apr 1999 |
| *Phyciodes graphica* | 41-2 | U.S.A.: Texas, Val Verde Co. | TX | 21 Apr 1999 |
| *Phyciodes graphica* | 67-9 | Mexico: Mexico State, Jilotepec | MX | 01 Apr 2001 |