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The phylogeny of the *Hyles euphorbiae* complex (Lepidoptera: Sphingidae): Molecular evidence from sequence data and ISSR-PCR fingerprints

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Abstract

The evolutionary history of the *Hyles euphorbiae* complex (HEC) was studied using mitochondrial DNA sequences comprising about 2300 bp derived from the genes cytochrome c oxidase subunits I (COX I) and II (COX II) and tRNA-leucine. In addition, we collected genomic fingerprinting data by ISSR-PCR to assess if and how the bi-parentally inherited nucleome may have diverged differently to the maternally inherited mitochondria. The COX sequences revealed a clear geographical pattern of genetic differentiation of the HEC into two main lineages, *H. euphorbiae* and *H. tithymali*. Our results provide no evidence that *H. dahlii* falls within a HEC s. str., although a sister-group relationship cannot be ruled out. The sequence data indicated intraspecific subdivisions and gene flow patterns, and possibly detected both introgression and a major contact zone on Mediterranean islands between these two evolutionary lineages. *Hyles tithymali* is hypothesized to have been able to retain ancient polymorphisms until the present, whereas *H. euphorbiae* appears to have (re)colonized its current distribution range after the Ice Ages from a few (or even only one) refugial populations by leptokurtic dispersal, resulting in low diversity. The ISSR-PCR data showed much higher variability among individuals of the HEC than did mtDNA sequence data. They provided insights into the genomic distribution of the simple sequence repeat $(GACA)_4$ and appear to describe a more complex pattern of introgression in the HEC. Our data revealed the HEC as a very young species complex, in which we have detected two distinct mitochondrial lineages, corresponding to *H. tithymali* (including the *deserticola*, *mauretanica* and *himyarensis* lineages) and *H. euphorbiae* (including *H. robertsi*), respectively.

Keywords: *Hyles euphorbiae* complex; Mitochondrial DNA; Molecular phylogeny; Ice ages; Introgression

Introduction

The hawkmoth genus *Hyles* Hübner comprises about 30 species (Kitching and Cadiou 2000), with representatives found on all continents except Antarctica, as well as on Madagascar and the Hawaiian Islands. Within *Hyles*, there is a group of species closely related to *Hyles*

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euphorbiae (Linnaeus) and *H. tithymali* (Boisduval), that collectively has become known as the *Hyles euphorbiae* complex (HEC). Meerman (1993) made the first attempt to resolve relationships within the HEC when he conducted a phenetic analysis based mainly on colour characters and ratios derived from measurements of various aspects of the male and female genitalia. The resulting phenogram (Fig. 1a) recognized two major species complexes, centred on *H. euphorbiae* which is distributed in central and eastern Europe, and *H. tithymali* which is distributed in Macaronesia and northwestern Africa, with an extension (as *H. t. himyarensis*) east to the Arabian Peninsula (the distribution in northeastern Africa is unclear, see Fig. 2a). Shortly afterwards, Derzhavets (1994) published a phylogeny of *Hyles* based on characters similar to those used by Meerman. The HEC comprised his "Section *euphorbiae*", but also included *H. nicaea* (de Prunner) (Fig. 1b). There are significant discrepancies between the patterns of relationship proposed by Meerman and Derzhavets, much of which are attributable to the different analytical approaches adopted by the respective authors. However, the lack of explicit data matrices in both papers makes evaluation of the conflict difficult. More recently, Danner et al. (1998) divided *Hyles* into eight subgenera (not recognized by Kitching and Cadiou 2000), without proposing any further resolution among them. This classification, which was not based on any explicitly stated and objective methodology, used only colour pattern characters of the adults and larvae. The HEC was split among three subgenera (Fig. 1c).

A major problem with all these attempts to classify *Hyles* is that the morphological features commonly used to separate and group species-level taxa in Lepidoptera, such as genital structure, show very little consistent variation within this genus. Those features that do vary, such as adult wing pattern or larval coloration, often do so with considerable overlap among the various currently recognized taxa, which makes their coding for phylogenetic analysis at best extremely difficult and at worst impossible. For this reason, Hundsdoerfer et al. (2005b) chose to reconstruct the phylogeny of *Hyles* based on a novel data source derived from the mitochondrial genes, cytochrome c oxidase subunits I (COX I) and II (COX II), and the ribosomal transfer RNA for leucine (tRNA-leu). Analysis of these sequences provided considerable resolution among the various *Hyles* lineages, while clustering most conspecific samples together in monophyletic units. In particular, they suggested that, contra Derzhavets (1994) and Danner et al. (1998), *H. nicaea* is not a member of the HEC and that the relationship of *H. dahlii* (Geyer) with the HEC is equivocal. This led Hundsdoerfer et al. (2005b) to define a HEC s. str. that excluded *H. dahlii*, and a HEC s. l. including this latter species, together with *H. hippophaes*, *H. siehei* and possibly even *H. vespertilio*. Within the HEC, two groups were recovered that centred on *H. euphorbiae* and *H. tithymali*, but there was still considerable ambiguity.

The present study attempts to produce a more detailed and informative evolutionary history of the HEC. We have used a substantially larger number of mtDNA sequences, comprising about 2300 bp derived from the genes COX I, COX II and tRNA-leu, than were analysed by Hundsdoerfer et al. (2005b) to examine maternal mitochondrial divergence further. We also included specimens from multiple populations of the HEC taxa, as well as sequences from as many closely related nominal taxa as possible, and of taxa that may plausibly share haplotypes with HEC members due to introgressive gene flow, ancestral polymorphism or incomplete taxonomy, as suggested by Funk (1999). Our study of the phylogeny of *Hyles* (Hundsdoerfer et al. 2005b) suggested that these phenomena may be important in the study of the HEC. In addition, we collected genomic fingerprinting data by Inter-simple sequence repeat (ISSR)-PCR to examine the genetic relationships based on the nucleome. ISSR amplification is a relatively novel technique that can rapidly differentiate closely related individuals (Gupta et al. 1994; Zietkiewicz et al. 1994; Tsumura et al. 1996). Unlike nucleotide sequences, ISSR markers describe DNA characteristics at several, mostly nuclear, chromosomal loci and thus avoid the use of gene trees as surrogates of species trees (Martin and Salamini 2000). Revealing discontinuous markers, ISSR-PCR can provide a measure of genetic differences dispersed across the entire nuclear genome. The absence of a band is interpreted as primer divergence or the loss of a locus through either the deletion of the SSR site or a chromosomal rearrangement (Wolfe and Liston 1998).

Until recently, the use of ISSR markers was restricted to cultivated plant species (e.g. Tsumura et al. 1996; Fang and Roose 1997; Assefa et al. 2003), their pests (e.g. Kumar et al. 2001a) or other animals of economic importance (e.g. Reddy et al. 1999). However, it is now being applied increasingly in population-level to interspecific studies of natural populations of plants and animals (e.g. King and Ferris 2000; Culley and Wolfe 2001; Guicking et al. 2002a, b; Luque et al. 2002; Nagy et al. 2002; Kauserud and Schumacher 2003; Treutlein et al. 2003; Sudupak 2004). ISSR markers have proven an efficient method for detecting hybridization in natural populations of plants (Wolfe et al. 1998) and animals (Wink et al. 2001; Fritz et al. 2005), and their use has been widened to include an application for determining the sex of birds (Wink et al. 1998).

ISSR marker analysis involves PCR amplification of DNA using a single primer composed of a microsatellite sequence such as $(GACA)_4$. The primer can be anchored at either the 3′ or 5′ end by 2–4 arbitrary, often degenerate, nucleotides. The amplification products are

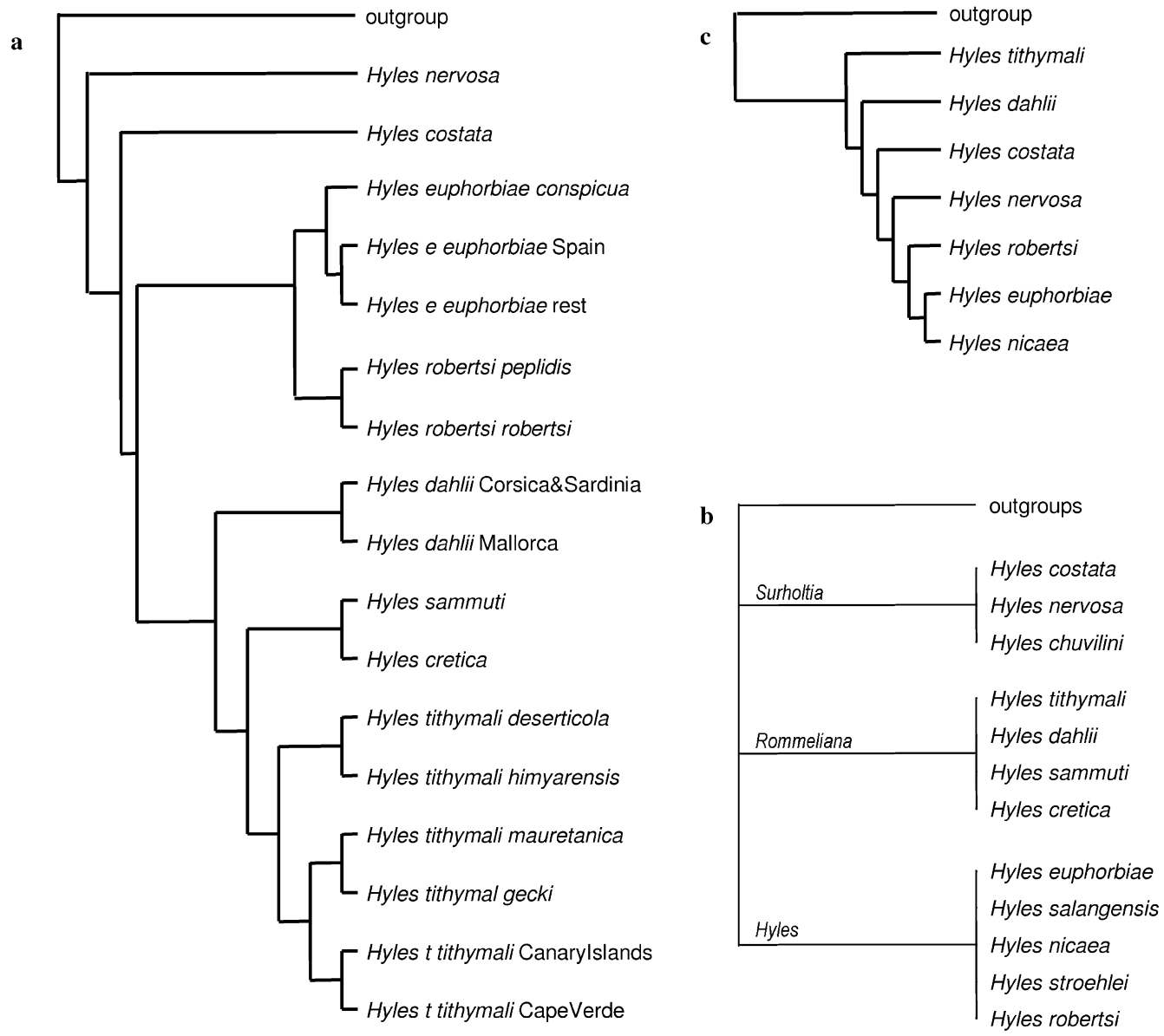

Fig. 1. Phylogeny of the *Hyles euphorbiae* complex (HEC) based on morphological characters. Species definitions follow Kitching and Cadiou (2000), and may differ from the ones used by the authors whose hypotheses are presented. (a) Phylogeny of the HEC according to Meerman (1993). (b) Phylogeny of the section '*euphorbiae*', which corresponds to the HEC, according to Derzhavets (1994). (c) The three *Hyles* subgenera (labelled above branches) of Danner et al. (1998), that include the members of the HEC.

then separated by Polyacrylamide Gel Electrophoresis (PAGE) or on an agarose gel. ISSR-PCR can reveal a large number of fragments, and thus many potentially polymorphic loci, in one PCR with good reproducibility. These characteristics are great advantages compared to RAPD amplification. However, the major advantage of ISSR-PCR over microsatellite analyses is the cost efficiency, because no initial investment in primer design is necessary. A disadvantage is that loci are usually interpreted as dominant markers, so no genotypic allele information is acquired, as it is in microsatellite analyses.

Material and methods

Taxon sampling

We attempted to sample as many species of the HEC as possible. Populations in Europe, North Africa, the Middle East and the Arabian Peninsula were sampled (Fig. 2), and special attention was paid to the two nominotypical subspecies, *Hyles e. euphorbiae* (continental Europe) and *Hyles t. tithymali* (Canary Islands). Specimens were acquired from numerous collectors (see Acknowledgements) and deposited in the following

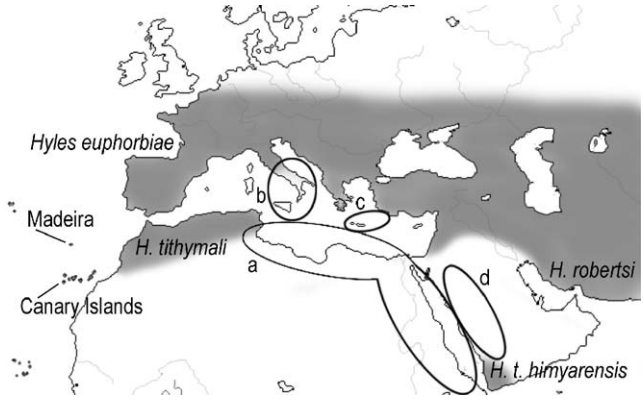

Fig. 2. Distribution ranges of *Hyles euphorbiae, H. robertsi* and *H. tithymali*. The distribution of *H. tithymali* in north-eastern Africa (a) is unclear; the HEC populations on Sicily, Malta, and in southern Italy (b), on Crete and Rhodes (c), and in the Asir mountains of Saudi Arabia (d) are postulated to be of hybrid origin.

three institutions: the Museum für Tierkunde, Staatliche Naturhistorische Sammlungen Dresden, Germany (MTD); The Natural History Museum, London, UK (BMNH); and the Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, Germany (ZFMK). The material was kept frozen at −20 °C, or in alcohol (ethanol or methanol), until extraction. Specimen and collection data are listed in Table 1. Sequences have been deposited in GenBank under the accession numbers AJ749444–AJ749548 (the sequences of some samples were divided between two accession numbers).

DNA extraction

The total DNA extraction procedure follows Hundsdoerfer et al. (2005b), to which the reader is referred for a detailed description. However, if the DNA solution still contained PCR inhibitors after this procedure, it was cleaned over NucleoSpin® Tissue columns (Macherey-Nagel), adapting the first step by mixing 30 µl DNA solution with 180 µl of the lysis buffer T1 and then following the standard protocol. A small number of DNA samples were isolated directly with this kit.

COX-PCR amplification, sequencing and alignment

Amplification, sequencing and alignment protocols again follow Hundsdoerfer et al. (2005b), to which the reader is referred for detailed descriptions of the methods. However, we provide here an overview of the sequences of the primers used (Table 2; nomenclature of Simon et al. 1994).

ISSR-PCR

The fragments between the microsatellites consisting of the tetra-repeat $(GACA)_n$ and its complement, $(TGTC)_n$, were amplified using the non-anchored primer $(GACA)_4$ (i.e. GACAGACAGACAGACA; Epplen et al. 1992). Each PCR was performed with about 500 ng template DNA in a 25 µl volume (10 pmol of the primer and 0.625 nmol of each dNTP, except dATP: 0.28 nmol cold dATP plus 0.1 µl radioactive α-^{33}P-dATP solution (370 MBq/ml, Amersham Biosciences), 0.1 units of Taq-Polymerase (SIGMA) and water, buffered with 10 mM Tris–HCl, 50 mM KCl, 0.5% Triton X-100, 1.5 mM $MgCl_2$) and covered by two drops of mineral oil. Thermo-cycling was performed with a Trio Thermo block TB1 (Biometra, Göttingen). Following the initial 5 min denaturation at 94 °C, the program consisted of 28 cycles of 50 s at 94 °C, 30 s at 55 °C, 120 s at 72 °C and 25 min at 72 °C for final elongation. The DNA fragments were separated by PAGE in a vertical apparatus (Base Acer Sequencer, Stratagene) for 4 h at 65 W. The denaturing gel (6 M Urea, 100 ml Long Ranger Solution, Biozym (PA), 100 ml TBE-Buffer (10 ×: 1 M Tris, 0.83 M Boric Acid, 10 mM EDTA, pH 8.6)) had a size of 45 × 30 cm and a thickness of 0.25 mm. After drying, the gel was exposed to an X-ray film (Hyperfilm-MP, Amersham) for at least 12 h and developed (Kodak). The film was then scanned with a resolution of 300–600 dpi. The bands were analysed visually on the film itself, but marked on an A3-sized print of the film. The bands of the ISSR-PCR fingerprints were visually scored into a data matrix as either absent (“0”) or present (“1”) following the guidelines of Jeffreys et al. (1991), Zietkiewicz et al. (1994), Bornet and Branchard (2001), Culley and Wolfe (2001), and Assefa et al. (2003).

Sequence characterization

Nucleotide composition (HEC samples only) and pairwise distances (*H. sammuti* Eitschberger, Danner & Surholt samples excluded due to apparent gene introgression phenomena that would distort the distance measures) of the COX sequence data were calculated with PAUP* 4.0b10 (Swofford 1998). MEGA 2.1 (Kumar et al. 2001b) was used to calculate the nucleotide and amino-acid substitution frequencies. The program ForCon 1.0 (Raes and van de Peer 1998) was used to convert input files between formats.

Phylogenetic analysis of the full mtDNA sequence data

A total of 100 samples (10–16 representing outgroup taxa, depending on the definition of the HEC, see above)

Table 1. List of specimens studied; for each taxon the taxonomic author, sample no., institution of deposit, and collection data are provided

Taxon	Author	No.	Institution[a]	Collector/provider	Date	Origin	Locality
Hyles nicaea castissima	Austaut	23208	MTD	O. Niehuis	27.06.2002	Morocco	High Atlas, SW Midelt, nr. Cirque de Jaffar; N 32°33′ W 4°54′
Hyles gallii	Rottemburg	0027	BMNH	M. O'Neill	vi.2000	Czechia	ex bred stock
Hyles gallii	Rottemburg	23189	MTD	H. Harbich	v.1999	Germany	Sonnenberg, Thüringen
Hyles livornica	Esper	16135	MTD	H. Harbich	1996	Corsica	ex bred stock
Hyles livornica	Esper	696071	BMNH	M. O'Neill	2000	France	ex bred stock, Toulon
Hyles hippophaes	Esper	695817	BMNH	M. O'Neill	2000	France	Hautes Alpes, San Crépin
Hyles vespertilio	Esper	0007	BMNH	M. O'Neill	vi.2000	Austria	South, ex bred stock
Hyles vespertilio	Esper	0114	BMNH	A.R. Pittaway	2000	France	Provence, nr. Toulon
Hyles siehei	Püngeler	16137	MTD	H. Harbich	1998	Turkey	Taurus
Hyles siehei	Püngeler	695862	BMNH	A.R. Pittaway	vi.1999	Turkey	Bolkar Daglari
Hyles dahlii	Geyer	0030	BMNH	M.R. Honey	1999	Mallorca	—
Hyles dahlii	Geyer	16138	MTD	H. Harbich	1996	Sardinia	—
Hyles dahlii	Geyer	23192	MTD	A. Meier	spring 2002	Sardinia	—
Hyles dahlii	Geyer	23193	MTD	A. Meier	spring 2002	Sardinia	—
Hyles dahlii	Geyer	23195	MTD	A. Meier	spring 2002	Sardinia	—
Hyles dahlii	Geyer	695815	BMNH	A. Meier	spring 2002	Sardinia	—
Hyles robertsi peplidis	Christoph	695835	BMNH	A.R. Pittaway	xi.2001	Iran	nr. Esfahan, bred 2001, emerged (with heat) xi.2001
Hyles r. peplidis	Christoph	695842	BMNH	A.R. Pittaway	xi.2001	Iran	as 695835
Hyles r. peplidis	Christoph	695872	BMNH	A.R. Pittaway	xi.2001	Iran	as 695835
Hyles sammuti	Eitschberger et al.	23239	MTD	M. Geck	autumn 2002	Sicily	Zafferana
Hyles sammuti	Eitschberger et al.	0013	BMNH	P. Sammut	iv.2000	Malta	—
Hyles sammuti	Eitschberger et al.	0051	BMNH	P. Sammut	iv.2000	Malta	—
Hyles sammuti	Eitschberger et al.	0052	BMNH	P. Sammut	iv.2000	Malta	—
Hyles sammuti	Eitschberger et al.	0053	BMNH	P. Sammut	iv.2000	Malta	—
Hyles sammuti	Eitschberger et al.	0054	BMNH	P. Sammut	iv.2000	Malta	—
Hyles sammuti	Eitschberger et al.	0055	BMNH	P. Sammut	iv.2000	Malta	—
Hyles sammuti	Eitschberger et al.	0065	BMNH	P. Sammut	iv.2000	Malta	—
Hyles costata	von Nordman	23245	MTD	A. Belik	12.06.1999	Russia	S. Transbaikal, Chita region, Kyra district, NE of Hentei Mts., nr. Kyra
Hyles t. mauretanica	Staudinger	23200	MTD	O. Niehuis	25.06.2002	Morocco	Median Atlas, betw. El-Kbab and Boumia; N 32°42.46′ W 5°11.19′
Hyles t. mauretanica	Staudinger	23201	MTD	O. Niehuis	25.06.2002	Morocco	as 23200
Hyles t. mauretanica	Staudinger	23202	MTD	O. Niehuis	25.06.2002	Morocco	as 23200
Hyles t. mauretanica	Staudinger	23204	MTD	O. Niehuis	27.06.2002	Morocco	High Atlas, SW Midelt, nr. Cirque de Jaffar; N 32°33′ W 4°54′
Hyles t. mauretanica	Staudinger	23205	MTD	O. Niehuis	27.06.2002	Morocco	as 23204
Hyles t. mauretanica	Staudinger	23211	MTD	O. Niehuis	28.06.2002	Morocco	Median Atlas, Mischliffen; N 33°24′ W 05°04′

Table 1. (*continued*)

Taxon	Author	No.	Institution[a]	Collector/provider	Date	Origin	Locality
Hyles t. mauretanica	Staudinger	23213	MTD	O. Niehuis	28.06.2002	Morocco	as 23211
Hyles t. mauretanica	Staudinger	23215	MTD	O. Niehuis	28.06.2002	Morocco	as 23211
Hyles t. mauretanica	Staudinger	23216	MTD	O. Niehuis	28.06.2002	Morocco	as 23211
Hyles t. mauretanica	Staudinger	23218	MTD	O. Niehuis	28.06.2002	Morocco	as 23211
Hyles t. mauretanica	Staudinger	23220	MTD	O. Niehuis	28.06.2002	Morocco	as 23211
Hyles t. mauretanica	Staudinger	041e	MTD	A. Hundsdörfer	24.05.2003	Morocco	Ifrane; N 33°29′11″ W 05°040′7″
Hyles t. mauretanica	Staudinger	042a	MTD	A. Hundsdörfer		Morocco	
Hyles t. mauretanica	Staudinger	23167	MTD	A. Hundsdörfer	19.06.2001	Algeria	ex bred stock
Hyles t. deserticola	Staudinger	055b	MTD	A. Hundsdörfer	25.05.2003	Morocco	South of the High Atlas; N 31°44′32″ W 04°11′54″
Hyles t. deserticola	Staudinger	055c	MTD	A. Hundsdörfer	25.05.2003	Morocco	as 055b
Hyles t. deserticola	Staudinger	055d	MTD	A. Hundsdörfer	25.05.2003	Morocco	as 055b
Hyles t. deserticola	Staudinger	16144	MTD	H. Harbich	17.03.1997	Algeria	ex bred stock, El Bayed
Hyles t. gecki	de Freina	0163	BMNH	A. Wakeham-Dawson, M. Salmon	11.10.2001	Madeira	Porto Santo
Hyles t. gecki	de Freina	0164	BMNH	as 0164	11.10.2001	Madeira	Porto Santo
Hyles t. gecki	de Freina	23236	MTD	S. Kalyabina-Hauff	15.01.2003	Madeira	Ribeira Brava, Miradouro
Hyles t. gecki	de Freina	23237	MTD	S. Kalyabina-Hauff	21.01.2003	Madeira	Ponta do Sol, coast
Hyles t. gecki	de Freina	23238	MTD	S. Kalyabina-Hauff	21.01.2003	Madeira	Ponta do Sol, coast
Hyles t. tithymali	Boisduval	008c	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Lanzerote; N 29°06′27″ W 13°28′08″
Hyles t. tithymali	Boisduval	009a	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Fuerteventura; N 28°42′53″ W 13°51′02″
Hyles t. tithymali	Boisduval	012	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Fuerteventura; N 28°42′22″ W 13°51′10″
Hyles t. tithymali	Boisduval	013a	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Fuerteventura; N 28°42′21″ W 13°51′09″
Hyles t. tithymali	Boisduval	695856	BMNH	A.R. Pittaway	2001	Canary Islands	ex bred stock, Gran Canaria
Hyles t. tithymali	Boisduval	077f	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Gran Canaria; N 28°06′59″ W 15°39′16″
Hyles t. tithymali	Boisduval	084a	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Gran Canaria; N 27°59′00″ W 15°41′26″
Hyles t. tithymali	Boisduval	094a	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Gran Canaria; N 27°27′52″ W 15°25′06″
Hyles t. tithymali	Boisduval	696099	BMNH	M. O'Neill	vi.2001	Canary Islands	Tenerife
Hyles t. tithymali	Boisduval	001	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Tenerife; N 28°03′10″ W 16°34′39″

Hyles t. tithymali	Boisduval	059a	MTD	A. Hundsdörfer	spring 2002	Canary Islands	Tenerife; N 28°04′03″ W 16°42′44″
Hyles t. tithymali	Boisduval	067c	MTD	A. Hundsdörfer	spring 2002	Canary Islands	La Gomera; N 28°06′31″ W 17°19′01″
Hyles t. tithymali	Boisduval	074e	MTD	A. Hundsdörfer	spring 2002	Canary Islands	La Gomera; N 28°02′16″ W 17°13′34″
Hyles t. tithymali	Boisduval	035a	MTD	A. Hundsdörfer	spring 2002	Canary Islands	La Palma; N 28°30′05″ W 17°51′49″
Hyles t. tithymali	Boisduval	053	MTD	A. Hundsdörfer	spring 2002	Canary Islands	La Palma; N 28°42′34″ W 17°45′07″
Hyles t. tithymali	Boisduval	100	MTD	A. Hundsdörfer	spring 2002	Canary Islands	El Hierro; N 27°40′32″ W 18°01′22″
Hyles t. tithymali	Boisduval	105	MTD	A. Hundsdörfer	spring 2002	Canary Islands	El Hierro; N 27°45′15″ W 18°08′36″
Hyles t. himyarensis	Meerman	23223	ZFMK	C.M. Naumann	13.06.2001	Yemen	Province Amrah, north
Hyles t. himyarensis	Meerman	23224	ZFMK	C.M. Naumann	13.06.2001	Yemen	Province Amrah, north
Hyles t. himyarensis	Meerman	23225	ZFMK	C.M. Naumann	?	Yemen	—
Hyles t. himyarensis	Meerman	16173a	MTD	C.M. Naumann	vii.2001	Yemen	—
Hyles euphorbiae ssp.	Linnaeus	23280	MTD	D. Guicking	20.05.2003	Kasachstan	West of Aralsea; N 46°18,64′ E 58°40,85′
Hyles euphorbiae ssp.	Linnaeus	23284	MTD	K.D. Milto via S. Kalyabina-Hauff	27.07.2003	Armenia	Northern coast of Sevan lake nr. Djil
Hyles euphorbiae ssp.	Linnaeus	23285	MTD	K.D. Milto via S. Kalyabina-Hauff	27.07.2003	Armenia	Northern coast of Sevan lake nr. Djil
Hyles euphorbiae ssp.	Linnaeus	23286	MTD	K.D. Milto via S. Kalyabina-Hauff	27.07.2003	Armenia	Northern coast of Sevan lake nr. Djil
Hyles euphorbiae ssp.	Linnaeus	23287	MTD	K.D. Milto via S. Kalyabina-Hauff	27.07.2003	Armenia	Northern coast of Sevan lake nr. Djil
Hyles e. euphorbiae	Linnaeus	0112	BMNH	A.R. Pittaway	2000	France	Provence, nr. Toulon
Hyles e. euphorbiae	Linnaeus	022p	MTD	A. Hundsdörfer	2002	France	South–West, nr. Leucate
Hyles e. euphorbiae	Linnaeus	695876	BMNH	M. O'Neill	viii.2001	France	South
Hyles e. euphorbiae	Linnaeus	695887	BMNH	D. Arthurs via M. O'Neill	vii.2001	France	South
Hyles e. euphorbiae	Linnaeus	23168	MTD	A. Hundsdörfer	27.04.2000	France	Narbonne, Aire des "Chavaliers de Cathares"
Hyles e. euphorbiae	Linnaeus	23169	MTD	A. Hundsdörfer	27.04.2000	France	Narbonne, Aire des "Chavaliers de Cathares"
Hyles e. euphorbiae	Linnaeus	23170	MTD	A. Hundsdörfer	21.08.2001	Germany	Viernheim, nr. Heidelberg
Hyles e. euphorbiae	Linnaeus	23171	MTD	A. Hundsdörfer	17.08.2001	Germany	Viernheim, nr. Heidelberg
Hyles e. euphorbiae	Linnaeus	23172a	MTD	A. Hundsdörfer	22.08.2001	Germany	Viernheim, nr. Heidelberg
Hyles e. euphorbiae	Linnaeus	23293	MTD	C. Anderssohn	01.07.2003	Germany	Peschenfeld, nr. Berlin
Hyles e. euphorbiae	Linnaeus	23197	MTD	A. Hundsdörfer	vi.2002	Italy	Naturns, nr. Meran, north
Hyles e. euphorbiae	Linnaeus	005k	MTD	A. Hundsdörfer	05.09.2002	Spain	Patja de Malgresa, Ebro Delta, Catalonia; N 40°46′06″ E 00°47′29″
Hyles e. euphorbiae	Linnaeus	008a	MTD	A. Hundsdörfer	06.09.2002	Spain	Maella, nr Caspe, betw. Zaragosa and Tortosa; N 41°05′43″ E 00°07′08″

Table 1. (*continued*)

Taxon	Author	No.	Institution[a]	Collector/provider	Date	Origin	Locality
Hyles e. euphorbiae	Linnaeus	010	MTD	A. Hundsdörfer	09.09.2002	Spain	Altura, NE of Sagunto/ Valencia; N 39°50′41″ E 0°32′16″
Hyles e. euphorbiae	Linnaeus	014f	MTD	A. Hundsdörfer	10.09.2002	Spain	Rojáles, Murcia; N 38°5′36″ E 00°42′43″
Hyles e. euphorbiae	Linnaeus	019e	MTD	A. Hundsdörfer	11.09.2002	Spain	Torreblanca, betw. Tortosa and Castellón; N 40°14′50″ E 00°14′21″
Hyles e. euphorbiae	Linnaeus	020c	MTD	A. Hundsdörfer	13.09.2002	Spain	Alió, Catalonia; N 41°17′35″ E 01°17′6″
Hyles e. euphorbiae	Linnaeus	021b	MTD	A. Hundsdörfer	02.09.2002	Spain	betw. Platja d'Aro and S'Agaro; N 41°18′14″ E 03°03′13″
Hyles e. euphorbiae	Linnaeus	030d	MTD	A. Hundsdörfer	spring 2003	Spain	Malaga; N 36°39′31″ E 04°28′30″
Hyles e. conspicua	Rothschild & Jordan	695864	BMNH	M.V.L. Barclay, D.J. Mann	8./10.05.2001	Turkey	Anatolia, Akyaylar tunnel; N 36°46′14″ E 30°34′21″ or Yalniz; N 36°25′3″ E 30°08′29″
Hyles e. conspicua	Rothschild & Jordan	695886	BMNH	M.V.L. Barclay, D.J. Mann	8/10.05.2001	Turkey	as 695864
Hyles e. conspicua	Rothschild & Jordan	696106	BMNH	F. Doganlar	29.09.2002	Turkey	Bolkar Daglari, Nigde, Maden Köyü; N 37°27′17″ E 34°38′22″
Hyles e. conspicua	Rothschild & Jordan	696107	BMNH	F. Doganlar	29.09.2002	Turkey	Bolkar Daglari, Nigde, Maden Köyü; N 37°27′17″ E 34°38′22″

[a]BMNH: Department of Entomology, The Natural History Museum, London, UK; MTD: Museum für Tierkunde, Staatliche Naturhistorische Sammlungen Dresden, Germany; ZFMK: Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, Germany.

Table 2. Sequences of primers used in this study to amplify and sequence the COX I + II and t-RNA-Leu genes

Name of primer	5′-sequence-3′
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC
C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC
C1-J-2183	CAACATTTATTTTGATTTTTTGG
TL2-N-3019mod	TTAAATCCAWTNCAYRWAATCTGCC
TL2-J-3037mod2	TAATATGGCAGATTWYRTGNAWTGG
TK-N-3782	GAGACCATTACTTGCTTTCAGTCATCT

The nomenclature follows Simon et al. (1994); if primers were modified or specifically designed for this study, they are marked by 'mod'.

were analysed using maximum parsimony (MP; equal weighting, EW) and maximum likelihood (ML), as implemented in PAUP* 4.0b10 (Swofford 1998). The MP analysis was undertaken using the following options: addition sequence = closest (one tree held at each step), TBR branch swapping, and no limit to the maximum number of most parsimonious trees. For the ML analysis, the majority rule consensus tree of the first 1000 most parsimonious trees was used as starting tree. The best evolutionary model for the data was established by hierarchical likelihood testing, performed with Modeltest (Posada and Crandall 1998). Branch support was estimated by two separate bootstrap analyses (1000 replicates), calculated with the MP and neighbour-joining (NJ) algorithms, respectively.

Timing of mtDNA diversification events

In our study of the phylogeny of *Hyles* (Hundsdoerfer et al., 2005a), we estimated whether rates of mtDNA molecular evolution were equivalent among taxa of the genus, a condition required for the dating of cladogenetic events. Our results validated the assumption of clock-like evolution for the sequences studied. In this paper we have not added any highly divergent sequences and thus consider the above result as also applicable to the present expanded mtDNA data set. Approximate times of diversification for selected clades were calculated by converting pairwise genetic distances into units of time, following Brower's (1994) inferred molecular clock for mtDNA sequence divergence. The underlying assumption then is a constant mutation rate of 1.1–1.2% per million years per lineage, leading to 2.3% sequence divergence between pairs of species per million years of separation.

Analysis of ISSR-PCR fingerprints

ISSR-PCR fingerprint data were acquired for 72 of the 100 samples from which mtDNA sequence data had been obtained. We therefore constructed a second, reduced mtDNA data set comprising these 72 samples for comparison with the ISSR data. Variation in the sequence data set was characterized in the same manner as the variation in the ISSR-PCR fingerprint data (following Culley and Wolfe 2001), with PAUP* 4.0b10 (Swofford 1998). Both data sets were then subjected to several analyses. First, to compare the variability of our ISSR-PCR data to that of other fingerprint studies, we calculated average population distances based on the coefficient of Nei and Li (1979). These pairwise distances were generated using the program RAPDistance (version 1.04; Armstrong et al. 1996) and the population means calculated using MS-Excel. Second, cluster analyses of the two data sets were performed using NJ (p-distances), as implemented in PAUP* 4.0b10 (Swofford 1998). These calculations were performed without an a priori defined outgroup taxon.

Both data sets were also subjected to two MP analyses. The first applied EW to all characters, as implemented in PAUP* 4.0b10 (Swofford 1998), using the options given above, except that the maximum number of most parsimonious trees held had to be set to 1000 due to run-time limitations. However, the results of the ISSR analysis were highly conflicting, showing that these data were highly homoplastic (see below). Consequently, we considered it appropriate to differentially weight the characters and downweight those that were most homoplastic. We therefore also analysed both data sets using implied weights (IW) as implemented in the program Parsimony and Implied Weights (PIWE), version 3.0 for Windows (Goloboff 1997). We used the strongest weighting function available (the concavity constant, $K = 1$) because of the high levels of homoplasy and conflict in the data. Both analyses were performed by heuristic search, using 20,000 replications (mult*20,000), holding 15 cladograms per replicate (hold/15), and a maximum of 100,000 trees (hold = 100,000), followed by extended branch-swapping (max*) of the resultant fittest trees. The strict consensus tree of the fittest trees is presented.

Finally, to examine the genetic structure among populations and regions, an Analysis of MOlecular Variance (AMOVA) of both data sets was performed using the program Arlequin (Schneider et al. 2000; ISSR matrix defined as 'standard' data; loci of the sequence data with >5% missing data excluded), following Kauserud and Schumacher (2003). Populations were defined as specimens from the geographically grouped localities of a particular subspecies, not differentiating populations from different islands or countries (e.g. all localities from the seven Canary Islands were treated as one population of *H. t. tithymali*; all *H. e. euphorbiae* from Spain, France, Germany, Italy, etc. were treated together). The 'groups' were defined as the species following Kitching and Cadiou (2000), with the

modification that the *H. robertsi* (Butler) samples were assigned to the *H. euphorbiae* species group.

Results

Sequence characterization

The sequence data (HEC only) showed a nucleotide composition of 34.5% A, 13.5% C, 39.5% T and 12.5% G. Most nucleotide sites were conserved (2143/2295). Of the 152 variable nucleotide sites 80 were parsimony informative and 72 represented autapomorphies. When translated to amino acids 734/765 were conserved and 28 were variable. Eleven of the amino acid substitutions were parsimony informative and seventeen represented autapomorphies.

Phylogenetic analysis

The ML tree of the 100 *Hyles* mtDNA sequences, with branch lengths proportional to evolutionary difference between taxa, is shown in Fig. 3a. The MP analysis resulted in 67,192 most parsimonious trees (CI = 0.656, RI = 0.894), the strict consensus tree of which is shown in Fig. 3b. Based on the phylogenetic results of Hundsdoerfer et al. (2005b), we chose *H. nicaea*, *H. gallii*, *H. livornica*, *H. vespertilio*, *H. hippophaes*, *H. siehei* and *H. dahlii* as outgroups for this more detailed study of the HEC s. str. The first four of these species are more distantly related to, and the latter three more closely associated with, the HEC s. str. (Fig. 3). In the ML tree (Fig. 3a), *H. hippophaes* clusters with three *H. sammuti* samples, and this cluster is the sister group of *H. siehei*. Together, these form the sister group of *H. dahlii*, with which they combine to form the sister group of the HEC s. str. In the MP strict consensus tree, the *H. hippophaes*/*H. sammuti* group is unresolved with respect to both *H. dahlii* and the HEC s. str. (Fig. 3b), and the sister group of this clade is *H. siehei*.

In addition to the five samples of *H. sammuti* from our previous study (Hundsdoerfer et al. 2005b), three more are included here. The possible hybrid origin of this taxon is indicated again (for additional references supporting this possibility, see Hundsdoerfer et al. 2005b). The *H. sammuti* sample from Sicily clearly clusters within *H. tithymali*, where it forms a distinct subclade with two *H. t. mauretanica* samples and one sample of *H. e. euphorbiae* from Spain (#008a, Table 1). The Maltese *H. sammuti* samples fall into two very different clusters. Four samples group within *H. euphorbiae*, where they are unresolved in the MP result (Fig. 3b). In the ML tree (Fig. 3a) they form a weakly demarcated clade with *H. euphorbiae* samples from Italy, Turkey and Spain. The three remaining *H. sammuti* samples fall outside of the HEC s. str. altogether, clustering with *H. hippophaes* (and *H. siehei* in the ML reconstruction) as discussed above, although with low bootstrap support (<50%).

The HEC s. str. (Fig. 3) divides into two distinct groups, corresponding to the *H. tithymali*- and *H. euphorbiae* groups. Both optimality criteria produce rather similar clade structures (Fig. 3), thus the following refers to both reconstructions, unless otherwise specified. The *H. euphorbiae* group forms one highly uniform cluster, in which the populations are largely unresolved (Fig. 3). Neither of the two subspecies, *H. e. conspicua* (Rothschild & Jordan) and *H. e. euphorbiae*, is recovered, indicating a high level of gene flow between them. The three sequences of *Hyles robertsi* (Iran) are also nested unresolved within this cluster.

Within the *H. tithymali* group, two main clades can be distinguished (Fig. 3a). The subspecies *H. t. tithymali* (Canary Islands) (except #094a) and *H. t. gecki* de Freina (Madeira) form a Macaronesian clade, and *H. t. deserticola* (Staudinger) (south of the High Atlas; except #16144) and *H. t. himyarensis* Meerman (Yemen) form a Saharo-Arabian clade (Fig. 2). Most *H. t. mauretanica* (except #200) show an affinity with the latter group (Fig. 3a), this clade then comprising a mainland African group but with the inclusion of *H. sammuti* (#239) and *Hyles e. euphorbiae* (#008a) as mentioned above, or else are unresolved with respect to the aforementioned two *H. tithymali* subgroups (Fig. 3b). The single sample of *Hyles costata* (western Siberia) clusters consistently and closely with the *H. tithymali* group (Fig. 3).

Timing of mtDNA diversification events

Dates of selected cladogenetic events, using Brower's (1994) inferred molecular clock for mtDNA sequence divergence, are presented in Table 3. Excluding *H. sammuti*, *H. dahlii*, and *H. e. euphorbiae* #008a, the oldest split within the HEC appears to have occurred just over 4 my (million years) B.P., i.e. that between *H. costata* and *H. euphorbiae* (including *H. robertsi*). The split between *H. costata* and *H. tithymali* is younger, which is consistent with the former species clustering with *H. tithymali* rather than *H. euphorbiae* (Fig. 3). The split of *H. tithymali* and *H. euphorbiae* (including *H. robertsi*) is dated to 3.0–1.4 my B.P. The maximum divergence time between representatives of *H. tithymali* is hypothesized to be 1.5–1.4 my B.P. Differentiation events within *H. euphorbiae* (including *H. robertsi*) are slightly younger, with a maximum divergence time of around one my B.P. Finally, the separation of *H. euphorbiae* and *H. robertsi* is estimated to be no more

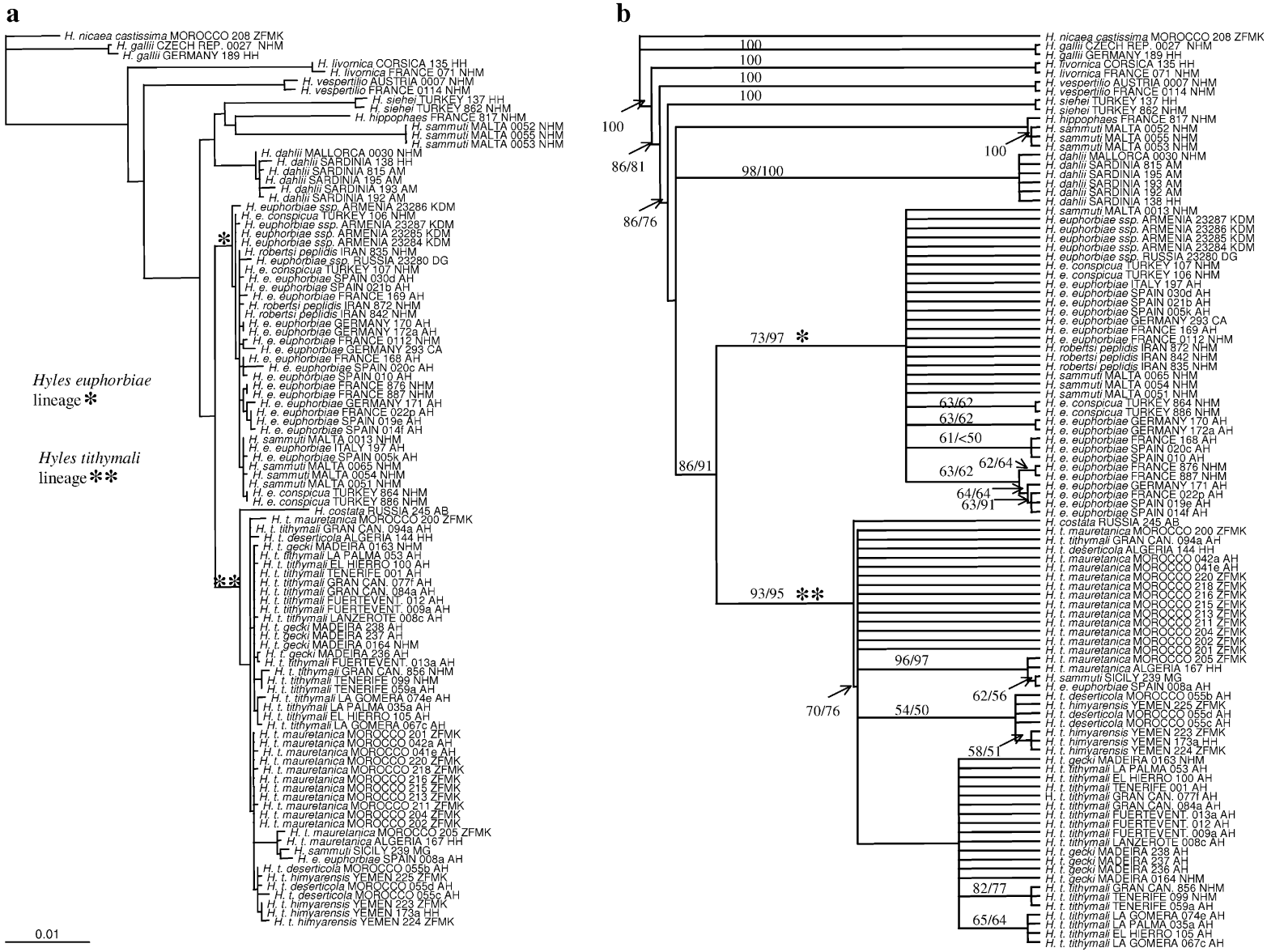

Fig. 3. Phylogeny of the *Hyles euphorbiae* complex (HEC) based on analyses of about 2300 bp mtDNA sequence data (COX I, t-RNA-Leu and COX II). (a) Maximum likelihood reconstruction calculated with the following parameters (evaluated by MODELTEST), corresponding to the TrN + G + I model: base = (0.3442 0.1349 0.1257), Nst = 6, Rmat = (1.0000 9.3050 1.0000 1.0000 29.1334), Γ-shape = 1.1395, Pinvar = 0.7550. Scale bar = 0.01 evolutionary changes; branch lengths are proportional to evolutionary difference between taxa. (b) Strict consensus tree of 67,192 most parsimonious trees found by heuristic search with the optimality criterion equally weighted maximum parsimony (CI = 0.656, RI = 0.894). Bootstrap support is given above branches, based on heuristic maximum parsimony/NJ-ml-distance (the latter not presented if equal to MP bootstrap).

Table 3. Approximate timing of selected diversification events

A

	Mean		St. deviation		Minimum		Maximum	
Split	*p*	ml	*p*	ml	*p*	ml	*p*	ml
HEC excl. *H. sammuti* and *H. dahlii*	0.0050	0.0054	0.0035	0.0039	0.0000	0.0000	0.0157	0.0185
H. euphorbiae to *H. robertsi*	0.0011	0.0011	0.0006	0.0007	0.0000	0.0000	0.0031	0.0032
H. euphorbiae + *H. robertsi* to *H. tithymali*	0.0081	0.0088	0.0009	0.0011	0.0062	0.0066	0.0115	0.0132
within *H. euphorbiae* + *H. robertsi*	0.0016	0.0016	0.0009	0.0009	0.0000	0.0000	0.0045	0.0047
within *H. tithymali*	0.0016	0.0017	0.0012	0.0013	0.0000	0.0000	0.0062	0.0067
H. costata to *H. tithymali*	0.0099	0.0110	0.0007	0.0009	0.0089	0.0098	0.0122	0.0139
H. costata to *H. robertsi* + *H. euphorbiae*	0.0133	0.0152	0.0006	0.0008	0.0124	0.0141	0.0157	0.0185

B

	p-distance		ml-distance	
Split	Range begin	Range end	Range begin	Range end
HEC excl. *H. sammuti* and *H. dahlii*	0.000	3.604	0.000	4.260
H. euphorbiae to *H. robertsi*	0.000	0.708	0.000	0.738
H. euphorbiae + *H. robertsi* to *H. tithymali*	1.424	2.645	1.520	3.036
within *H. euphorbiae* + *H. robertsi*	0.000	1.026	0.000	1.088
within *H. tithymali*	0.000	1.426	0.000	1.546
H. costata to *H. tithymali*	2.045	2.799	2.247	3.195
H. costata to *H. robertsi* + *H. euphorbiae*	2.861	3.604	3.252	4.260

(A) Distances between selected splits of clades (all *H. sammuti*, all *H. dahlii*, and sample *H. euphorbiae* #008a excluded); both the uncorrected (*p*-)distances and the model-corrected (ml-)distances (of the total sequence data) are presented. (B) Range of divergence times of the selected splits, assuming an underlying constant mutation rate of 1.1–1.2% per million years per lineage, leading to 2.3% pairwise sequence divergence per million years (Brower 1994); a range from the minimal (range begin) to the maximal (range end) pairwise distance is calculated; times are given in million years from the present.

than 0.7 my B.P., indicating very low genetic differentiation.

ISSR-PCR fingerprints

Since no sizing mechanism was available to determine the molecular weights of the fragments, comparisons were enabled by including common samples on the gels. The bands with high molecular weight were excluded due to non-reproducibility. Only bands that could be compared across all gels were scored, giving a data set without missing entries. A total of 28 fragments from 72 samples were scored for analysis.

Sequences versus ISSR-PCR fingerprints

Variability of ISSR-PCR fingerprint data compared to mtDNA sequence data is presented in Table 4. In all characteristics examined, ISSR-PCR fingerprint data are more variable than sequences. Nevertheless, the absolute number of differences is generally higher for sequence data. An exception to this trend is observed within *H. euphorbiae* and *H. tithymali*, respectively, where there is a higher number of shared, parsimony informative sites in the ISSR-PCR fingerprints than the sequence data (Table 4).

Average interpopulation distances based on the coefficient of Nei and Li (1979) are presented in Table 5. Maximum genetic distance, with no shared bands, is indicated by a value of 1, whereas a value of 0 represents no genetic difference with all bands shared between the two populations. Many average distances within populations are not significantly smaller (i.e. there is no overlap of the ranges defined by the standard deviations) than between populations, indicating very high intra-population or inter-individual variation.

The results of the NJ cluster analyses of the two data sets, mtDNA and ISSR-PCR, are presented in Fig. 4. Although based on fewer samples, the sequence data show a well supported branching pattern (Fig. 4a) broadly similar to that obtained previously (Fig. 3). Of particular note are the three Maltese *H. sammuti* samples at the base of the HEC s. str. on a relatively long branch. The ISSR-PCR data present a very different topology (Fig. 4b). Bootstrap support was generally low, which is expected in an analysis using few, very variable markers. *Hyles dahlii* is corroborated as a

Table 4. Comparison of the variability of the two sources of molecular data

A					
Characteristic	Nucleotide positions COX sequence data	Bands of ISSR fingerprint			
Total	2295	28			
Fixed, constant	2157 (94%)	0 (0%)			
Polymorphic, variable	138 (6%)	28 (100%)			
Shared, parsimony informative	83 (3.6%)	26 (92.9%)			
Unique, parsimony uninformative	55 (2.4%)	2 (7.1%)			
Characteristic	*H. dahlii*	*H. sammuti*	*H. robertsi*	*H. euphorbiae*	*H. tithymali*
B					
Fixed, constant	2289 (99.7%)	2232 (97.3%)	2294 (100.0%)	2255 (98.3%)	2211 (96.3%)
Polymorphic, variable	6 (0.3%)	63 (2.7%)	1 (0.0%)	40 (1.7%)	84 (3.7%)
Shared, parsimony informative	0 (0.0%)	46 (2.0%)	0 (0.0%)	10 (0.4%)	10 (0.4%)
Unique, parsimony uninformative	6 (0.3%)	17 (2.7%)	1 (0.0%)	30 (1.3%)	74 (3.2%)
C					
Fixed, constant	20 (71.4%)	14 (50.0%)	23 (82.1%)	8 (28.6%)	8 (28.6%)
Polymorphic, variable	8 (28.6%)	14 (50.0%)	5 (17.9%)	20 (71.4%)	20 (71.4%)
Shared, parsimony	2 (7.1%)	11 (39.3%)	0 (0.0%)	15 (53.6%)	16 (57.1%)
Unique, parsimony uninformative	6 (21.4%)	3 (10.7%)	5 (17.9%)	5 (17.9%)	4 (14.3%)

(A) Whole data sets ($n = 73$) consisting of *H. dahlii* ($n = 4$), *H. sammuti* ($n = 8$), *H. robertsi* ($n = 3$), *H. euphorbiae* ($n = 21$) and *H. tithymali* ($n = 37$). (B) Sequence data of the five species separately. (C) ISSR data of the five species separately.

lineage separate from the *H. tithymali*/*H. euphorbiae* group (although *H. euphorbiae* #010 from Spain is included within it), but the latter does not form the two distinct branches seen in the mtDNA tree (Fig. 4a). Although the *H. tithymali* samples are largely found within one large (but poorly supported) cluster, many *H. euphorbiae* samples are associated with that same group. The remaining *H. euphorbiae* lineage samples form several clusters below the one that includes those of *H. tithymali*, none of which, however, is supported by bootstrap values >50%. Interestingly, the *H. robertsi* samples form a single cluster near the base of the phenogram.

The results of the mtDNA data EW-MP analysis (not shown) show a branching pattern similar to that found previously (Figs. 3a and 4a). However, EW-MP analysis of the ISSR-PCR data (not shown) yielded a strict consensus tree with very little resolution, indicating a high amount of internal data conflict. Application of IW to the mtDNA data also produced a strict consensus tree (Fig. 5a) corresponding closely to previous results. In contrast, however, the IW-MP analysis of the ISSR-PCR data (Fig. 5b) produced much more resolution than that with EW, although still less than for the mtDNA data (Figs. 3a, 4a and 5a) and the ISSR NJ tree (Fig. 4b). Surprisingly, in the IW-MP ISSR tree, the *H. dahlii* sample #195 clusters with *H. tithymali*, and another (#193) with *H. euphorbiae*. The Spanish *H. e. euphorbiae* #010 is again associated with *H. dahlii*; together they form the sister group of the remaining HEC s. str. There are no mixed clusters of *H. euphorbiae* and *H. tithymali*, as in the NJ tree (Fig. 4b), except, interestingly, the Spanish *H. euphorbiae* #008a, which clusters with *H. tithymali* in the mtDNA results (Figs. 3, 4a and 5a) as well.

AMOVA analyses of the sequence data (of all samples for which ISSR data are also available; Table 6a) resulted in the greatest proportion (62.65%) of the variance being among species. Significant genetic structuring into the species *H. dahlii*, *H. sammuti*, *H.*

Table 5. Matrix of Nei and Li (1979) average pairwise genetic distances (∅) with standard deviations (δ) for the ISSR-PCR data of the ten populations of the HEC sampled, calculated with RAPDistance v. 1.04; populations defined as follows: *H. dahlii* ($n = 4$), *H. robertsi* ($n = 3$), *H. e. euphorbiae* ($n = 17$), *H. e. conspicua* ($n = 4$), *H. sammuti* ($n = 8$), *H. t. mauretanica* ($n = 11$), *H. t. deserticola* ($n = 2$), *H. t. gecki* ($n = 5$), *H. t. tithymali* ($n = 15$), *H. t. himyarensis* ($n = 4$); values along center diagonal (in boldface): within populations, values below diagonal: between populations

	H. dahlii		*H. robertsi*		*H. euphorbiae*		*H. conspicua*		*H. sammuti*		*H. mauretanica*		*H. deserticola*		*H. gecki*		*H. tithymali*		*H. himyarensis*	
	∅	δ	∅	δ	∅	δ	∅	δ	∅	δ	∅	δ	∅	δ	∅	δ	∅	δ	∅	δ
H. dahlii	**0.440**	**0.108**																		
H. robertsi	0.714	0.181	**0.363**	**0.180**																
H. euphorbiae	0.623	0.176	0.691	0.120	**0.528**	**0.153**														
H. conspicua	0.768	0.158	0.727	0.103	0.576	0.174	**0.544**	**0.148**												
H. sammuti	0.716	0.167	0.681	0.141	0.633	0.136	0.676	0.139	**0.544**	**0.171**										
H. mauretanica	0.791	0.123	0.706	0.117	0.657	0.160	0.523	0.168	0.574	0.169	**0.455**	**0.210**								
H. deserticola	0.796	0.099	0.781	0.019	0.620	0.154	0.490	0.053	0.595	0.172	0.400	0.189	**0.333**	**n.a.**						
H. gecki	0.842	0.169	0.889	0.137	0.660	0.178	0.642	0.151	0.658	0.189	0.553	0.140	0.427	0.159	**0.285**	**0.104**				
H. tithymali	0.779	0.144	0.795	0.126	0.662	0.175	0.561	0.150	0.618	0.168	0.478	0.157	0.405	0.131	0.439	0.137	**0.422**	**0.128**		
H. himyarensis	0.802	0.174	0.779	0.199	0.628	0.201	0.553	0.147	0.630	0.206	0.464	0.165	0.399	0.128	0.297	0.113	0.447	0.136	**0.305**	**0.040**

euphorbiae (incl. *H. robertsi*) and *H. tithymali* is observed. Variance among populations is rather low, indicating high gene flow between populations within species. Nevertheless, genetic structuring at this level is significant at the <0.01 level. The ISSR data show much higher variance (Table 6b) within populations than among either populations or species. This corroborates the above observation that the level of variability from this source of molecular data is much higher than in the COX sequences. Genetic structure among populations within species is observed with a higher significance level in the ISSR-PCR than the sequence data (Table 6).

Discussion

Sequence characterization

Nucleotide composition of the three codon positions of the COX genes reveals an AT bias, which is not unusual for insect mtDNA (Simon et al. 1994). However, this feature of the data necessitates more complex models in ML calculations and distance correction procedures to correct for the effect of multiple hits than under the assumption of equal base frequencies. Therefore, we place more confidence in the results of the dating processes based on ML-corrected distances.

Phylogeny and biogeography of the HEC

In a more extensive analysis of *Hyles*, Hundsdoerfer et al. (2005b) concluded that *H. nicaea* is not closely related to the HEC s. str. and excluded it from this group. The same relationship was recovered in the present study (Fig. 3), corroborating our rejection of Danner et al.'s (1998) subgenus *Hyles* (Fig. 1c). Rather, the HEC s. str. is restricted to just those species most closely related to *H. euphorbiae* and *H. tithymali*, together with those placed by Danner et al. (1998) in subgenus *Surholtia*: *H. costata, H. chuvilini* Eitschberger, Danner & Surholt, and *H. nervosa* (Rothschild & Jordan, 1903). Unfortunately, samples of the last two species were unavailable for sequence analysis, and none of the three were available for ISSR analysis.

The position of *H. dahlii*

More closely associated with the HEC are *H. vespertilio*, *H. hippophaes*, *H. siehei* and *H. dahlii*. It is the position of the last of these species that conflicts most with previous hypotheses of relationship based on morphological characters (Fig. 1). Meerman (1993) included *H. dahlii* within the HEC (as part of the *H. tithymali* subgroup) (Fig. 1a). Derzhavets (1994), too, placed *H. dahlii* within the HEC s. str., between *H.*

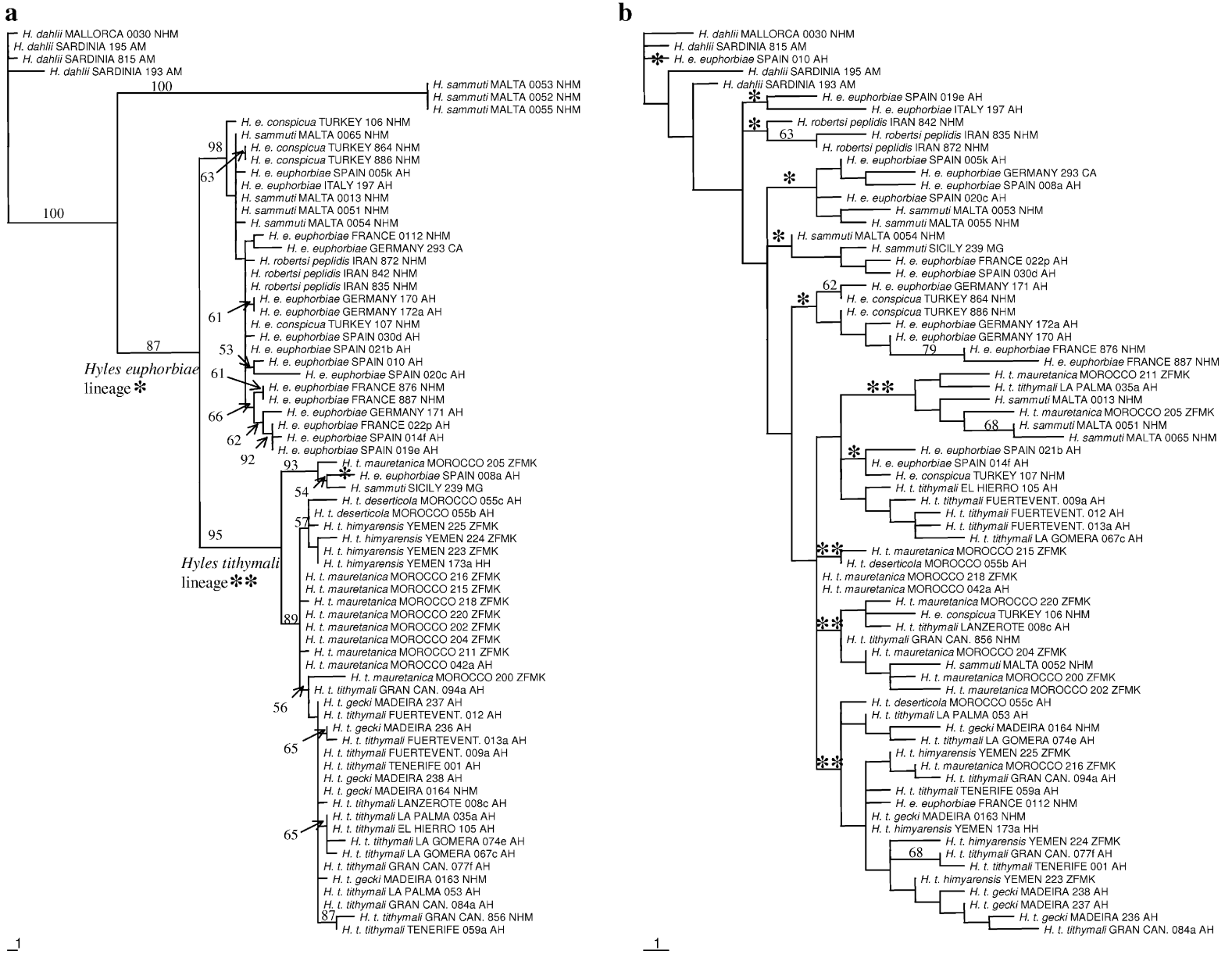

Fig. 4. Phenograms of 72 HEC samples derived from NJ cluster analyses (p-distance). (a) About 2300 bp mtDNA sequence data (COX I, t-RNA-Leu and COX II). (b) ISSR-PCR data. Bootstrap support is given above branches, which are proportional to phenetic distance. Scale bar indicates one character difference for comparison with branch lengths.

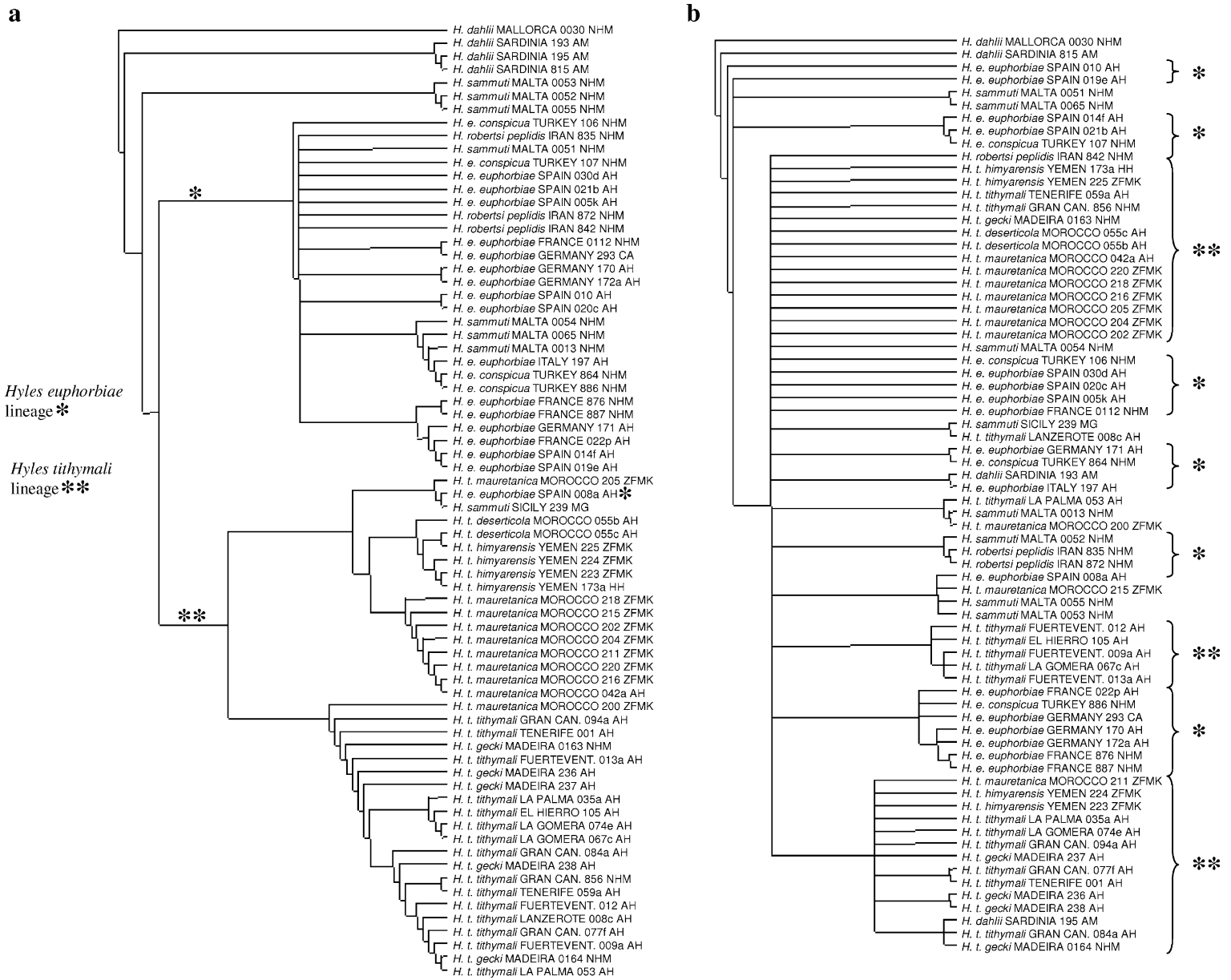

Fig. 5. Strict consensus trees derived from maximum parsimony analysis using implied weights (IW) as implemented in PIWE (see text for details). (a) About 2300 bp mtDNA sequence data (COX I, t-RNA-Leu and COX II; $n_{trees} = 5$, fit = 683.5). (b) ISSR-PCR data ($n_{trees} = 100{,}000$, fit = 102.4).

Table 6. Results of AMOVA calculations (10,100 random permutations) of the data; groups were defined as species following Kitching and Cadiou (2000), except that samples of *H. robertsi* were included in the species group of *H. euphorbiae*

Source of variation	d.f.	Sum of squares	Variance components	% of variation	Fixation indices	*p*-value
A						
Among groups	3	324.157	6.75547	62.65	$F_{CT} = 0.62653$	0.0000***
Among populations within groups	6	33.212	0.28870	2.68	$F_{SC} = 0.07169$	0.0031**
Within populations	63	235.508	3.73822	34.67	$F_{ST} = 0.65330$	0.0002***
Total	72	592.877	10.78239			
B						
Among groups	3	44.777	0.58408	16.53	$F_{CT} = 0.16526$	0.0018**
Among populations within groups	6	31.288	0.43339	12.26	$F_{SC} = 0.14690$	0.0000***
Within populations	63	158.565	2.51691	71.21	$F_{ST} = 0.28788$	0.0000***
Total	72	234.630	3.53438			

(A) Sequence data. (B) ISSR-PCR data.***Significant at the <0.001 level, **significant at the <0.01 level to contain genetic structure.

tithymali and the remaining species (Fig. 1b). Danner et al. (1998) included *H. dahlii*, together with *H. tithymali* (the subspecies of which they treated as species), *H. cretica* Eitschberger, Danner & Surholt and *H. sammuti*, within subgenus *Rommeliana* Eitschberger & Zolotuhin (Fig. 1c). In the present study, the ML tree (Fig. 3a) indicates a position for *H. dahlii* outside the bulk of the HEC s. str., in a sister-group relationship with species that no previous author has considered close relatives. If the HEC were expanded to incorporate *H. dahlii*, then *H. siehei* (Fig. 3a) and *H. hippophaes* (Fig. 3) would also have to be included. However, in the MP trees (Fig. 3b) *H. siehei* forms a separate branch at the base of the group, with *H. dahlii* and a cluster comprising *H. hippophaes* and three *H. sammuti* samples forming a trichotomy with the HEC s. str. Hundsdoerfer et al. (2005b) hypothesized that the *H. sammuti* samples were of hybrid origin and therefore should be treated with caution, as should the position of the single *H. hippophaes* sample, because singleton samples may show artefactual clustering due solely to the lack of other samples with similar characteristics. Furthermore, Nei and Li (1979) genetic distances (of the ISSR data) between the *H. dahlii* and HEC s. str. samples (Table 5) are only slightly higher than those within the HEC s. str. itself, indicating a rather close relationship. Thus, a sister-group relationship between *H. dahlii* and the HEC s. str. cannot be entirely ruled out on the basis of our present data. In contrast to its position in all three morphological studies (Fig. 1), our results provide no evidence that *H. dahlii* falls within the HEC s. str., and it certainly is not most closely related to a monophyletic *H. tithymali*. Consequently, the rejection of subgenus *Rommeliana* by Hundsdoerfer et al. (2005b) as a valid, monophyletic taxon is corroborated here, a conclusion also suggested by de Freina and Geck (2002) based on a reanalysis of morphological characters.

Possible introgression zones of HEC lineages

Our phylogeny of the HEC s. str. (Fig. 3) differs from those derived from morphology (Fig. 1) in several other respects that corroborate the observations described by Hundsdoerfer et al. (2005b). The additional samples of *H. sammuti* further support a scenario of hybridization on Malta (Fig. 2b). Larvae of the HEC population on Crete (currently treated as *H. cretica*) are reported to show morphological characteristics of *H. tithymali* (see Meerman 1988; Meerman and Smid 1988; Harbich 1989), and this population should be examined with molecular methods. We would hypothesize that all populations on the Mediterranean islands (Malta, Crete, Sicily, possibly Rhodes; Fig. 2b and c) may be of hybrid origin. In addition, contact zones between *H. tithymali* and *H. euphorbiae* lineages are hypothesized for central and southern Italy by Mazzei et al. (2004; Fig. 2b) and for the Asir mountains in Saudi Arabia by Pittaway (in Wiltshire 1990; Fig. 2d). A closer examination of the HEC populations in Italy is in preparation.

Divergence of the HEC in comparison to other insect groups

Hyles costata (from Mongolia and the Russian Far East; Derzhavets 1979) is placed at the base of *H. tithymali*, as found by Hundsdoerfer et al. (2005b), to which we refer the reader for a detailed discussion of the corresponding systematics. In the present study, the mean genetic distance between *H. costata* and *H. tithymali* is slightly higher than in our previous estimate (1.2–1.9% vs. about 1%). However, a close relationship between these two species remains evident, and the genetic distance still is less than that detected between species in studies of other Lepidoptera based on the same genes as used here (Blum et al. 2003; Brown et al. 1994; Sperling and Hickey 1994). Thus, we maintain our previous assessment that the classification of these two

species by Danner et al. (1998) into separate subgenera is excessive.

The same assessment applies to *H. tithymali* and *H. euphorbiae* (including *H. robertsi*, see below). Their mitochondrial genetic divergence is 0.6–1.3%, a range that is almost identical to that reported within species of some Lepidoptera (e.g. 0.6–1.2% reported by Aagaard et al. (2002), but much lower than that observed between closely related species of other butterfly genera (Blum et al. 2003; Brown et al. 1994; but see also Sperling and Hickey 1994). Sperling et al. (1999) reported within-lineage variation of COX haplotypes in a geometrid moth of about 2%, and values up to 2.4% were found in a species group of chrysomelid beetles by Funk (1999). Divergences of as much as 3.4% have been described for morphologically distinct subspecies of the butterfly, *Heliconius erato* (Brower 1994). Thus, we found a relatively extremely low level of divergence between the two main lineages of *H. tithymali* and *H. euphorbiae*, more akin to within-species variation in other insects. These results suggest a very recent cladogenetic event. Consequently, we regard the classification of *H. tithymali* and *H. euphorbiae* by Danner et al. (1998) into different subgenera, and the treatment of their subclades as species, as excessive.

The *H. tithymali* lineage

Within the *H. tithymali* subgroup, there is a suggestion of differentiation into two clades (Fig. 3a), although bootstrap support is very weak. Leaving aside three unresolved samples, one group comprises the Macaronesian populations (*H. t. tithymali*, Canary Islands; *H. t. gecki*, Madeira; Fig. 2), and the other includes the Saharo-Arabian taxa (*H. t. mauretanica*, mountains of North Africa; *H. t. deserticola*, northern Sahara Desert; *H. t. himyarensis*, mountains of Yemen; Fig. 2). Within this latter group (Fig. 3a), and for now leaving aside the included samples of *H. sammuti* and *H. euphorbiae* (see above), the Saharan *H. t. deserticola* clusters with the Yemeni *H. t. himyarensis*. Our results support a very close relationship between these two taxa and corroborate the hypothesis of Pittaway (1993) that during the last Ice Age, *H. t. deserticola* ranged east as far as the Arabian Peninsula. When the ice retreated, so did *H. t. deserticola*, leaving an isolated population in the mountains of Yemen that then diverged slightly to become *H. t. himyarensis*. At present, it is unclear how far across North Africa *H. t. deserticola* actually ranges (Meerman 1988; Fig. 2a), although Gatter and Gatter (1977) reported a migration through the Sahara and Pittaway (1983a) mentioned its occurrence in western Egypt. As we previously observed (Hundsdoerfer et al. 2005b), but based on fewer samples, the samples of the Canary Islands/Madeira cluster are very poorly resolved (Fig. 3a), providing evidence that recognition of *H. t. gecki* as a subspecies separate from *H. t. tithymali* (e.g. de Freina 1991; Harbich 1992) may not be warranted. Furthermore, this study did not detect any genetic difference between the samples from La Palma, Gran Canaria and Fuerteventura, which differ slightly in morphological features (Harbich 2000; Gil-T. 2002). In particular, our data provide no evidence to support the observation that *H. t. deserticola* has established on Fuerteventura (Gil-T. 2002). Our three specimens (#009a, #012, #013a; Table 1) from the "sand-dunes of Corralejo" in the northeast of the island were collected in 2002 (Gil's specimens were caught in 1997). On the basis of their mtDNA sequences, they cluster with the other samples of *H. t. tithymali* and *H. t. gecki* (Fig. 4). Unless Gil collected the only three *H. t. deserticola* at this locality, we might have expected to detect evidence of the progeny of these pioneers in our analysis. However, mitochondrial sequences track only the maternal lineage, so hybridization between male *H. t. deserticola* and female *H. t. tithymali* would not be detected. Nevertheless, ISSR data, which are free from this limitation, also consistently place these Fuerteventuran samples in a group with *H. t. tithymali* and *H. t. gecki* (Figs. 4 and 5), whereas the *H. t. deserticola* samples continue to cluster with those of *H. t. himyarensis* (Figs. 4a and 5). Thus, the genomic data provide no evidence that these Fuerteventura samples are *H. t. deserticola*. A detailed morphological analysis of the Canary Islands populations can be found in Hundsdoerfer et al. (2005a).

The *H. euphorbiae* subgroup and within-species comparisons

The previously observed close relationship between *H. robertsi* and *H. euphorbiae* (see Hundsdoerfer et al. 2005b) is corroborated (Figs. 3 and 4a). Within this clade, uncorrected genetic distances between samples were 0.0–0.45% (max. 0.47% ML-corrected; mean: 0.16%; Table 3). Within *H. tithymali* lineages, the maximum observed value was 0.62% (0.67% ML-corrected), with a mean of 0.16%. These values are slightly higher than the maximum distances for some conspecific butterfly mtDNA haplotypes based on the same genes (Blum et al. 2003), which were at most 0.13%. However, within-species variation of COX I and II as high as 1.03% has been recorded, although sequence divergence between species was just 0.5% in one case (Eastwood and Hughes 2003). The latter incongruence was interpreted as a scenario in which morphological and mitochondrial evolutionary rates were not in step. We consider that the same interpretation is valid for the HEC. High morphological variability in this group of hawkmoths has led to the description of many species by several authors, most notably Danner et al. (1998) and Eitschberger and Surholt (1999). However, this high rate of morphological evolution is not matched by that of the mtDNA. The

latter indicates divergence of no more than two main lineages: *H. euphorbiae* (including *H. robertsi*) and *H. tithymali* (including *H. costata*), within which there has been little genetic differentiation. In the light of these results, we consider the actions of Danner et al. (1998; see also Eitschberger and Surholt 1999), who raised all subspecies within the HEC s. str. to species status, to be inappropriate. Nevertheless, ISSR-PCR fingerprints have provided possible evidence for high nucleomic variability, which could be reflected in the high morphological variability.

The HEC and the ice ages

Hewitt (1996, 1999, 2000) theorized that southern Europe is richer in diversity of species haplotypes than central and northern Europe. When the latter areas were depopulated by the Ice Ages, the former acted as refugia. Central and northern Europe was recolonized from these gene pools, and due to leptokurtic (leading-edge) dispersal diversity is low. For the HEC s. str., modification of this pattern is necessary to explain current genetic structure with respect to the timing of the Ice Ages. At 1.4–3.0 my B.P., the split between *H. euphorbiae* and *H. tithymali* dates to the (end of the) Pliocene. Whether proto-HEC populated Europe is uncertain, for the continent was covered by subtropical forest then, and most current HEC food plants (*Euphorbia*) need open habitats. Nevertheless, as it is also not certain whether the proto-HEC population was dependent on the same food plants, we will assume for the purposes of this discussion that the proto-HEC populated at least southern Europe towards the end of the Tertiary (Pliocene). Then, a split in the proto-HEC population may have led to a northern (European) lineage, *H. euphorbiae*, and a southern (North African) lineage, *H. tithymali* (Fig. 2). Subsequent Pleistocene glaciations would have caused many species in central Europe to become extinct (Hewitt 1996, 1999, 2000, and references therein), including, most probably, the *H. euphorbiae* populations. It may even have been too cold for this species in large parts of southern Europe, so that it was forced into very small Mediterranean refugia, possibly the Mediterranean Islands, and the Middle East. Within the southern range of the HEC in North Africa, the populations of *H. tithymali* were able to survive the glaciations. This hypothesis may explain the subclade formation within *H. tithymali*, but not in *H. euphorbiae*: The former was able to retain ancient polymorphisms until the present, whereas the latter (re)colonized its current distribution range after the Ice Ages from a few (or even only one) refugial populations by leptokurtic dispersal, resulting in low diversity.

The lack of differentiation within *H. euphorbiae/conspicua/robertsi* leads to two considerations. First, the observed pattern suggests that there is no effective barrier to mitochondrial gene flow anywhere within the range of the three taxa (Fig. 2). Second, this pattern might be the result of the Pleistocene Ice Ages. Relict populations of many organisms recolonized central Europe after each Ice Age, a process that could have proceeded very rapidly. Different organisms dispersed from different refugia; three main patterns have been discerned (Hewitt 1999, 2000), exemplified by *Chorthippus parallelus* (a grasshopper), *Erinaceus europaeus/concolor* (hedgehog) and *Ursus arctos* (brown bear). The *H. euphorbiae* pattern seems to resemble most that of the grasshopper. Only the grasshoppers in the Balkans refugium recolonized central Europe, a process that apparently occurred very rapidly. In the case of *H. euphorbiae*, we postulate that relict populations in southern Europe would have been rather small, and that it was another relict population, with a range much further southeast in the region of what today is Iran (Fig. 2: *H. robertsi*), that recolonized (central) Europe, for *H. robertsi* shows no mtDNA differentiation from European *H. euphorbiae*. A difference from the grasshopper pattern that results from this hypothesis is that there do not appear to be distinct haplotypes or subspecies of *H. euphorbiae* in either Iberia or Italy, although more samples need to be examined to corroborate this assessment.

However, it is unclear how old this recolonization event is. During the interglacials, rich vegetation could grow in central Europe (Küster 1995) but we do not know how far north the HEC and their food plants were able to spread. From about 18,000 years B.P., the climate began to moderate (Küster 1995) and that, from about 10,000 years B.P. onwards, allowed increasing numbers of thermophile plants to recolonize the central European steppe. Euphorbias are often pioneer plants, so we expect that these HEC food plants recolonized Europe rapidly, allowing *H. euphorbiae* populations to follow closely. They may have spread sufficiently fast from east to west to reach Iberia before Europe became almost completely covered in forest, at about 7000 B.P. The caterpillars, as well as the food plants, need open habitats with high insolation to thrive, so it is expected that their populations declined once more during this phase. It is unknown whether small populations were able to survive locally until, with the spread of Neolithic culture from 6500 years B.P. onwards (Küster 1995), humans once again created open habitats. Because the early farming techniques came from the Middle East and spread north and west (to Iberia; Küster 1995), as we postulate is the case with *H. euphorbiae*, it may be possible that the moths followed their route. Cultivated steppe replaced woodland, which allowed both euphorbias and moths to thrive.

The present study dates the oldest split within *H. euphorbiae* to about 1 my B.P., i.e. within the Pleistocene Ice Ages, indicating that gene pools other than the one in the Middle East may have contributed to the current

population. This implies that the species had relict populations within Europe both during the Ice Ages and during the forested phases. However, these dates should be regarded with caution, because they are based on less than 1% sequence variation, an amount close to the error limits of data acquisition. Furthermore, they are based on a relatively small part of the total DNA. The challenge will be to find and develop molecular techniques that can reliably detect recent differentiation within the HEC and thus allow clarification of these possible scenarios. Also, more samples need to be studied to allow the application of phylogeographic methods, such as nested-clade analysis, to infer population history precisely. Priority should be given to Italy and the eastern Mediterranean Islands, because their HEC populations show morphological differences from the recognized subspecies (Meerman and Smid 1988; Harbich 1989; Mazzei et al. 2004) and might represent hybrids (Fig. 2b and c).

HEC dispersal barriers

No differences were detected in mtDNA between populations north and south of the Pyrenees respectively, the Alps and the Caucasus, and only comparatively small differences were found between the east and west of the Sahara Desert (Fig. 2). Thus, the Mediterranean Sea appears to be the only effective barrier to the HEC, separating *H. euphorbiae* and *H. tithymali*. However, the Mediterranean Sea is unlikely to be a barrier that is never crossed, particularly at the Strait of Gibraltar where the distance between Europe and North Africa is only 13 km. This would pose no problem to adult HEC moths. After all, given suitable weather conditions *H. euphorbiae* regularly, if rarely, cross the English Channel which, at a minimum distance of 34 km, is over twice as far. Gómez-Bustillo and Fernández-Rubio (1976) mention *H. t. deserticola* as an occasional immigrant in continental Spain. Our study may have detected progeny of a *H. tithymali* immigrant to Spain. The Spanish sample #008a, collected within the range of *H. euphorbiae*, shows an mtDNA sequence typical of *H. tithymali*, and its ISSR-PCR profile also shows greater similarity to *H. tithymali* (Fig. 5b). However, the morphology of this caterpillar does not show any of the features usually considered typical of *H. tithymali*.

This is not surprising, however, because controlled crossing and backcrossing experiments have shown that the genome of the parent species contributing most to a hybrid and its backcrosses (presumably *H. euphorbiae* in this case) determines the morphological appearance of the progeny (Harbich 1976). Mitochondrial genes are concerned with energy production and do not contribute to morphology. Therefore, *H tithymali* mitochondria may persist in a (maternal) lineage that otherwise resembles *H. euphorbiae*. The phenomenon of mitochondrial polyphyly in *H. sammuti* can be explained similarly. Although morphologically similar, the Maltese samples are divided into two mitochondrial haplotypes, one that clusters within the *H. euphorbiae* lineage and another that is very different (Fig. 3). The Sicilian sample resembles the Maltese morphologically, but exhibits a *H. tithymali* mitochondrial haplotype. The HEC population on Malta has been described as morphologically very variable (Valletta 1973), so a combined morphological and molecular study focused on the HEC populations of the Mediterranean Islands (Fig. 2) promises interesting evolutionary and biological insights.

Interestingly, the Sahara desert does not appear to be a serious barrier to *H. tithymali* (see discussion above). Our data show that genetic differentiation between Moroccan *H. t. deserticola* and Yemeni *H. t. himyarensis* is very low, despite them being separated by thousands of kilometres. This is in marked contrast to the larger differences observed between *H. t. deserticola* and the geographically much closer *H. t. tithymali* population on the Canary Islands, which are separated by just over 100 km of sea (Fig. 2). Furthermore, Gatter and Gatter (1977) reported migration of *H. t. deserticola* across the Sahara Desert. These observations again indicate that water is a more effective barrier to *H. tithymali* than desert. However, HEC populations from eastern and western Africa may not even have to cross the desert to remain in contact; we expect suitable habitats with the foodplant *Euphorbia paralias* to exist on beaches along the Mediterranean coast (as observed by AKH in Tunisia).

The mtDNA sequences of *H. t. tithymali* are indistinguishable from those of *H. t. gecki* from Madeira (see discussion above), indicating either a very recent colonization of Madeira from the Canary Islands (Fig. 2), or ongoing genetic exchange following an earlier colonization event. In view of the detectable separation of mainland African populations from those on the Canary Islands, we tend towards the first hypothesis. Favourable weather conditions could have allowed passive transport of *H. t. tithymali* from the Canary Islands to Madeira rather recently (and might continue to do so). We prefer this scenario to one in which moths flew (and continue to fly) the distance actively, because we can see no plausible reason why they would not fly to mainland Africa as well.

H. dahlii (Balearic Islands, Corsica and Sardinia) has been reported in mainland Africa (Hundsdoerfer in press) as well as continental Europe (Masó Planas et al. 1979), northeast of Barcelona in Spain. The former finding was interpreted as a rare dispersion event, in accordance with the interpretation of the Mediterranean Sea representing an effective dispersal barrier to the HEC.

The present study may have detected progeny of a male *H. dahlii* in mainland Spain (Masó Planas et al.

1979). Based on ISSR-PCR data (Figs. 4b and 5b), the Spanish *H. euphorbiae* sample #010 clusters among or near *H. dahlii*, although this relationship is not supported by the (maternally inherited) mtDNA data (Figs. 4a and 5a; see discussion below). This hypothesis of differential barriers to dispersal should be examined using additional samples and phylogeographic methods (as suggested above) to infer population history more precisely.

HEC taxonomy

It was Pittaway (1983) who first placed *H. t. mauretanica* as a subspecies of *H. tithymali*, although Oberthür (1881) had recognized their affinity over a century earlier, treating them as conspecific. Kitching and Cadiou (2000) concurred with this systematic position, but *H. t. mauretanica* has been placed as a subspecies of *H. euphorbiae* by other authors (de Freina and Witt 1987; Harbich 1997a). The latter interpretation can be understood if larval morphology is emphasized, for *H. t. mauretanica* has the double row of white spots as in *H. euphorbiae*, not a single row as usual in *H. tithymali* (for illustrations see Danner et al. 1998). However, our data clearly indicate that *H. t. mauretanica* is part of the *H. tithymali* subgroup, at least with regard to the maternal, mitochondrial line. Similarly, *H. t. himyarensis* was first described as a subspecies of *H. tithymali* by Meerman (1988), but later interpreted as a subspecies of *H. euphorbiae* by both Harbich (1991) and Meerman (1991). This latter interpretation was later supported by data from the number of cells in the micropylar rosette of the egg (Harbich 1996, 1997b). However, as with *H. t. mauretanica*, our data clearly place *H. t. himyarensis* within the *H. tithymali* subgroup. We agree with Harbich that the HEC is a very young species complex, but nevertheless we have detected two distinct mitochondrial lineages within it, corresponding to *H. tithymali* (including the *mauretanica* and *himyarensis* lineages) and *H. euphorbiae* (including *H. robertsi*), respectively. Hybrids between these two subgroups are fertile and healthy (Harbich 1988, 1989, 1991, 1994, 1997b), as far as the F8 generation in some cases (M. O'Neill, pers. comm.), and those reproductive isolation mechanisms that have been described for hybrids between *H. euphorbiae* and more distant species (e.g. *H. gallii*; Harbich 1976) do not appear to apply.

Thus, whether the *H. euphorbiae* and *H. tithymali* lineages should each continue to be treated as a species, or as subspecies of a more inclusive *H. euphorbiae*, becomes a question of what species concept is applied. For the present, we retain the classification of Kitching and Cadiou (2000), as this seems to describe the lineage structure best (except for the species status of *H. robertsi*). We categorically reject the classification of Danner et al. (1998), in which all morphologically distinguishable populations (including putative hybrids) are treated as species. Given the apparent absence of any of the reproductive isolation mechanisms that apply to other hybrid combinations in *Hyles*, there is no evidence to support a HEC that is genetically differentiated to such a high degree.

Sequences versus ISSR-PCR fingerprints

A comparison of the trees in Figs. 4 and 5 shows discrepancy between the relationships indicated by the mitochondrial and nuclear data. For example, the ISSR-PCR data do not recover the *H. euphorbiae* and *H. tithymali* lineages intact. Rather, the samples form mixed clusters (especially in Fig. 4b) or show little resolution (Fig. 5b). However, we interpret the associations in the phenetic NJ tree (Fig. 4b) as artefactual, caused by the low number of highly variable characters in the ISSR-PCR data. Weighted parsimony analysis, which decreases the contribution of the more homoplastic characters, eliminates many of these associations but produces fewer groups (Fig. 5b). The only *H. euphorbiae* sample that clusters with *H. tithymali* is #008a (Fig. 5b), the mitochondrial sequence of which also shows this relationship (Fig. 5a).

In contrast, the ISSR-PCR data show a different pattern of relationships between *H. euphorbiae* and *H. robertsi*. In the NJ tree (Fig. 4b), the three samples of the former lineage form a distinct cluster towards the base of the tree, but they are unresolved within a large polytomy of *H. euphorbiae* samples based on mtDNA data (Fig. 5a). Genetic distances (Nei and Li 1979) among the *H. robertsi* samples are significantly lower than those between these samples and any other HEC population. The closest populations are *H. dahlii*, *H. euphorbiae* (both subspecies), *H. sammuti*, and *H. t. mauretanica,* to which the genetic distances all are in the same range. This indicates that the Iranian HEC may be more differentiated from the European HEC than the mtDNA data suggest. Phylogenetically informative nuclear fragments should be sequenced to clarify this relationship.

The hybrid status of Maltese *H. sammuti* suggested by the mitochondrial data (Figs. 3 and 4a) is reflected differently by the ISSR data (as we have only a single sample from Sicily, #239, we cannot make similar general observations on this population; however, it should be noted that the maternal lineage of this sample appears to have a *H. tithymali* origin). The mitochondrial data place one cluster of samples (#0052, #0053, #0055) outside the HEC s. str., and the rest (#0013, #0051, #0054, #0065) within *H. euphorbiae* (Figs. 2a and 4a). In contrast, the ISSR data (Fig. 4b) place samples #0053, #0054 and #0055 with *H. euphorbiae*, and #0013, #0051, #0052 and #0065 with *H. tithymali*. Maternal inheritance (in most organisms) and lack of recombina-

tion in organellar DNA make it useful for identifying the maternal parent of hybrids and introgressants (e.g. Ferris et al. 1997). We may have observed this effect in our mitochondrial data, where *H. e. euphorbiae* sample #008a (Spain) clusters with *H. tithymali* and the Maltese *H. sammuti* samples cluster in three different clades (Figs. 3, 4a and 5a). The former appears to be the result of recent secondary contact between *H. euphorbiae* and *H. tithymali*, in which the latter appeared within the range of the former for an unknown reason (Fig. 2). Whether the situation in *H. sammuti* is the result of primary or secondary hybridization is difficult to assess because insufficient samples have been analysed from these populations (particularly that in Sicily, where only a single sample was available). Primary hybridization would imply that the Mediterranean Islands represent an ancient natural hybrid zone between *H. euphorbiae* and *H. tithymali* and indicate that the observed polymorphisms reflect processes predating the Ice Ages. Secondary hybridization could imply that the Mediterranean Islands are a contact zone between the two lineages caused by the Ice Ages. Hybrids may have formed as northern *H. euphorbiae* populations (central Europe) extended south and North African *H. tithymali* populations extended north during climate oscillations (Fig. 2).

In plants, maternal inheritance of, and lack of recombination in, organellar DNA has resulted in some extreme situations. In some regions, chloroplasts of morphologically distinct species of alder (*Alnus*) appear to be distributed according to geographic locality, not systematics (King and Ferris 2000). However, due to the biparental mode of inheritance of nuclear DNA, these species-specific markers will tend to be combined additively in hybrids of recent origin. Accordingly, investigations into genomic relationships using ISSR-PCR of sympatric populations of plant species that appear to hybridize (e.g. King and Ferris 2000) have revealed discordance between phylogenies based on nuclear (ISSR) versus organelle (chloroplast) sequences. This also appears to be the case in the HEC. The ISSR data and mitochondrial sequences indicate very different relationships between the individuals. Whereas COX sequences revealed a clear geographic pattern of genetic differentiation (leaving aside the exceptional samples) – *H. euphorbiae* in central Europe and *H. tithymali* in Macaronesia, northern Africa and Saharo-Arabia – ISSR-PCR has provided insight into a very variable nuclear genome, leading to clusters that show neither geographic nor taxonomic structure.

Two opposing hypotheses may account for this situation. The first is that nuclear ISSR data reflect systematic relationships among samples more realistically than mitochondrial data. There are several reasons for this, one of which is that HEC mitochondria behave like the aforementioned alder chloroplasts (King and Ferris 2000), that is, they are distributed geographically by introgression, rather than according to phylogeny. A second explanation is lineage sorting, which would imply that the ancestral HEC population had several mitochondrial haplotypes. These would have segregated geographically by chance as the two species diverged, whereas the nuclear genome would reflect the "true" relationships. A third cause may be mutations in the coding regions of the organellar genome conferring a selective advantage to the organisms possessing them, which could give rise to a selective sweep and result in dominance of a single haplotype in any given geographical area, while maintaining variation in the nuclear genome. However, the selective force acting on a favourable mutation would have to be very strong and long-acting to produce the observed geographical pattern of mtDNA variation. A mutation may be advantageous during a glacial period and disadvantageous during an interglacial, but the rapid climate changes that have characterized recent Ice Age cycles may be too fast to allow dominance of one or a few haplotypes to become established in a given geographical area. Furthermore, as described above, the climatic conditions in many parts of the two lineages' distributions are not extremely different. This scenario was postulated for *Alnus* chloroplast genes, but rejected as unlikely by King and Ferris (2000), and we also consider it unlikely for the HEC.

The second hypothesis is that mitochondrial data reflect systematic relationships among samples more realistically than do nuclear ISSR data. Since ISSR-PCR variability is very high, and only relatively few characters could be coded with certainty, we favour this alternative. Nevertheless, the ISSR-PCR data may indicate a closer relationship between the *H. euphorbiae* and *H. tithymali* lineages than do the mtDNA data. The high individual genomic variability of the HEC, as reflected by the ISSR data, is surprising. Although polymorphic microsatellite loci had been assumed to be rather rare in Lepidoptera, because they were difficult to find (Meglécz and Solignac 1998), an increasing number of studies have found otherwise (Reddy et al. 1999; Harper et al. 2000; Kumar et al. 2001b; Luque et al. 2002). Our study shows that the tetrarepeats $(GACA)_n$ and $(TGTC)_n$ are present in large numbers in the genome of HEC moths. In almost every individual, they seem to be dispersed differently in the genome. A Nei and Li (1979) pairwise genetic distance of 1.0 (indicating no bands are shared) was found 116 times in our data set, whereas a distance of 0.0 (identical band patterns) was observed only twice. Whether this high variability reliably reflects such a low degree of phylogenetic relationship, and whether this level of variability is an HEC peculiarity or a general characteristic of Lepidoptera genomes, is discussed in a separate paper (Hundsdoerfer and Wink, in press).

The available computer packages implement many distance measures, based on different coefficients, but being a relatively new technique, ISSR data analysis is not yet standardized in this regard. In this study, we chose to use Nei and Li (1979) distances because this is a widely used measure that excludes shared absences as uninformative. This enabled us to compare our results with those of other workers, although some difficulties remained. In their study of the population genetic structure of *Viola pubescens* Aiton (Violaceae), Culley and Wolfe (2001) also found a surprisingly high ISSR variability (reflected by 100% polymorphic bands, as in our study) compared to other species of *Viola*. Although Culley and Wolfe (2001) used software different from ours, (!WAVSIML, as opposed to RAPDistance), the mathematical algorithm is the same, and should yield comparable results. The maximum Nei and Li genetic distance observed between *V. pubescens* populations, 0.672, is slightly higher than the 0.576 we found between subspecies (or populations, as defined in Table 5) of either *H. euphorbiae* or *H. tithymali*. Using yet another program (MVSP-pc) to calculate Nei and Li distances among natural populations of chickpea species, the values recently reported by Sudupak (2004) do not appear comparable. However, Sudupak (2004) applied a logarithmic transformation whereby the genetic distance between two samples a and b, GD_{ab}, is given as $-\ln(S_{ab})$, where S_{ab} is the Nei and Li (1979) "similarity", or "distance coefficient", as determined using MVSP-pc. Our distance estimates are equivalent to Sudupak's (2004) "distance coefficients" prior to transformation; thus, a direct comparison is not possible. However, a qualitative comparison can be made with Sudupak's results. Overall, he reported generally low intraspecific, population-level variability with two intraspecific distance averages of zero. In our data, intrapopulation averages were rather high, and there were only two single pairwise comparisons of zero distance (indicating identical band patterns), but no distance averages of zero. Thus, compared to Sudapak's study, we observed a relatively high level of variation.

This is the first report of partitioning of genetic variability within and among populations of the HEC. The AMOVA calculations indicate that both sources of molecular data corroborate a genetic structure that divides the HEC into two species, *H. euphorbiae* (probably including *H. robertsi*) and *H. tithymali*. Whether *H. euphorbiae* and *H. robertsi* should be united into a single species, undifferentiated into subspecies, cannot be answered definitively by this study. Although the mitochondrial data suggest this would be appropriate, the nuclear ISSR data reveals more variability. We do not fully understand the genetics of ISSR markers yet, thus other sources of nuclear data should be sought to elucidate the relationship between *H. euphorbiae* and *H. robertsi* before any taxonomic changes are undertaken. The same argument applies for *H. sammuti*. More samples should be examined using nuclear markers (preferably sequences or microsatellites) to explore its hypothesized hybrid status. Overall, ISSR markers show greater promise for elucidating relationships among individuals within populations and between closely related populations than between species, even if these are incipient.

Conclusions

The COX sequences revealed a clear geographical pattern of genetic differentiation in the HEC into two main lineages, *H. euphorbiae* and *H. tithymali*. The sequence data also indicated intraspecific subdivisions and gene flow patterns, and possibly detected both introgression and a major contact zone on the Mediterranean Islands between these two evolutionary lineages. *Hyles tithymali* is hypothesized to have been able to retain ancient polymorphisms until the present, whereas *H. euphorbiae* (re)colonized its current distribution range after the Ice Ages from a few refugial populations (or even only one) by leptokurtic dispersal, resulting in low diversity. The ISSR-PCR data provided further insights into the genomic distribution of the simple sequence repeat $(GACA)_4$ among individuals of the HEC and appear to describe higher variability and a more complex pattern of introgression. However, further sources of data are required to test the phylogenetic hypothesis and hybridization scenario presented here. In addition to morphological data, other molecular data from the nuclear genome should be investigated.

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