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No genetic differentiation in the rose-infesting fruit flies *Rhagoletis alternata* and *Carpomya schineri* (Diptera: Tephritidae) across central Europe

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Abstract. After the last glacial retreat in Europe, multiple recolonizations led to intraspecific differentiation in many of the recolonizing taxa. Here we investigate the genetic diversification across central Europe in two recolonizing taxa, the tephritid fruit flies *Rhagoletis alternata* (Fallén, 1814) and *Carpomya schineri* (Loew, 1856), which attack rose hips. Analysis of amplified and sequenced fragments of the mitochondrial genes encoding cytochrome oxidase I (800 bp), cytochrome oxidase II (470 bp) and cytochrome *b* (450 bp), indicate that all the individuals of *R. alternata* (n = 21) collected from across Europe share the same haplotype. Two individuals of *C. schineri* form Berlin, which is further north of the range than previously reported in the literature, differ from the other individuals (n = 13) in one nucleotide position on the cytochrome oxidase II gene fragment. This level of genetic variation in sequences with a summed length of 1720 bp is unexpectedly lower than in other insect taxa (n = 63). This might have been caused by a selective sweep by a cytoplasmic symbiont such as *Wolbachia,* or a recent range expansion associated with a host shift or a single recolonization event.

INTRODUCTION

Many European species survived the last ice age in one or several refugia. Owing to the isolation of these regions during this period, the taxa in the refugia differentiated (Hewitt, 1996). With the warming of the climate, species ranges expanded again and populations from different refugia came into secondary contact (Hewitt, 1996), which resulted in many taxa in Europe having complex genetic suture zones (Taberlet et al., 1998).

Here, we analyse the genetic diversification in two tephritid fruit flies that attack rose hips: *Rhagoletis alternata* (Fallén) and *Carpomya schineri* (Loew). The former, *R. alternata* is distributed throughout the Palaearctic region (Kandybina, 1977; Smith & Bush, 2000). In contrast, *C. schineri* has a more restricted distribution in southern Europe, southern Asia, and northern Africa (White & Elson-Harris, 1992). In southern Europe, these two species occur sympatrically. The larvae of both fruit flies develop in rose hips, where they feed not on the seeds but on the hypanthium. Adult females of *R. alternata* lay their eggs under the surface of hips, which they mark with a pheromone that deters other females. Around October, the larvae leave the hips and pupate in the soil (Bauer, 1986, 1998). The larvae of *C. schineri* leave the hips around August and the adult flies emerge around June the following year (Hendel, 1927).

Both flies are host specific at the generic level and their distribution depends on that of their host plants, mainly the dog roses, *Rosa* section *Caninae*. Members of this rose section are the most common rose species in Europe and western Asia. They are evolutionarily young, originated by hybridization (Wissemann, 2002; Ritz et al., 2005) and expanded their range into central and northern Europe after the last ice age (Dingler, 1907). The distribution of the roses provided a platform for the flies to recolonize Europe from certain refugia. If the flies came from Mediterranean refugia there should be genetic suture zones in central Europe. We therefore screened the genetic variation in samples of flies collected from populations across this area.

MATERIAL AND METHODS

Collection of larvae

Infested rose hips were collected in September 2004 from the canton Valais in Switzerland and in 2006 across Europe (Fig. 1; Supplementary material: Table S1), sent to our laboratory in perforated plastic bags and then stored at 15°C. The larvae were allowed to leave the hips and pupate within the bags. Pupae were stored in 95%-alcohol at 5°C for DNA analysis. Pupae parasitized by *Utetes magnus* (Fischer, 1958) are brown (T. Hoffmeister, pers. commun.), which allowed us to select non-parasitized individuals for DNA extraction. As a control, some adult *R. alternata* were allowed to emerge from each sample.

DNA extraction and amplification of mtDNA fragments

DNA was extracted from pupae using spin columns (DNeasy tissue kit, Qiagen, Hilden, Germany). Three mitochondrial DNA (mtDNA) fragments were chosen for amplification: 800 bp of cytochrome oxidase I (COI) amplified with primers C1-J-2183 (forward, 5′-CAA CAT TTA TTT TGA TTT TTT GG-3′) and TL-N-3014 (reverse, 5-′TCC ATT GCA CTA ATC TGC CAT ATT A-3′) (Simon et al., 1994), 470 bp of cytochrome oxidase II (COII) amplified with primers C2-J-3291 (forward, 5′-GAA ATA ATT TGA ACA ATT CTA CCA GC-3′) and TK-N-3772 (reverse, 5′-GAG ACC ATT ACT TGC TTT CAG TCA TCT-3′) (Smith & Bush, 1997), and 450 bp of the 3′end of cytochrome *b* (Cyt *b*) amplified with primers CB-J-10933 (forward, 5′-TAT GTA CTA CCA TGA GGA CAA ATA TC-3′) and CB-N-11367 (reverse, 5′-ATT ACA CCT CCT AAT TTA TTA GGA AT-3′) (Simon et al., 1994); all positions as in the mitochondrial genome of *Drosophila yakuba* (Clary & Wolstenholme, 1985). A thermocycler (Eppendorf Mastercycler, Hamburg, Germany) was used for the amplifications following the protocol: initial denaturation cycle at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1

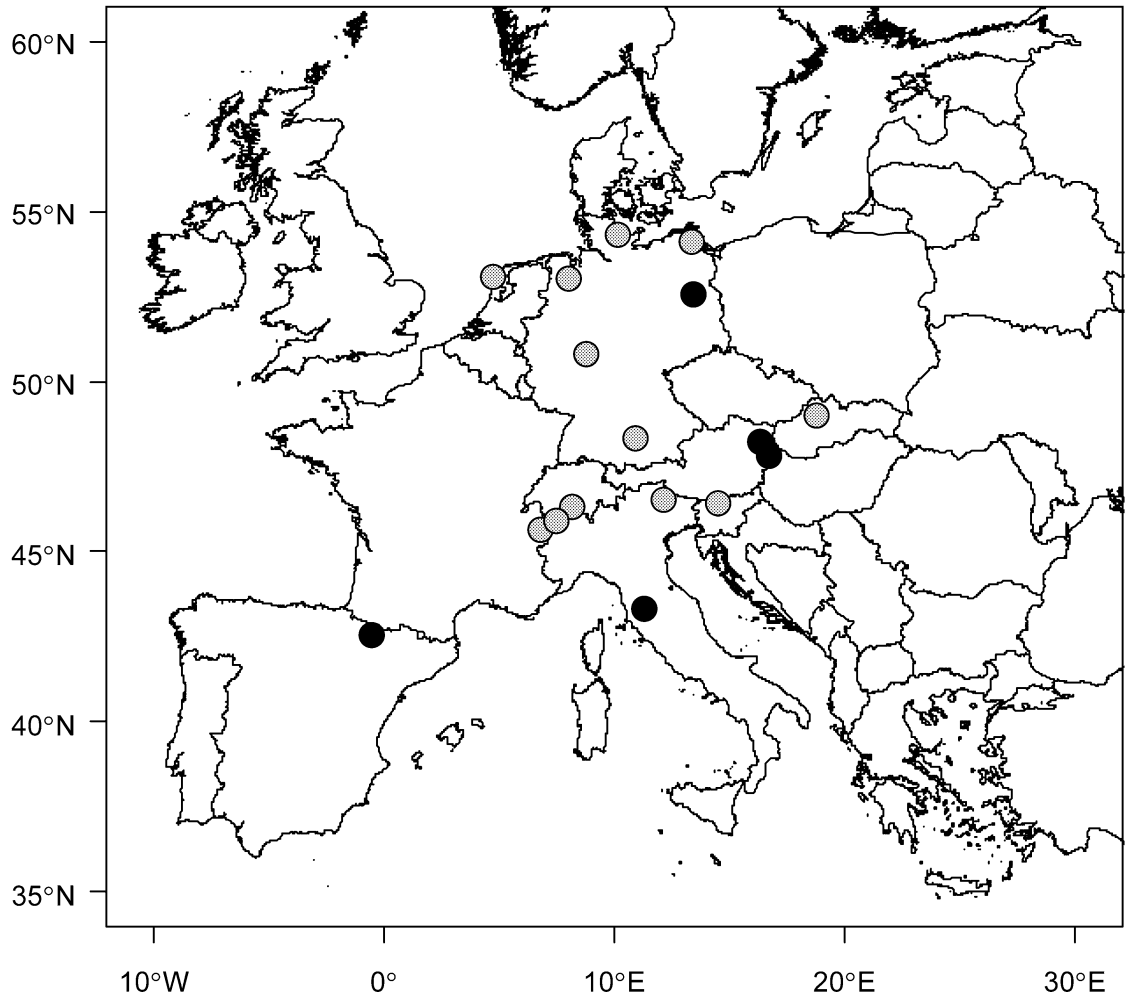

Fig. 1. Sites at which *Rhagoletis alternata* (grey circles) and *Carpomya schineri* (black circles) were sampled in Europe.

min at 60°C for the COI primers, 58°C for COII primers, and 45°C for the Cyt *b* primers and then extension at 72°C for 1 min. The final extension step was for 10 min at 72°C.

The amplification reactions (20 µl) contained 20–100 ng of template DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 80 µM dNTP, 10 µM of each of the two primers and 1 unit of *Taq* DNA polymerase (Sigma, Taufkirchen, Germany). The products were purified using a Qiagen MinElute PCR purification kit. The purified PCR products were sequenced directly in both directions by Sequencing Laboratories Göttingen GmbH, Germany.

Sequence analysis

We compared the variability in mtDNA with that recorded for other insect populations. We searched for population genetic studies of insect taxa in the ISI Web of Knowledge. The aim of our ISI web search was not to construct a complete data set, but to retrieve sufficient information for statistical analysis. We used the keywords "Insect", "Diptera", "Lepidoptera", "Coleoptera" or "Hymenoptera", in combination with "genetic structure" and/or "genetic differentiation". We selected these keywords after some testing. These preliminary searches showed that the selected keyword combinations retrieved sufficient but a manageable number of papers. Furthermore, to obtain the most recent publications only the first 100 were selected. Finally, we excluded studies on parthenogenetic and social insects as they are known to show low genetic variability. From the published reports on these insect taxa, we extracted the number of haplotypes found and the number and length of the sequences (Supplementary material: Table S2). All variables were $\log_{10}$-transformed before analysis using a general linear model in STATISTICA (Version 6.1). Both fly species were included in the analyses. The number of expected haplotypes was calculated for both fly species using the coefficients of the model (Table 1).

TABLE 1. Estimated coefficients and analyses of variance of the general linear model.

	Estimates	df	SS	MSS	F	*P*
Intercept	–0.765	1	0.206	0.206	1.526	0.22
log (length of sequence)	0.232	1	0.182	0.182	1.347	0.25
log (number of sequence)	0.744	1	5.72	5.72	42.25	< 0.001
Residuals		60	8.12	0.135		

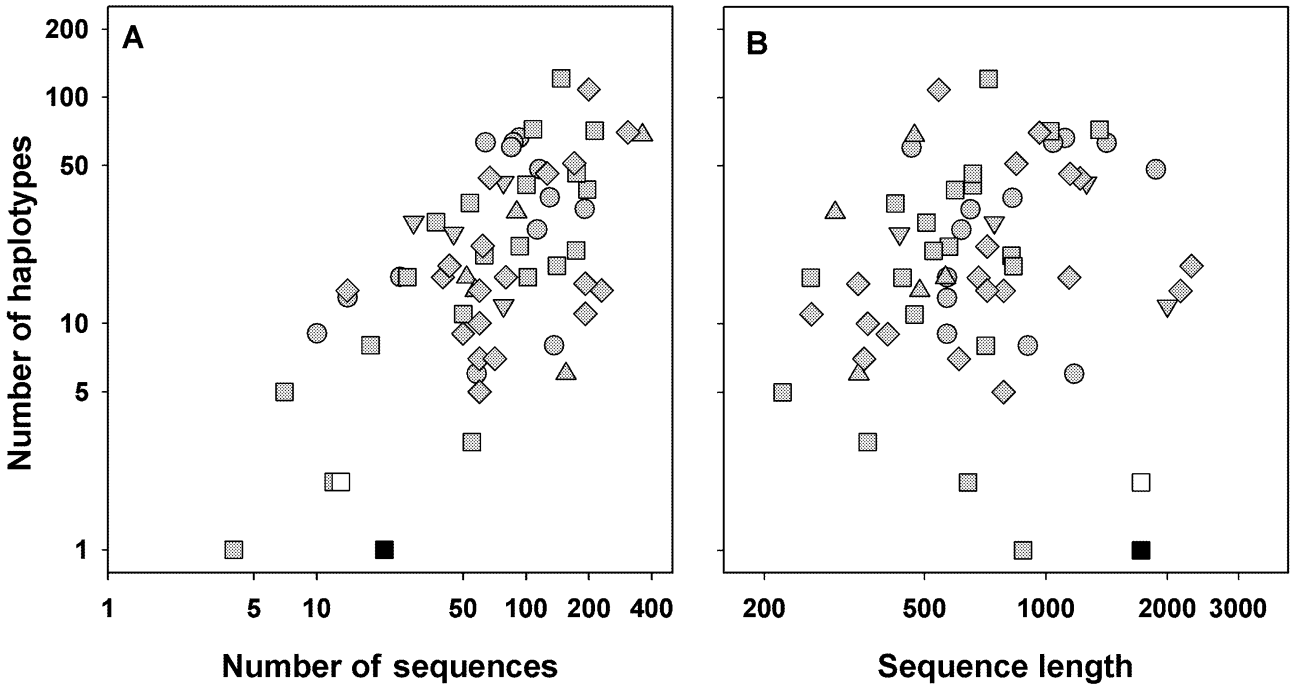

Fig. 2. Number of haplotypes in relation to (A) the number of sequences analysed (all variables $\log_{10}$ transformed) and (B) sequence length for 63 insect taxa (Supplementary material obtained from the literature cited in Table S2). Twenty-one sequences from *Rhagoletis alternata* (black square) and 13 from *Carpomya schineri* (white square) are included in this analysis. Grey squares, Diptera; grey circles, Coleoptera; grey triangles, Hymenoptera; grey diamonds, Lepidoptera; grey inverted triangles, other insect orders.

RESULTS AND DISCUSSION

We sequenced the chosen mtDNA fragments of 21 individuals of *R. alternata* from 12 localities across central and southern Europe (Fig. 1). None of the 1720 nucleotide positions from COI, COII, and Cyt *b* (accession numbers FJ571363, FJ571366, and FJ571369, respectively) were variable, i.e. there is only one haplotype throughout the area sampled. The nucleotide sequences of the COII gene fragment were identical to the COII sequence of *R. alternata* available in GenBank (acc. no. U53260). We also found no differences in the sequences of the amplified COI (acc. nos. FJ571364 and FJ571365) and Cyt *b* gene fragments (acc. nos. FJ571370 and FJ571371) from 13 individuals of *C. schineri* from 4 localities, and the nucleotide sequences of the COI gene fragment were identical to the published sequence for this species (acc. no. U53267). In contrast to the situation in *R. alternata*, the COII gene fragment sequences from 2 individuals of *C. schineri* (acc. no. FJ571367) differed at one nucleotide position from those in the other 11 individuals (acc. no. FJ571368), which shared one haplotype. The 2 individuals with the second haplotype were collected in Berlin, far north of the range of *C. schineri* reported in the literature (Kandybina, 1977; White & Elson-Harris, 1992; Smith & Bush, 2000).

The mtDNA genes chosen for our analyses, those encoding cytochrome oxidase I and II subunits and cytochrome *b,* are protein-encoding genes with considerable variability even between closely related species and populations of the same species (Rokas et al., 2002; Simon et al., 1994). Furthermore, the chosen fragments of these genes include the most variable positions of the genes and are therefore often used for population genetic studies of animals, especially insects (e.g. Lunt et al., 1998; Rokas et al., 2003; Arias et al., 2005; Pramual et al., 2005; Sezonlin et al., 2006). These criteria indicate that the selected mtDNA gene fragments should be suitable for elucidating the biogeographic history of these fly species.

We compared our findings with mtDNA sequence data for other insect populations (Fig. 2). For 21 sequences with a summed length of 1720 bp (Fig. 2) 9–10 expected haplotypes were calculated and 6–7 haplotypes for 13 sequences of the same summed length. The standardized residual of the model for *R. alternata* was –2.93 (P = 0.0016) and for *C. schineri* –1.62 (P = 0.053).

Compared to the results for other insect populations, the genetic variation in *R. alternata* and *C. schineri* was significantly lower than expected. This could have at least two explanations. One possibility is that symbionts, such as *Wolbachia,* shape mtDNA evolution (Hurst & Jiggins, 2005), which would constrain the suitability of mtDNA sequences for molecular biogeographic studies of insects. During the initial phase of symbiont invasion, selective sweeps may reduce mtDNA diversity, thereby producing a genetic signal similar to that produced by a population bottleneck with subsequent expansion (Hurst & Jiggins, 2005). *Wolbachia* is known to infect members of the genus *Rhagoletis* (Riegler & Stauffer, 2002) but not members of the genus *Carpomya* (Kittayapong et al., 2000). The second possibility is that the flies recently and rapidly expanded their range from one source population. Such an expansion could be induced by colonization or a host-shift; in both cases, lower levels of genetic variation would be expected due to founder effects (Harrison, 1991). Both of the fly species studied are specialists and therefore dependent on the distribution of their host, members of the genus *Rosa* section *Caninae.* These dog roses originated by hybridization during the last ice age (Wissemann, 2002; Ritz et al., 2005) prior to the recolonization of Europe (Dingler, 1907). Founder individuals of the two fly species may have shifted to this new host, which would provide an explanation for the low genetic variability.

Today, the distribution and density of roses is influenced by humans. *Rosa rugosa,* for example, was introduced from East Asia about 100 years ago (Hegi, 1975) and is now cultivated in parks, gardens and along roads all over Central Europe. Also its rose hips are attacked by *R. alternata*, and this increase in available hosts may have triggered the spread of these flies across Europe. *R. alternata* disperses well, and even the Alps do not seem to be an effective geographical barrier (Vaupel et al., 2007). This increase in distribution can also be partly explained by the behaviour of the females. After oviposition, the females mark the rose hips with a pheromone (Bauer, 1986, 1998). Often, a high proportion of the hips, up to 100%, are infested and marked. Females leave such locations and search for rose shrubs with a lower proportion of infested hips. These observations and our results indicate that a recent range expansion of the flies from an unknown source area may account for their low genetic variability. Nuclear markers of *R. alternata*, e.g. allozyme genes, also show little variability, which suggests a high level of gene flow between European populations (Leclaire & Brandl, 1994; Vaupel et al., 2007). Note also that our finding *C. schineri* near Berlin extends the known range of this species northwards. The range expansion of this fly species could also be influenced by humans, who may have transported the larvae in plant material.

In conclusion, we found a surprisingly low level of genetic variability in tephritid fruit fly populations across central Europe. The reasons remain uncertain, but a recent and single colonization from an unknown source or the host shift to *Rosa* section *Caninae* are plausible explanations. The reporting of such findings is likely to facilitate a pluralistic understanding of the biogeography of plants and animals living in Europe.

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TABLE S1. Localities at which *Rhagoletis alternata* and *Carpomya schineri* were sampled in Europe.

Species	No. of specimens	Country	Locality	Lat.	Long.	Collector
R. alternata	2	Austria	Seesattel	46.4261	14.5305	H. Rieger-Hager
	1	Austria	Corte	46.5333	12.1333	M. Brändle
	2	France	St. Bernhard	45.6278	6.8304	M. Brändle
	2	Germany	Oldenburg	53.0000	8.0000	Botanical Garden
	2	Germany	Marburg	50.8167	8.7667	A. Kohnen
	2	Germany	Augsburg	48.3500	10.9000	Botanical Garden
	1	Germany	Greifswald	54.0833	13.3833	S. Starke
	2	Germany	Kiel	54.3333	10.1333	A. Kohnen
	2	Italy	Aosta	45.8992	7.4839	M. Brändle
	1	Netherlands	De Koog	53.0833	4.7500	W. Koopman
	1	Slovakia	Martin	49.0189	18.7891	M. Brändle
	3	Switzerland	Ulrichen	46.3044	8.1778	A. Vaupel
C. schineri	4	Austria	Vienna	48.2167	16.3667	F. Tod
	1	Austria	Sandeck	47.8333	16.7500	R. Brandl
	2	Germany	Berlin	52.5333	13.4167	M. Raddatz
	3	Italy	Siena	43.3167	11.3167	P. Castagnini
	3	Spain	Jaca	42.5667	–0.5500	H. Zimmermann

TABLE S2. Studies on the number of haplotypes in insects used in the comparison with those found in this study on *Rhagoletis alternata* and *Carpomya schineri*.

Species	Locus	Length (bp)	No. of specimens sequenced	No. of haplotypes	Reference
Aedes aegypti	NADH 4	361	55	3	da Costa-da-Silva et al. (2005)
Aedes vexans	NADH 5	423	54	34	Szalanski et al. (2006)
Agathis sp. n.	CO I – CO II	1260	78	42	Althoff et al. (2001)
Aglais urticae	CO I	1216	67	44	Vandewoestijne et al. (2004)
Aglais urticae	D-loop	715	62	22	Vandewoestijne et al. (2004)
Andricus quercustozae	Cytb	433	45	25	Rokas et al. (2003)
Anopheles albimanus	Cytb	222	7	5	Airas et al. (2005)
Anopheles arabiensis	NADH 5	595	196	39	Themu et al. (2005)
Anopheles flavirostris	CO I	261	102	16	Foley et al. (2006)
Anopheles stephensi	CO I	877	4	1	Oshaghi et al. (2006)
Anopheles stephensi	CO II	640	12	2	Oshaghi et al. (2006)
Anopheles superpictus	CO I	708	18	8	Oshaghi et al. (2007)
Aphidus ervi	CO I – CO II	2000	78	12	Hufbauer et al. (2004)
Bactrocera depressa	CO I	821	63	20	Mun et al. (2003)
Bactrocera dorsalis	CO I	505	37	28	Shi et al. (2005)
Bactrocera oleae	NADH 1	574	93	22	Nardi et al. (2005)
Baetis rhodani	CO I	472	360	68	Williams et al. (2006)
Busseola fusca	Cytb	965	307	70	Sezonlin et al. (2006)
Carpomya schineri	COI, COII, Cyt b	1720	10	1	Present study
Chorthippus parallelus	CO I	300	90	31	Lunt et al. (1998)
Culicoides imicola	CO I	472	50	11	Dallas et al. (2003)
Dendroctonus rufipennis	CO I	1114	93	66	Maroja et al. (2007)
Diglyphus isaea	CO I	745	29	28	Sha et al. (2006)
Drosophila arizonae	CO I	658	100	41	Reed et al. (2007)
Drosophila lacertosa	NADH 2	1026	213	71	He et al. (2007)
Drosophila mojavensis	CO I	658	174	46	Reed et al. (2007)
Drosophila montanta	CO I + CO II	1358	108	72	Mirol et al. (2007)
Epirrita autumnata	D-loop	542	200	108	Snall et al. (2004)
Erebia palarica	CO I	786	60	5	Vila et al. (2004)
Erebia palarica	D-loop	354	60	7	Vila et al. (2004)
Erebia palarica	D-Loop + CO I	1142	40	16	Vila et al. (2004)
Erebia triaria	CO I	786	60	14	Vila et al.(2005)
Erebia triaria	CO I – CO II	1147	126	46	Vila et al. (2004)
Erebia triaria	D-loop	361	60	10	Vila et al. (2004)
Gonioctena olivacea	CO I	1176	58	6	Mardulyn et al. (2005)
Gonioctena olivacea	D-loop 1	1875	116	48	Mardulyn et al. (2005)
Gonioctena olivacea	D-loop 2	649	191	32	Mardulyn et al. (2005)
Homalodisca coagulata	CO I	486	57	14	Smith (2005)
Hyalopterus	CO I	343	155	6	Lozier et al. (2007)
Hyles tithymali	CO I – CO II	2295	43	18	Hundsdoerfer et al. (2006)
Ips typographus	CO I	902	136	8	Stauffer et al. (1999)
Moneilema appressum	CO I	1413	64	63	Smith et al. (2005)
Ochlerotatus caspius	CO II	526	173	21	Porretta et al. (2007)
Orchesella cincta	CO II	563	52	16	Timmermans et al. (2005)
Ostrinia nubilalis	CO I – CO II	2156	14	14	Coates et al. (2004)
Phlebotomus papatasi	Cytb-NADH1	441	27	16	Esseghir et al. (1997)
Pissodes strobi	CO I	826	130	36	Laffin et al. (2004)
Plutella xylostella	CO I	681	80	16	Li et al. (2006)
Polyura eudamippus	CO II	405	50	9	Long et al. (2006)
Rhagoletis alternata	COI, COII, Cyt b	1720	10	1	Present study
Simulium tani	CO I	720	147	121	Pramual et al. (2005)
Spodoptera frugiperda	CO I – CO II	608	71	7	Lewter et al. (2006)
Synemon plana	CO II	715	230	14	Clarke et al. (2003)
Tarphius canariensis	CO I + CO II	1041	87	63	Emerson et al. (2000)
Tegeticula yuccasella	CO I	844	170	51	Leebens-Mack et al. (2004)
Tephritis bardanae	CO I – CO II	830	140	18	Diegisser et al. (2004)
Thanasimus dubius	CO I	464	85	60	Schrey et al. (2005)
Thaumetopoea wilkinsoni	CO I	262	192	11	Simonato et al. (2007)
Thaumetopoea wilkinsoni	CO II	342	192	15	Simonato et al. (2007)
Tomicus destruens	CO I – CO II	617	113	26	Vasconcelos et al. (2006)
Trypocopris alpinus	CO I	568	10	9	Carisio et al. (2004)
Trypocopris pyrenaeus	CO I	568	25	16	Carisio et al. (2004)
Trypocopris vernalis	CO I	568	14	13	Carisio et al. (2004)