

Phylogenetic evaluation of the taxonomic status of *Timandra griseata* and *T. comae* (Lepidoptera: Geometridae: Sterrhinae)

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Abstract. The sterrhine loopers *Timandra griseata* and *T. comae* have been treated as distinct species since 1994. However, morphological differences between the taxa are minor and therefore their status has often been disputed. Here, we present a molecular phylogenetic study, which separates *T. griseata* and *T. comae* into different clades. Altogether, 43 *Timandra* specimens from eight European countries were studied. The phylogeny is based on a comparative sequence analysis of mitochondrial genes coding for the cytochrome C oxidase subunit I (COI) and NADH dehydrogenase subunit 1 (ND1). Nevertheless, a single individual of both species was assigned to the “wrong” clade. The symplesiomorphy of *T. griseata* and *T. comae* is considered to be a result of introgressive hybridization. Conditions that could lead to the hybridization of *T. griseata* and *T. comae* are discussed, as well as the likely distribution history of these taxa in Northern Europe. Results of the current analysis are in favour of retaining the species status of *T. griseata* and *T. comae*.

INTRODUCTION

The sterrhine genus *Timandra* Duponchel, 1829 is distributed mainly in the Eastern Palaearctic and Oriental region (Kaila & Albrecht, 1994). Only three species occur in the Western Palaearctic (Müller, 1996) and one in the Nearctic (Prout, 1935–38). The total number of species in this genus is 21 (Scoble, 1999). Most species in this genus have similar wing pattern, but differences in genitalia enable the species to be distinguished quite easily (Inoue et al., 1982).

Kaila & Albrecht (1994) defined the *Timandra griseata* group as including species that have a short and blunt sacculus and sclerotized costa of the valva branching at three quarters from the base, forming two branches of about equal length. Three species were considered to belong to this group: sibling species *T. griseata* (Petersen, 1902) and *T. comae* (Schmidt, 1931) in the Western Palaearctic and *T. recompta* (Prout, 1930) in the Eastern Palaearctic. Of these species, *T. recompta* clearly differs from the others, especially in the characters of the female genitalia. *T. griseata* and *T. comae* were treated as separate species for the first time by Kaila & Albrecht, 1994, who also used the name *comai*. The name *comae* by Schmidt (1931), which originally was given in honour of Mr. Pedro Coma, was found to be an incorrect original spelling (Kaila & Albrecht, 1994). Their emendation from *comae* to *comai* was based on the third edition of the Code ICZN (1985), article 31(a)(ii), which states that if a noun in the genitive case is formed directly from a modern personal name of a man, *-i* has to be added to the stem of that name; see details also in Kullberg et al. (2002). The name *comai* was subsequently used in sev-

eral publications (eg. Kaila & Albrecht, 1995; Palmqvist, 1997; Kaila et al., 1999; Sihvonen, 2001; Kullberg et al., 2002). On the other hand, an alternative interpretation is possible, as article 31(a)(i) in Code ICZN (1985) and article 31.1.1. in Code ICZN (1999) state that a noun in the genitive case formed from a personal name that is Latin or has been latinized, is to be formed in accordance with the rules of Latin grammar. Following this paragraph results in *comae* as used in three major recent moth catalogues by Müller (1996), Scoble (1999) and Hausmann (2004). Based on this interpretation and the principle of priority, we consider *comae* to be the valid name and *comai* to be an unnecessary emendation.

Morphological differences between *T. griseata* and *T. comae* are often regarded as insufficient to treat them as separate species. Therefore, their specific status has been questioned by lepidopterists and criticized in literature (Hausmann, 1997, 2004). The external morphology of *T. griseata* and *T. comae* differs as follows: ground colour of the forewing is whitish in *T. griseata*, yellowish in *T. comae*, dusting on wings is dense and grey in *T. griseata*, sparse and brownish-grey in *T. comae*, average wingspan is bigger and sexual size dimorphism more accentuated in *T. griseata* (Kaila & Albrecht, 1994). There are small differences in the position of the junction between the anterior and posterior parts of corpus bursae and in the angle of appendix bursae of the females. The external genitalia of male *T. griseata* and *T. comae*, however, are indistinguishable (Kaila & Albrecht, 1994). On the other hand, a detailed study of everted vesicae by Sihvonen (2001) supports the original hypothesis of Kaila & Albrecht (1994).

In addition to the differences in morphology, the distribution of *T. griseata* and *T. comae* is also different (Kaila & Albrecht, 1994). These species are allopatric over most of their geographic range, with a narrow contact zone in Fennoscandia and the northern part of the Baltic countries. *T. comae* is widely distributed in the Western Palaearctic – its range covers all of western, southern and eastern Europe, reaching southern Finland, central Sweden and southern Norway in the north and Turkmenistan in the east (Müller, 1996; Viidalepp, 1996; Aarvik et al., 2000; Huldén et al., 2000; Hausmann, 2004). *T. griseata* is restricted to northern Europe. This species is widely distributed in Finland and Sweden (Huldén et al., 2000; Hausmann, 2004) and has a wider range in southern Norway than *T. comae* (Aarvik et al., 2000). *T. griseata* has also been recorded in northwestern Russia (Kaila & Albrecht, 1994), but is rare in Estonia and Latvia (Kaila et al., 1999; Savenkov & Šulcs, 2004).

The only report of *T. griseata* from Denmark (Larsen, 1995), as well as notes that this species occurs in Belorussia, Ukraine, Crimea, the Ural Mountains, Caucasus and Trans Caucasus (Viidalepp, 1996) have been interpreted as erroneous (Hausmann, 2004). Moreover, *T. griseata* and *T. comae* differ in their phenology. In Finland, where *T. griseata* and *T. comae* are sympatric, the first imagoes of the bivoltine *T. comae* appear about two weeks earlier than the earliest specimens of the usually univoltine *T. griseata*. The first generation of *T. comae* is less abundant than the second, therefore, this species is most numerous in August, while the number of *T. griseata* peaks in late June (Kaila & Albrecht, 1994).

Recent short reports on the differences in mitochondrial NADH dehydrogenase subunit 1 (*ND1*) (Miller et al., 2001) and cytochrome C oxidase subunit I (*COI*) (Trusch et al., 2002) sequences of *T. griseata* and *T. comae* also indicate that these taxa might represent separate species. However, since Miller et al. (2001) used only one specimen of *T. griseata* and two of *T. comae* and Trusch et al. (2002) have not published the details of their study, an additional and more extensive molecular survey is needed to resolve the taxonomic status of *T. griseata* and *T. comae*; the need for further investigation is emphasized also by Hausmann (2004).

In this study, we analysed specimens of *T. griseata* and *T. comae* from Estonia and Finland, where these taxa are sympatric (Kaila & Albrecht, 1994; Müller, 1996; Kaila et al., 1999), and a few additional individuals from other European countries (Latvia, Sweden, Germany, Belorussia, Ukraine and Bulgaria). Partial sequences from mitochondrial *COI* and *ND1* genes were used for phylogenetic inference, since mitochondrial genes are reported to be more useful than nuclear genes in most cases for resolving the boundaries of recently diverged species (Caterino et al., 2000; Wiens & Penkrot, 2002).

MATERIAL AND METHODS

Moths

Forty-three specimens of the European *T. griseata* group from 27 localities were studied (Table 1). Most of the material (36 individuals) was collected from 20 localities in Estonia and Fin-

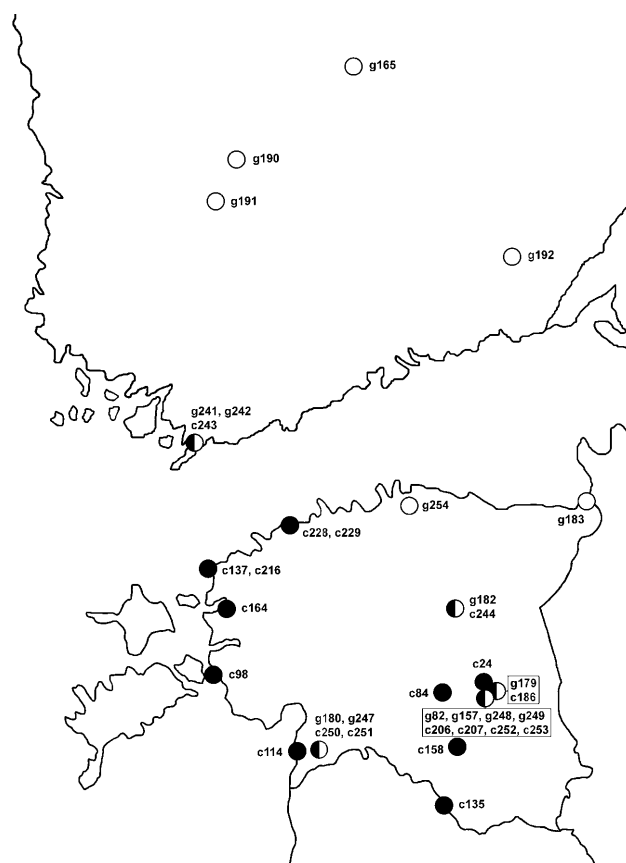


Fig. 1. Sampling localities of *T. griseata* (open circles), *T. comae* (filled circles), and both species (half-filled circles) in Estonia and Finland. Specimens, their numbers and localities are presented in accordance to the Table 1.

land (Fig. 1). Both older pinned moths from the collection of the Institute of Agricultural and Environmental Sciences (individuals collected 1990–2000, Table 1) and fresh specimens were studied. All specimens were identified following the criteria given by Kaila & Albrecht (1994) and Kaila et al. (1999). L. Kaila kindly re-examined the critical specimens. Abdomens of the moths were removed, cut into two parts and stored at -20°C . Two to three anterior segments of the abdomen were used for DNA extraction; posterior parts with genitalia were retained at -20°C as vouchers and as a backup of the genetic material. Thoraces with head, wings and legs were pinned and kept in the IAE collection as vouchers, except for *T. griseata* 241, *T. griseata* 242 and *T. comae* 243, which are in the collection of the Finnish Museum of Natural History.

DNA purification, amplification and sequencing of gene fragments

Purification of the total genomic DNA was performed by using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions for isolating nucleic acids from mammalian tissue, with the exception that the first incubation step was 55°C for 12 h rather than 1 h.

For the amplification of the mtDNA gene *COI*, primers RON (5' GGA TCA CCT GAT ATA GCA TTC CC 3', see Caterino & Sperling, 1999) and a novel primer NAN (5' CCC GGT AAA ATT AAA ATA TAA ACT TC 3') were used. Novel primers NF (5' CGA TAT CGA GGT AAT GTT CCA CGA 3') and NR (5' CTG GTT GGT CAT CTA ATT CTA ATT ATG C 3') were

TABLE 1. Specimens, collecting localities and dates, names of collectors and identifiers; and the NCBI accession codes for the mtDNA sequences. *T. gri* – *Timandra griseata*; *T. com* – *Timandra comae*. (Est – Estonia, Swe – Sweden, Fin – Finland, Lat – Latvia, Bul – Bulgaria, Ukr – Ukraine, Ger – Germany, Blr – Belorussia).

Specimens	Country	Locality	Date	Sex	Collector	Identifier	NCBI acc. code for COI	NCBI acc. code for NDI
<i>T. com</i> 24	Est	Tartu	03.06.01	M	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. gri</i> 82	Est	Tatra Valley	10.07.01	F	E. Õunap	E. Õunap	AY870298	AY870310
<i>T. com</i> 84	Est	Vaibla	09.08.01	M	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. com</i> 98	Est	Puhtu	10.08.01	F	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. com</i> 114	Est	Kabli	03.08.01	M	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. com</i> 135	Est	Mustjõe suue	14.07.01	M	I. Taal	E. Õunap	AY870293	AY870305
<i>T. com</i> 137	Est	Põõsaspea	28.07.01	M	I. Taal	E. Õunap, L. Kaila	AY870300	AY870311
<i>T. com</i> 144	Swe	Öland, Runsbäck	26.07.01	F	M. Lindeborg	E. Õunap	AY870293	AY870305
<i>T. com</i> 150	Swe	Smaland, Kalmar	26.07.01	M	M. Lindeborg	E. Õunap	AY870293	AY870307
<i>T. gri</i> 157	Est	Tatra Valley	10.07.01	F	T. Tammaru	E. Õunap	AY870299	AY870310
<i>T. com</i> 158	Est	Puka	18.08.01	M	J. Viidalepp	E. Õunap	AY870293	AY870305
<i>T. com</i> 164	Est	Kapa	07.09.01	M	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. gri</i> 165	Fin	Tikkakoski	July 01	M	O. Lahtinen	E. Õunap	AY870299	AY870310
<i>T. gri</i> 179	Est	Kurepalu	07.07.94	M	R. Pedmanson	L. Kaila	AY870298	AY870310
<i>T. gri</i> 180	Est	Nigula Nature Reserve	08.06.90	M	R. Ülemaante	L. Kaila	AY870294	AY870306
<i>T. gri</i> 182	Est	Endla Nature Reserve	18.–25.06.95	M	K. Kimmel	L. Kaila	AY870303	AY870312
<i>T. gri</i> 183	Est	Narva-Jõesuu	06.07.96	M	T. Tammaru	L. Kaila	AY870304	AY870312
<i>T. com</i> 186	Est	Kurepalu	11.08.93	M	R. Pedmanson	E. Õunap	AY870293	AY870305
<i>T. com</i> 188	Lat	Zentene	24.05.93	F	T. Tammaru	E. Õunap	AY870293	AY870305
<i>T. com</i> 189	Bul	Rupite	21.05.95	F	T. Tammaru	E. Õunap	AY870293	AY870305
<i>T. gri</i> 190	Fin	Eh: Ruovesi	–02.09.01	M	M. Rantala	E. Õunap	AY870302	AY870310
<i>T. gri</i> 191	Fin	Tampere Iidesjärvi	23.07.01	M	J. Salokannel & K. Mattila	E. Õunap	AY870299	AY870310
<i>T. gri</i> 192	Fin	Es: Savitaipale	30.06.00	M	M. Hirvonen	E. Õunap	AY870302	AY870310
<i>T. com</i> 206	Est	Tatra Valley	30.08.02	M	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. com</i> 207	Est	Tatra Valley	30.08.02	F	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. com</i> 216	Est	Põõsaspea	16.08.02	M	I. Taal	E. Õunap	AY870293	AY870305
<i>T. com</i> 228	Est	Muraste	22.08.01	M	M. Kruus	E. Õunap	AY870297	AY870305
<i>T. com</i> 229	Est	Muraste	22.08.01	M	M. Kruus	E. Õunap	AY870293	AY870305
<i>T. com</i> 237	Ukr	Kiev	10.08.02	M	I. Kostjuk	E. Õunap	AY870293	AY870305
<i>T. com</i> 239	Ger	Wedermark-Brelingen	05.–15.08.02	F	Eckard O. Krüger	E. Õunap	AY870293	AY870305
<i>T. com</i> 240	Blr	Berezina Nature Reserve	09.08.02	M	E. Õunap	E. Õunap	AY870296	AY870305
<i>T. gri</i> 241	Fin	Tammisaari, Gullö	30.06.02	M	K. Mikkola	L. Kaila	AY870301	AY870310
<i>T. gri</i> 242	Fin	Tammisaari, Gullö	07.08.02	F	K. Mikkola	L. Kaila	AY870301	AY870310
<i>T. com</i> 243	Fin	Tammisaari, Gullö	12.08.02	M	K. Mikkola	L. Kaila	AY870293	AY870309
<i>T. com</i> 244	Est	Endla Nature Reserve	11.–18.08.96	M	K. Kimmel	L. Kaila	AY870295	AY870305
<i>T. gri</i> 247	Est	Nigula Nature Reserve	13.–16.07.03	M	K. Kübar	E. Õunap	AY870301	AY870310
<i>T. gri</i> 248	Est	Tatra Valley	02.07.03	F	E. Õunap	E. Õunap	AY870298	AY870310
<i>T. gri</i> 249	Est	Tatra Valley	02.07.03	M	E. Õunap	E. Õunap	AY870304	AY870312
<i>T. com</i> 250	Est	Nigula Nature Reserve	03.–05.08.03	M	K. Kübar	E. Õunap	AY870293	AY870305
<i>T. com</i> 251	Est	Nigula Nature Reserve	03.–05.08.03	M	K. Kübar	E. Õunap	AY870293	AY870308
<i>T. com</i> 252	Est	Tatra Valley	11.09.03	M	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. com</i> 253	Est	Tatra Valley	11.09.03	F	E. Õunap	E. Õunap	AY870293	AY870305
<i>T. gri</i> 254	Est	Palmse	15.–20.06.99	M	M. Kruus	E. Õunap	AY870304	AY870313

designed and used for the analysis of the mtDNA *NDI* gene. A 392-bp fragment was amplified from *COI* and 398-bp fragment from *NDI* gene. PCR was performed in a total volume of 20 µl. The reaction mixture contained 1X BD Advantage 2 PCR buffer, 1U BD Advantage 2 Polymerase mix (BD Biosciences,

San Jose, USA), 0.2 mM dNTP (Fermentas, Vilnius, Lithuania); 1.5 mM MgCl₂, 4 pmol of primers and 20–80 ng of purified genomic DNA.

PCR was performed on a T1 Thermocycler (Biometra, Goettingen, Germany), cycling parameters were a 2 min denaturing

TABLE 2. Nucleotide variation in mtDNA *COI* and *NDI* sequences of *T. griseata* and *T. comae*. The number of specimens for each haplotype and the number of localities for each haplotype are given in the last two columns. Dots indicate nucleotide identity to the *T. comae* 24 sequence. Numbering corresponds to the homologous sequence of *Bombyx mori* (AY048187). Substitution types are indicated as tv for transversion, ts for transition, and * for both transition and transversion.

Nucleotide position according to <i>B. mori</i>	<i>COI</i>													<i>NDI</i>										No. of specimens	No. of localities		
	3	4	4	4	4	5	5	5	5	5	6	6	6	7	4	4	4	5	5	6	6	6	7			7	7
Codon position	3	1	3	3	3	1	3	1	3	3	3	3	2	3	3	3	3	3	1	3	3	3	3	2	3		
Substitution type	tv	ts	ts	ts	ts	ts	ts	ts	ts	*	ts	ts	ts	tv	ts	ts	ts	ts	ts	ts	ts	ts	tv	ts	ts		
<i>T. com</i> 24	C	G	G	A	A	G	A	T	A	T	A	G	A	A	G	A	G	A	A	A	T	A	T	T	C	21	17
<i>T. com</i> 150	G	1	1
<i>T. com</i> 228	.	.	A	1	1
<i>T. com</i> 240	.	A	1	1
<i>T. com</i> 243	C	1	1
<i>T. com</i> 244	G	1	1
<i>T. com</i> 251	C	1	1
<i>T. gri</i> 82	A	.	.	G	G	.	.	.	G	G	.	A	.	C	A	G	A	G	.	G	.	G	A	.	T	3	2
<i>T. gri</i> 157	A	.	.	G	G	.	.	.	G	.	A	.	C	A	G	A	G	.	G	.	G	A	.	T	3	3	
<i>T. gri</i> 182	.	.	.	G	.	.	G	.	.	A	G	A	.	C	A	.	A	.	.	G	.	G	A	.	T	1	1
<i>T. gri</i> 183	.	.	.	G	.	.	G	.	.	A	.	A	.	C	A	.	A	.	.	G	.	G	A	.	T	2	2
<i>T. gri</i> 190	A	.	.	G	G	.	.	C	.	G	.	.	.	C	A	G	A	G	.	G	.	G	A	.	T	2	2
<i>T. gri</i> 241	A	.	.	G	G	A	.	.	.	G	.	A	.	C	A	G	A	G	.	G	.	G	A	.	T	4	3
<i>T. gri</i> 254	.	.	.	G	.	.	G	.	.	A	.	A	.	C	A	G	.	G	A	.	T	1	1

step at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 49°C and 60 s at 68°C with subsequent 7-min final extension at 68°C for *COI* amplification. *NDI* amplification was carried out under similar conditions, except for the annealing step, which was 30 s at 54°C. The presence of PCR products was checked on a 1.6% agarose gel. The PCR solution was treated with shrimp alkaline phosphatase and exonuclease I (USB, Cleveland, USA). One unit of both enzymes was added to the PCR solution, which was incubated for 30 min at 37°C, followed by 15 min inactivation at 80°C.

DNA cycle sequencing was performed using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Uppsala, Sweden). 33 cycles (15 s at 95°C, 15 s at 53°C and 60 s at 60°C for primer RON; 15 s at 95°C, 15 s at 47°C and 60 s at 60°C for primer NAN; 15 s at 95°C, 15 s at 54°C and 60 s at 60°C for primers NF and NR) were performed in a total volume of 10 µl. Both DNA strands were sequenced with 5 pmol of primers RON and NAN for *COI*, and NF and NR for *NDI*. Sequences were resolved using an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, USA).

Phylogenetic analysis

Consensus sequences were created with Consed (Gordon et al., 1998), using sequence data from both DNA strands. Sequences were double-checked by eye and aligned with Clustal W (Thompson et al., 1994). BioEdit was used as a sequence editor (Hall, 1999).

Homogeneity of the *COI* and *NDI* sequences was calculated using the partition homogeneity test in PAUP 4.0b10 (Swofford, 1998). The test was performed with the random addition heuristic search option, using 1000 replicates. Reduced median joining network was calculated with Netw 4106 (Bandelt et al., 1999). A neighbour-joining tree was constructed from the sequence data following the Kimura 2-parameter model using MEGA 2.1 (Kumar et al., 2001), branch supports were assessed by 1000 bootstrap pseudoreplicates. Modeltest 3.06 (Posada & Crandall, 1998) was used to search for the model of DNA sub-

stitution that best fitted the data. MrBayes 3.04b (Ronquist & Huelsenbeck, 2003) was used for the Bayesian estimation of phylogeny. Since the TrN+I+G model selected by Modeltest is not implemented in MrBayes, the GTR+I+G model was used for Bayesian inference. An initial run was performed to determine the burn-in value, which was used in further analyses to exclude all trees prior to the stable log likelihood estimate. Searches were conducted with four simultaneous Markov Chains over 2 million generations, sampling every 100 generations. To estimate posterior probabilities of recovered branches, 50% majority rule was applied. To ensure that Bayesian inference was not trapped in local optima, the analysis was performed three times, starting from different random trees. Average log-likelihood (lnL) values with standard deviation (SD) at a stationarity were calculated using MS Excel and compared for convergence. Phylograms were created as average-branch-length consensus trees in MrBayes and visualised with TreeView 1.6.6 (Page, 1996).

RESULTS

Altogether, 43 specimens of the *Timandra griseata* group were successfully analysed. Based on morphological characters, 16 of them were identified as *T. griseata* and 27 as *T. comae* (Table 1).

Alignment of *COI* and *NDI* sequences did not reveal any insertions or deletions. Sequences were also aligned to the complete sequence of the mitochondrial genome of *Bombyx mori* (Linnaeus, 1758) (AY048187). Sequenced *COI* gene fragment (392-bp) corresponds to positions 1808–2199, and *NDI* gene fragment (398-bp) to positions 12064–12461 of the *B. mori* mitochondrial genome.

Combined analysis of the *COI* and *NDI* sequences yielded 25 variable positions out of 790 (3.16%) (Table 2). Four substitutions were at the first, two at the second and twenty at the third codon position. The transition-

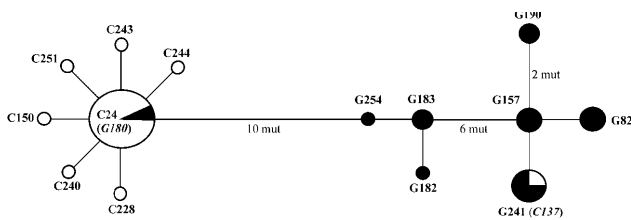


Fig. 2. Median joining network of *T. griseata* (black, haplotype name begins with G) and *T. comae* (white, haplotype name begins with C), based on *COI* and *ND1* sequences (790bp).

transversion rate was 22 : 4. Nineteen substitutions were synonymous and six non-synonymous. Intraspecific variation occurred at six positions (0.76%) in the *T. comae* and 12 positions (1.52%) in *T. griseata* sequences. Five transitions and two transversions (0.89%) distinguished *T. griseata* from *T. comae*. In the *COI* locus, homoplasmy was found at position 591, both A and G was recorded in *T. griseata* individuals, while all *T. comae* specimens carried T at this site.

On a minimum spanning network (Fig. 2), *T. griseata* is clearly separated from *T. comae*, the shortest distance between the closest haplotypes of these species is 10 mutations. *T. comae* form a single haplogroup with seven haplotypes. Haplotype C24 is the central and by far the most numerous one in this haplogroup (21 specimens, all other *T. comae* haplotypes are represented by a single specimen). *T. griseata* can be divided into two haplogroups: the first consists of haplotypes G183, G254 and G182. The second haplogroup, consisting haplotypes G157, G190, G82 and G241, is six mutations away from the haplotype G183. No haplotype of *T. griseata* was significantly more abundant than the others (Fig. 2).

The model (TrN+I+G) was selected by Modeltest according to the Akaike Information Criterion. As it is not possible to implement the TrN+I+G model in MrBayes, the GTR+I+G model was used. Three independent Bayesian analyses converged on statistically equivalent log-likelihood scores and reached an asymptotic level after no more than 20,000 iterations (not shown). Majority rule consensus trees of three rounds of Bayesian analysis were identical and posterior probability values highly correlated, indicating that the analyses converged on a single optima. Both neighbour-joining and Bayesian inference gave similar tree topologies, albeit with different statistical support (Fig. 3). A major feature of the phylogenetic analysis, strongly supported by both methods, is that it divides *T. comae* and *T. griseata* into separate clades. *T. griseata* has a more pronounced internal structure, consisting of two subclades. Three of the four individuals in subclade I were collected from northern Estonia (Palmse, Narva-Jõesuu and Endla Nature Reserve), and one from the Tatra Valley near Tartu in eastern Estonia (Fig. 1). Specimens in subclade II originate from a variety of localities in eastern, south-western and northwestern Estonia and southern Finland (Table 1).

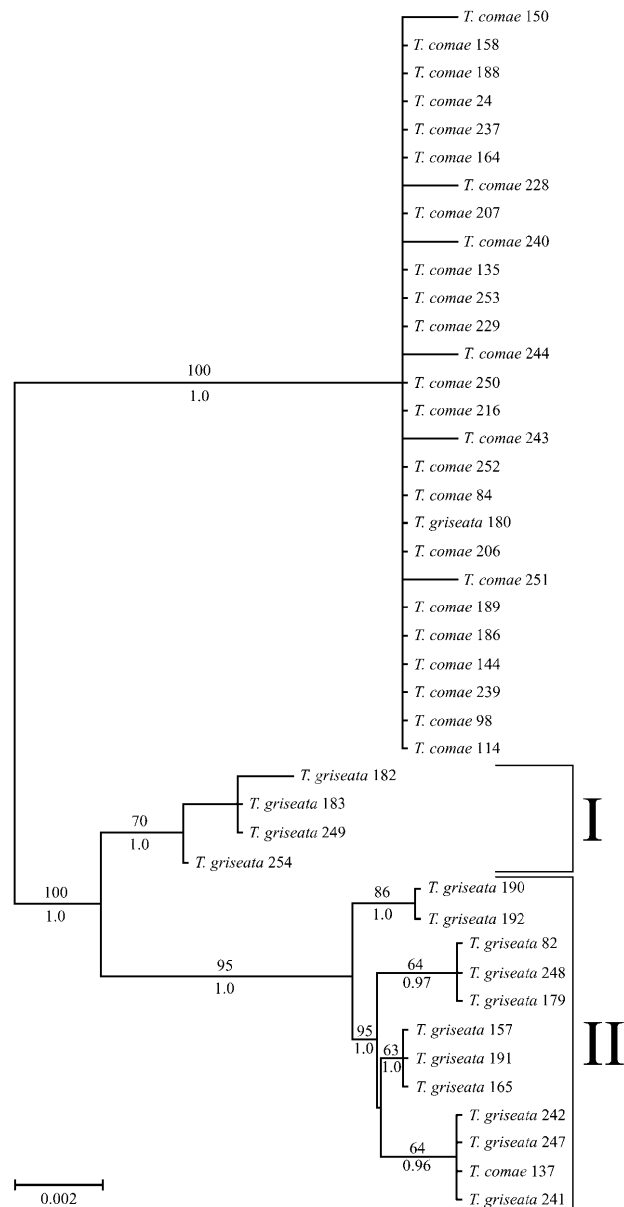


Fig. 3. Neighbour-joining tree of *T. griseata* and *T. comae*, based on sequences of the mtDNA gene fragments: *COI* (392 bp) and *ND1* (398 bp). As the Bayesian analyses yielded trees with similar topology, Bayesian posterior probabilities are included. Above the branches are bootstrap supports of the neighbour-joining tree (NJ) and below are posterior probabilities for Bayesian inference (BY). The average branch-length consensus tree was constructed from 9980 Bayesian trees recovered using the GTR+I+G model, lnL = -1335.49 ± 5.29.

DISCUSSION

The phylogenetic analysis separated *T. griseata* and *T. comae* into different clades (Fig. 3), supporting the suggestion that these taxa represent separate species (Kaila & Albrecht, 1994; Miller et al., 2001) or, as expressed by Trusch et al. (2002), “natural entities”. Nonetheless, two individuals, *T. griseata* 180 and *T. comae* 137, were included in the clade of the other species. In order to exclude the possibility of misidentification, the morphological characters of both individuals were re-examined;

our identifications were later confirmed by L. Kaila (University of Helsinki). To rule out mistakes in genetic analysis (picking wrong individual, contamination, sequencing errors) and subsequent misinterpretation of data, the whole analysis from dissection to DNA purification and sequencing was repeated for these two specimens, using the posterior parts of their abdomens as the source of genomic DNA (see also Material and Methods). As this re-examination confirmed the results of the first analysis, we conclude that the identity and positions of *T. griseata* 180 and *T. comae* 137 in the phylogenetic tree are valid.

Following the Mayr's (1963) biological species concept, it is generally assumed that species must be monophyletic entities (Harrison, 1998). However, as shown by Pamilo & Nei (1988) and summarized by Wahlberg et al. (2003) and Funk & Omland (2003) in various sources, both polyphyly and paraphyly may arise during the speciation process. Complete lineage sorting may or may not have occurred during speciation and it cannot be assumed as an ineluctable event (Wahlberg et al., 2003).

Incomplete lineage sorting can result either from a recent divergence of populations, or from hybridization, which does not allow complete segregation of lineages. In the case of recent divergence, not enough time has elapsed for mutations to become fixed; both species still share identical or very similar haplotypes (i.e. have retained the polymorphism of their ancestral population). However, the claim of incomplete lineage sorting holds only for the genetic markers under investigation, usually the genes of mtDNA. It is conceivable that complete lineage sorting has occurred at other loci in the genome; candidates are primarily genes related to reproduction and the formation of a reproductive barrier between diverged populations, either at a pre- or postzygotic level (genes associated with genitalia formation, pheromone system, etc.). To find the sorted locus or loci for animals other than model organisms is an unrealistic task at present. A statement solely in favour of incomplete lineage sorting between species presumes that hybridization is ruled out. If hybridization occurs and the hybrids yield fertile offspring, then successful backcrossing does not allow lineages to diverge. On the other hand, when diverged populations hybridize (i.e. after the disappearance of geographic barriers), but do not produce fertile offspring, then one can still observe individuals that carry haplotypes of another species. However, since they do not pass genes to the next generation, the parent population retains its genetic status.

Here, the closest mtDNA haplotypes of *T. griseata* and *T. comae* differ from each other by at least 10 substitutions (Fig. 2), indicating that these taxa did not separate from each other recently. Moreover, *T. griseata* has a significantly diverged intraspecific genetic structure (Fig. 3), which supports the hypothesis that intraspecific diversification of *T. griseata* has ancient history. However, it is unlikely that haplotypes of *T. comae* 137 and *T. griseata* 180 have retained their ancestral mtDNA sequence, while

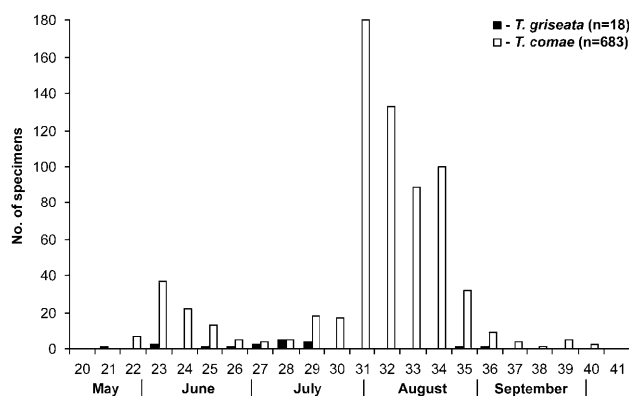


Fig. 4. Phenology of *T. griseata* and *T. comae* in Estonia.

a remarkable amount of mutations have accumulated in the population of *T. griseata* (and to lesser extent in *T. comae*). Therefore, we favour the scenario that the few atypical sequences in *T. griseata* and *T. comae* most likely originated from hybridization. Additional support for the hybridization scenario comes from the phenology of *T. griseata* and *T. comae*. *T. comae* is bivoltine, while *T. griseata* is usually univoltine. Both species are protandric (i.e. males appear earlier than females), as most insects with discrete generations (e.g. Carvalho et al., 1998). Flight periods of *T. griseata* and *T. comae* differ, but partly overlap in southern Finland (Kaila & Albrecht, 1994).

In Estonia, a similar pattern was observed (Fig. 4): the flight period of the first generation of *T. comae* ranges from late May to the end of June; the second and more abundant brood is on wing from late July to September. The flight period of *T. griseata* begins in late June and peaks in the middle of July, lasting until the end of the month. Therefore, the flight periods of the species overlap at least twice in each year, providing an opportunity for the species to hybridize. The earliest possible time when hybridization can occur is the end of June, when both sexes of the first generation of *T. comae* and only males of *T. griseata* are flying, but females of *T. griseata* have not emerged yet. Males of *T. griseata* not finding a receptive female of their own species can copulate with females of *T. comae*.

Hybridization may also occur at the end of July, when both sexes of *T. griseata*, but only males of the second generation of *T. comae*, are on wing and females of the second *T. comae* brood have not yet emerged. At this time, males of *T. comae* may hybridize with females of *T. griseata*. However, it is unlikely that there are many unfertilized females of either *Timandra* species searching for mates at the end of their flight period. Therefore the probability for hybridization is low, which can explain why only two *Timandra* specimens of possible hybrid origin were found during this study.

The hypothesis of hybridization between *T. griseata* and *T. comae* receives further support from the discussion of Pittaway (1993) for closely related sphingid species *Hyles euphorbiae euphorbiae* (Linnaeus, 1758) and *Hyles*



Fig. 5. Hypothetical colonisation routes for *T. griseata* in Estonia.

vespertilio (Esper, 1780). The flight period of *H. vespertilio* starts 8–14 days earlier than that of *H. e. euphorbiae*. When the earliest *H. e. euphorbiae* males emerge, there are no receptive conspecific females available for them; therefore, they sometimes hybridize with females of *H. vespertilio*. Although *H. e. euphorbiae* × *H. vespertilio* is the commonest sphingid hybrid in Europe, the parent species have remained distinct, because hybrids are unable to produce viable offspring when back-crossed with *H. vespertilio* (Pittaway, 1993). However, while *H. e. euphorbiae* and *H. vespertilio* are morphologically rather different and hybrids are easily distinguishable, *T. comae* 137 and *T. griseata* 180 show no intermediate morphological characteristics usually seen in F₁ generation hybrid sphingids. A likely explanation is that *T. comae* 137 and *T. griseata* 180 are not F₁ hybrids and the hybridization event is more ancient.

The eastern limit of *T. griseata* is unknown, but it is probably significantly further east than current data indicate. Notes that *T. griseata* occurs south of Scandinavia and the Baltic States (Viidalepp, 1996) should be interpreted with care until validated (as the identification was carried out before the recognition of *T. griseata* and *T. comae* as separate species, all individuals were identified as *T. griseata*).

T. griseata and *T. comae* became sympatric during the 20th century. *T. griseata* has been resident in Finland since the 19th century (the oldest records date back to 1869), while *T. comae* expanded its distribution into this area during the 20th century. The earliest Finnish specimen of *T. comae* was taken in 1920 (Kaisila, 1954; Kaila & Albrecht, 1994). Our survey of the Estonian insect collections gave similar results: all *Timandra* individuals, collected between 1874 and 1939, were identified as *T. griseata*; the earliest Estonian *T. comae* specimen was collected in 1940. The largely different geographic distributions and timing of colonization by these species supports Kaisila's hypothesis, that *T. griseata* (*Calothysanis amataria griseata* in his paper) and *T. comae* (*C. a. brykaria*) have different colonization histories in northern Europe: in addition to the differences in their time of arrival, they also came via different routes: *T. griseata* from the east and *T. comae* from the west.

The *T. griseata* clade is divided into two subclades (Fig. 3) that differ in geographic distribution. Most of the specimens from Estonia and Finland belong to the subclade II, but three haplotypes constitute the subclade I. Three specimens of the subclade I were from northern Estonia (i.e. all north Estonian individuals) and one from the Tatra Valley near Tartu in eastern Estonia (Fig. 1). Data on the phylogenetic structure and geographic distribution of the different haplotypes of *T. griseata* further indicate that this species may have come from two directions. The most likely scenario is that specimens of subclade I spread into Estonia via the northern side of Lake Peipsi, while ancestors of subclade II came via a southern route (Fig. 5). A similar colonization pattern is documented for another Lepidopteran, *Parnassius mnemosyne estonicus* Bryk 1922, which rapidly expanded its range, both in northern and southeastern Estonia, during the last two decades (Viidalepp, 2000).

The completely different intraspecific genetic structure of *T. griseata* (highly divergent, ancient radiation) compared to *T. comae* (limited divergence, rapid radiation) proves that the evolution of these taxa has been different. The complex phylogenetic structure and current geographic distribution of *T. griseata* in northern Europe indicates that this species colonized northern Europe from several (or at least two) refugia. An alternative possibility, that the population with high intraspecific genetic polymorphism came from a single refuge area, is less likely because of its current distribution pattern. Limited genetic divergence of the population of *T. comae* suggests that all current populations in Europe came from a single refuge area and descend from a population with a low polymorphism at the mtDNA loci. Another explanation that only part of the genetic diversity reached their current locations, i.e. the population has gone through a genetic bottleneck, deserves less credit, as the geographic range of the specimens studied was wide (from Bulgaria to Scandinavia).

For a sustainable identification of animals, Hebert et al. (2003) proposed a system that employs DNA sequences as taxon “barcodes”. They suggested that the mitochondrial *COI* gene could serve as the core of a global bio-identification system. Our results tend to support their proposal, although with reservations. As two *Timandra* specimens were placed into the “wrong” clade, we suggest that complete lineage sorting and lack of hybridisation are necessary preconditions to be fulfilled before the use of “barcoding” is justified.

CONCLUSIONS

The phylogenetic structure, colonisation history, phenology and current distribution of *T. griseata* and *T. comae* seems to be an example of the evanescence of geographic barriers between populations, which has resulted in hybridization, albeit with low frequency. As the populations nevertheless appear to retain their integrity, separation of *T. griseata* and *T. comae* into two separate species is warranted.

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