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Molecular phylogeny of the sawfly subfamily Nematinae (Hymenoptera: Tenthredinidae)

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Abstract. Nematinae is one of the largest subfamilies in the sawfly family Tenthredinidae, but internal relationships are unknown in the absence of any formal phylogenetic analysis. To understand the internal phylogeny of Nematinae, we sequenced a portion of the mitochondrial cytochrome oxidase I gene and the nuclear elongation factor-1α gene from thirteen outgroup taxa and sixty-eight nematine species, the ingroup taxa of which represent all major genera and subgenera within the subfamily. Maximum parsimony and Bayesian phylogenetic analyses of the DNA sequence data show that: (1) Nematinae are monophyletic in a broad sense which includes *Hoplocampa*, *Susana* and the tribe Cladiini, which have been classified often into separate subfamilies; together with *Craterocercus*, these taxa form a paraphyletic basal grade with respect to the remaining Nematinae, but among-group relationships within the grade remain weakly resolved; (2) the remainder of the ingroup, Nematinae s. str, is monophyletic in all combined-data analyses; (3) within Nematinae s. str, the 'Higher' Nematinae is divided into three groups, *Mesoneura* and the large tribes Nematini and Pristiphorini; (4) although the traditional classifications at the tribal level are largely upheld, some of the largest tribes and genera are obviously para- or polyphyletic; (5) according to rate-smoothed phylogenies dated with two fossil calibration points, Nematinae originated 50–120 million years ago. In addition, the results from all Bayesian analyses provide strong and consistent support for the monophyly of Tenthredinidae, which has been difficult to demonstrate in previous parsimony analyses of morphological and molecular data.

Introduction

The cosmopolitan sawfly family Tenthredinidae includes over 6000 species divided into six subfamilies (Goulet, 1992; Taeger & Blank, 1998). One of the largest is Nematinae, which comprises over 1000 species. Nematinae has a primarily northern distribution: by contrast with most other insect taxa, the diversity of nematines reaches its peak in the north temperate region (Marlatt, 1896; Kouki *et al.*, 1994; Kouki, 1999) and, in many Arctic areas, nematines are the only sawflies present (Benson, 1958; Smith, 1979; Gauld & Bolton, 1988). The southernmost naturally occurring nematine species occur in Brazil (Malaise, 1942; Smith, 2003) and Borneo (Benson, 1963). Nematine larvae rank amongst the principal insect herbivores in many habitats, and some species associated with trees are considered as serious pests. For example, *Hoplocampa* species damage fruits of orchard trees, and *Anoplonyx* and *Pristiphora* species can damage spruce and larch forests severely (Smith, 1979; Gauld & Bolton, 1988; Viitasaari, 2002).

Despite their ubiquity and ecological importance, the phylogenetic relationships amongst nematine taxa remain

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unknown. The subfamily has been divided traditionally into some eight to nine tribes and approximately forty genera (cf. Smith, 1979; Zhelochovtsev, 1988, 1994; Abe & Smith, 1991; Lacourt, 1998, 1999; Taeger *et al.*, 1998). Because the definition of Nematinae is ambiguous, some nematine groups (especially the tribe Cladiini, and the genera *Susana* and *Hoplocampa*) have been classified frequently as separate subfamilies (e.g. Yuasa, 1922; Ross, 1937, 1951; Maxwell, 1955; Goulet, 1992; Lacourt, 1998, 1999). To date, no formal morphology-based phylogenetic analyses of the Nematinae have been performed, partly because of the limited morphological divergence amongst groups and partly as a result of difficulties that follow from the broad Holarctic distributions of many nematine species and genera (Benson, 1958, 1962; Smith, 1979; Goulet, 1992). A phylogenetic hypothesis presented by Ross (1937), although not a formal analysis, was based on a philosophy of minimizing morphological changes along the tree (Fig. 1), and later Maxwell (1955) drew a tree on the basis of larval anatomy. However, the two phylogenies include few nematine genera and are directly contradictory in the placement of many taxa.

The purpose of this study was to gain a more robust hylogenetic hypothesis of the subfamily Nematinae. For this, we used DNA sequence data from the mitochondrial cytochrome oxidase I gene and the nuclear elongation factor-1α gene to reconstruct the relationships amongst thirteen outgroup taxa and sixty-eight nematine species that represent all tribes and all main genera within the Nematinae. Maximum parsimony and Bayesian phylogenetic analyses of the sequence data support the monophyly of the subfamily in a broad sense, give a relatively well-resolved overall view of the nematine radiation, and provide a framework for further studies.

Materials and methods

Taxon sampling

The three largest nematine genera, *Pristiphora*, *Nematus* and *Pachynematus* s.l., have proven to be particularly complex from a taxonomic perspective, because attempts to divide them into smaller genera or subgenera have led to difficulties in classifying many species (Benson, 1958; Smith, 1979; Viitasaari, 2002). Here, we follow mainly the nomenclature of the maximally divisive (sub)generic classifications of Zhelochovtsev (1988, 1994) and Lacourt (1998, 1999) to ensure a broad enough taxon sample. The sixty-eight ingroup taxa included in the phylogenetic analysis (Table 1) represent all major genera within Nematinae, and most subgenera of the large genera. The missing genera are rare and (near) monotypic (*Adelomos* Ross, *Neodineura* Taeger, *Megadineura* Malaise, *Katsujia* Togashi and *Nepionema* Benson), or their generic status is disputed (for example, Benson (1963) and Abe & Smith (1991) consider the Asian *Moricella* Rohwer a synonym of *Mesoneura*). The largest missing genus is *Kerita* Ross, which includes three Nearctic species (Smith, 1976).

To establish the phylogenetic position of Nematinae, we included thirteen outgroup taxa that supposedly represent different levels of divergence (Table 1). *Sterictiphora*, *Diprion* and *Abia* represent three other families from the superfamily Tenthredinoidea (Argidae, Diprionidae and Cimbicidae, respectively). Of these, *Sterictiphora* was used to root the trees, in accordance with results from phylogenetic analyses by Rasnitsyn (1988), Vilhelmsen (1997), Ronquist *et al.* (1999) and Schulmeister (2003). The ten non-nematine tenthredinid species represent all five other subfamilies within the family Tenthredinidae (Taeger & Blank, 1998). All specimens have been deposited as voucher

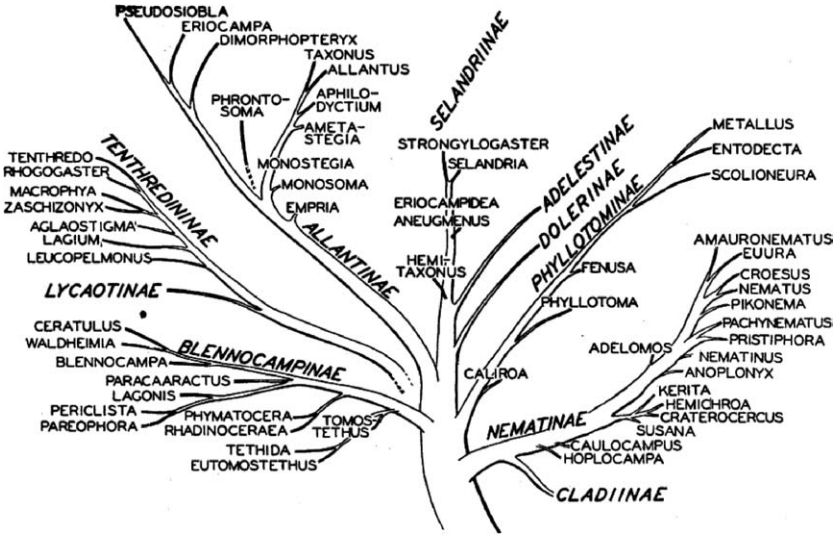

Fig. 1. Ross's (1937) phylogenetic hypothesis of the Tenthredinidae. The subfamily Nematinae (and Cladiinae) is on the lower right-hand side of the tree.

Table 1. Taxa and samples used in the study, and collection dates and localities. Classification follows mainly Zhelochovtsev (1988, 1994) and Lacourt (1998, 1999). Ingroup tribes that are para- or polyphyletic are indicated, and tribes that should be lowered to a subtribal rank in order to make Pristiphorini and Nematini monophyletic are given in parentheses (see Fig. 3).

Tribe	Genus (subgenus) species	Sex/stage (host, if larva)	Location	Date	Collector
'Susaninae' (included in ingroup)	*Susana annulata* Smith	♀	Butte County, CA, U.S.A.	iv.2002	H. J. Jacobson
Hoplocampini (polyphyletic)	*Hoplocampa oregonensis* (Ashmead)	♀	Elk mtn, BC, Canada	17.vii.2002	T. Nyman
	Hoplocampa marlatti Rohwer	♀	Cambridge, MA, U.S.A.	5.v.2002	T. Nyman
	Caulocampus acericaulis (MacGillivray)	♀	Aneaster, ON, Canada	17–20.v.1995	B. DeJonge
	Caulocampus matthewsi Smith	♀	Petersham, MA, U.S.A.	8–15.v.2002	T. Nyman
Cladiini	*Cladius comari* Stein	♂	Mekrijärvi, Finland	8.vii.2001	T. Nyman
	Priophorus pallipes (Serville)	Larva, *Betula pubescens*	Laboratory colony, Finland	12.iv.2000	A. Kause
	Trichiocampus aeneus (Zaddach)	Larva, *Salix phylicifolia*	Hausjärvi, Finland	2.ix.2001	V. Vikberg
Mesoneurini (polyphyletic)	*Mesoneura opaca* (Fabricius)	Larva, *Quercus* sp.	Overijse, Belgium	26.v.2001	J.-L. Boevé
	Craterocercus fraternalis (Norton)	♀	Hardy County, WV, U.S.A.	17.4.-3.v.2002	D. R. Smith
Pseudodineurini	*Pseudodineura fuscula* (Klug)	♀	Nana Aseme, Estonia	21.4.2002	M. Heidemaa
	Pseudodineura parva (Norton)	♂	Petersham, MA, U.S.A.	14–22.v.2002	T. Nyman
	Endophytus anemones (Hering)	Larva, *Anemone nemorosa*	Etzen, Austria	5.v.2002	E. Altenhofer
Dineurini	*Platycampus luridiventris* (Fallén)	♂	Joensuu, Finland	11.vi.2001	T. Nyman
	Anoplonyx apicalis (Brischke)	Larva, *Larix sibirica*	Janakkala, Finland	1.ix.2002	V. Vikberg
	Dineura virididorsata (Retzius)	Larva, *Betula pubescens*	Janakkala, Finland	7.vii.2002	V. Vikberg
	Hemichroa (*Hemichroa*) *crocea* (Geoffroy)	Larva, *Alnus glutinosa*	Grimminge, Belgium	4.ix.2002	J.-L. Boevé
	Hemichroa (*Varna*) *militaris militaris* (Cresson)	♀	Petersham, MA, U.S.A.	29.v.–4.vi.2002	T. Nyman
	Hemichroa (*Varna*) *militaris thoracicus* (Harrington)	♀	Petersham, MA, U.S.A.	7–16.vi.2003	T. Nyman
	Nematinus fuscipennis (Serville)	♀	Tohmajärvi, Finland	15.vi.2001	T. Nyman, M. Viitasaari
	Fallocampus americanus (Marlatt)	♀	Petersham, MA, U.S.A.	16.vi.2003	T. Nyman
Stauronematini	*Stauronematus compressicornis* (Fabricius)	Larva, *Populus tremula*	Suchowola, Poland	22.viii.1997	H. Roininen, A. Zinovjev
(Pristicampini)	*Pristicampus incisus* (Lindqvist)	Larva, *Potentilla fruticosa*	Turenki, Finland	18.vii.1997	A. Zinovjev, V. Vikberg
(Pristolini)	*Pristola macnabi* Ross	♀	Mt. Seymour, BC, Canada	13.vii.2002	T. Nyman
	Melastola ferruginosa Wong	♂	Mt. Seymour, BC, Canada	13.vii.2002	T. Nyman
	Melastola sp.	Larva, *Vaccinium parvifolium*	Burnaby, BC, Canada	9.vii.2002	T. Nyman
Pristiphorini (paraphyletic)	*Pristiphora* (*Pristiphora*) *geniculata* (Hartig)	Larva, *Sorbus aucuparia*	Etzen, Austria	28.viii.2000	E. Altenhofer
	Pristiphora (*Lygaeophora*) sp.	♀	Nuorgam, Finland	8.vi.2001	T. Nyman
	Pristiphora (*Lygaeonematus*) *erichsonii* (Hartig)	Larva, *Larix sibirica*	Janakkala, Finland	18.vii.2002	V. Vikberg
	Pristiphora (*Lygaeonematus*) *abietina* (Christ)	Larva, *Picea abies*	St. Petersburg, Russia	13.vii.1997	A. Zinovjev
	Pristiphora (*Lygaeotus*) *alpestris* (Konow)	♀ ex larva, *Betula pubescens* ssp. *czerepanovii*	Laboratory colony, Kevo, Finland	8.vi.2001	L. Kapari
	Pristiphora (*Lygaeotus*) *coactula* (Ruthe)	♀	Nuorgam, Finland	8.vi.2001	T. Nyman
	Pristiphora (*Sala*) *chlorea* (Norton)	♀	Petersham, MA, U.S.A.	4.vi.2002	T. Nyman
	Sharliphora nigella (Förster)	♀	Vellavere, Estonia	25.iv.2002	M. Heidemaa
	Micronematus monogyniae (Hartig)	Larva, *Prunus* sp.	Parikkala, Finland	21.vi.2002	H. Roininen
	Neopareophora litura (Klug)	♂	Hardy County, WV, U.S.A.	17.iv.–3.v.2002	D. R. Smith

Table 1. Continued.

Tribe	Genus (subgenus) species	Sex/stage (host, if larva)	Location	Date	Collector
(Bacconematini)	*Bacconematus pumilio* (Konow)	Larva, *Ribes nigrum*	Turenki, Finland	3.vii.2002	V. Vikberg
Nematini (paraphyletic)	*Pikonema scutellatum* (Hartig)	Larva, *Picea abies*	Parikkala, Finland	6.vii.1996	H. Roininen, A. Zinovjev
	Pikonema dimmockii (Cresson)	♀	Mt. Seymour, BC, Canada	13–17.vii.2002	T. Nyman
	Epicenematus montanus (Zaddach)	Larva, *Picea abies*	Parikkala, Finland	12.vii.1996	H. Roininen, A. Zinovjev
	Pachynematus kirbyi (Dahlbom)	♂	Mekrijärvi, Finland	8.vii.2001	T. Nyman
	Polynematus annulatus (Gimmerthal)	Larva, *Rumex longifolius*	Turenki, Finland	13.vi.2002	V. Vikberg
	Eitelius gregarius (Marlatt)	Larva, *Salix discolor*	Ithaca, NY, U.S.A.	1997	A. Zinovjev
	Craesus septentrionalis L.	Larva, *Alnus glutinosa*	Grimminge, Belgium	4.ix.2000	J.-L. Boevé
	Brachycoluma viduata (Zetterstedt)	Larva, *Salix myrsinifolia*	Parikkala, Finland	1.vi.2002	H. Roininen
	Brachycoluma sp.	Larva, *Salix pentandra*	Joensuu, Finland	12.vi.1996	H. Roininen
	Pontopristia sp.	Larva, *Salix myrsinifolia*	Joensuu, Finland	10.vi.2001	T. Nyman
	Pontopristia sp.	Larva, *Salix candida*	Churchill, MB, Canada	1.viii.2002	T. Nyman
	Amauronematus eiteli Saarinen	Larva, *Salix pentandra*	Parikkala, Finland	24.vi.2001	T. Nyman
	Amauronematus longicauda (Hellén)	♀	Nuorgam, Finland	8.vi.2001	T. Nyman
	Amauronematus amplus Konow	♀	Nuorgam, Finland	8.vi.2001	T. Nyman
	Nematus (*Pteronidea*) *melanaspis* Hartig	♀ ex larva, *S. pentandra*	Parikkala, Finland	24.vi.2001	T. Nyman
	Nematus (*Pteronidea*) *lipovskyi* Smith	♀	Petersham, MA, U.S.A.	7–14.v.2002	T. Nyman
	Nematus (*Pteronidea*) *miliaris* (Panzer)	Larva, *Salix caprea*	Joensuu, Finland	28.viii.2001	T. Nyman
	Nematus (*Kontuniemiana*) *ribesii* (Scopoli)	Larva, *Ribes rubrum*	Ruokolahti, Finland	10.vii.1994	H. Roininen
	Paranematus tulunensis (Vikberg)	♀	St. Petersburg, Russia	14.vii.1997	A. Zinovjev
	Larinematus imperfectus (Zaddach)	Larva, *Larix sibirica*	Janakkala, Finland	3.vi.2002	V. Vikberg
	Phyllocolpa excavata Marlatt	Larva, *Salix pentandra*	Kesälahti, Finland	16.vii.1998	H. Roininen
	Phyllocolpa tuberculata (Benson)	Larva, *Salix starkeana*	St. Petersburg, Russia	15.v.1997	A. Zinovjev
	Pontania (*Pontania*) *dolichura* (Thomson)	Larva, *Salix phylicifolia*	Keret, Russia	2.viii.1998	T. Nyman
	Pontania (*Pontania*) *bridgmanii* (Cameron)	Larva, *Salix starkeana*	Puhos, Finland	24.vii.1998	T. Nyman
	Pontania (*Eupontania*) *pustulator* Forsius	Larva, *Salix phylicifolia*	Taivalkoski, Finland	30.vii.2001	T. Nyman
	Pontania (*Eupontania*) *aestiva* (Thomson)	Larva, *Salix myrsinifolia* ssp. *borealis*	Kilpisjärvi, Finland	21.viii.2001	T. Nyman
	Pontania (*Eupontania*) *herbaceae* (Cameron)	Larva, *Salix herbacea*/*polaris*	Abisko, Sweden	16.viii.1998	T. Nyman
	Euura (*Euura*) *atra* –group sp.	Larva, *Salix starkeana*	Puhos, Finland	3.viii.2001	T. Nyman
	Euura (*Euura*) *venusta* (Zaddach)	Larva, *Salix aurita*	Joensuu, Finland	20.viii.2000	T. Nyman
	Euura (*Gemmura*) *lanatae* Malaise	♀ ex larva, *Salix lanata*	Kilpisjärvi, Finland	15.viii.1997	T. Nyman
Unplaced taxa	*Fagineura crenativora* Vikberg & Zinovjev	♀ ex larva, *Fagus* sp.	Mt. Tanzawa, Japan	24.iv.2003	A. Yamagami
Outgroup taxa					
DIPRIONIDAE: Diprioninae	*Diprion similis* (Hartig)	♂	Petersham, MA, U.S.A.	29.v–4.vi.2002	T. Nyman
CIMBICIDAE: Abiinae	*Abia candens* Konow	♀	Suida River, Russia	30.vi.1997	A. Zinovjev
ARGIDAE: Sterictiphorinae	*Sterictiphora* sp.	♂	Petersham, MA, U.S.A.	4.vi.2002	T. Nyman
TENTHREDINIDAE:					
Tenthredininae: Tenthredinini	*Tenthredo notha* Klug	♂	Joensuu, Finland	9.vii.1996	A. Zinovjev

Table 1. Continued.

Tribe	Genus (subgenus) species	Sex/stage (host, if larva)	Location	Date	Collector
Selandriinae: Strongylogastrini	*Strongylogaster tacita* (Norton)	♂	Petersham, MA, U.S.A.	29.v.2002	T. Nyman
Allantinae: Athaliini	*Athalia circularis* (Klug)	♀	Parikkala, Finland	12.vii.1996	A. Zinovjev, H. Roininen
Allantinae: Eriocampini	*Eriocampa ovata* (L.)	♀	Squamish, BC, Canada	10.vii.2002	T. Nyman, J. Joy
Heterarthrinae: Heterarthrini	*Heterarthrus nemoratus* (Fallén)	♀	Tohmajärvi, Finland	12.vi.2001	T. Nyman, M. Viitasaari
Heterarthrinae: Fenusini	*Scolioneura betuleti* (Klug)	Larva, *Betula pubescens* ssp. *czerepanovii*	Kilpisjärvi, Finland	10.viii.2001	T. Nyman, V. Vikberg
Heterarthrinae: Caliroini	*Caliroa* sp.	Larva, *Salix daphnoides* ssp. *acutifolia*	Salangriva, Latvia	22.viii.1997	H. Roininen, A. Zinovjev
Heterarthrinae: Caliroini	*Endelomyia aethiops* (Fabricius)	Larva, *Rosa* sp.	Mekrijärvi, Finland	23.viii.1997	T. Nyman, A. Zinovjev
Blennocampinae: Blennocampini	*Ardis brunniventris* (Hartig)	Larva, *Rosa* sp.	Parikkala, Finland	vii.1997	H. Roininen
Blennocampinae: Phymatocerini	*Phymatocera aterrima* (Klug)	♂	Parikkala, Finland	17.vi.1997	J. Sorjonen

samples at the Zoological Museum of the University of Oulu, Finland.

DNA extraction, polymerase chain reaction (PCR) and sequencing

Total genomic DNA was extracted from adults or larvae stored in ethanol at −20 °C using the DNeasy Tissue Kit (Qiagen, Valencia, California). PCRs (50 μl) contained 1 × Qiagen reaction buffer with $MgCl_2$ added to a final concentration of 2.5 mM, 0.2 μM of each primer, 0.15 mM of each dNTP and 1.5 units of *Taq* DNA polymerase.

An approximately 850-bp piece of the mitochondrial cytochrome oxidase I (CoI) gene was amplified with the primers sym-C1-J-1718 (5′-GGA GGA TTT GGA AAY TGA YTA GTW CC-3′; modified from Simon *et al*., 1994) and A2590 (5′-GCT CCT ATT GAT ARW ACA TAR TGR AAA TG-3′; Normark *et al*., 1999). The PCR programme consisted of an initial denaturing step at 94 °C for 5 min, followed by forty cycles of 1 min denaturing at 94 °C, 45 s annealing at 49 °C and 1 min extension at 72 °C; the last cycle was followed by a final 5 min extension step. Double-stranded PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), including a 35% guanidine-HCl step. Purified PCR products were sequenced in both directions with the BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and an ABI 3100 automated sequencer (Applied Biosystems). The internal primers sym-C1-J-1751 (5′-GGA GCY CCY GAT ATA GCA TTY CC-3′; modified from Simon *et al*., 1994) and C1-N-2442B (5′-GCT ART CAT CTR AAA ATT TTA ATT CCW GTD GG-3′; modified from Normark *et al*., 1999 by B. O'Meara) were used to confirm sequences when necessary. Sequences were read, edited and aligned using Sequencher version 4.1 (Gene Codes Corp., Ann Arbor, Michigan).

Hymenopteran genomes contain two paralogous copies (F1 and F2) of the nuclear elongation factor-1α (EF-1α) gene, but the copies can be separated because their introns are located differently (Danforth & Ji, 1998; Danforth *et al*., 1999). We used the primers M44-1 (Cho *et al*., 1995) and Cho10 (Danforth *et al*., 1999) to amplify and sequence a fragment of the F2 copy from a subset of the sawfly taxa, and then designed a new reverse primer (EF1a-r1200; Table 2), which partially overlaps with the 3′ recognition site of the third intron of the F2 copy (see Danforth & Ji, 1998). This intron is absent from the F1 copy and, consequently, the primer combination M44-1 + EF1a-r1200 amplified only the F2 copy from all included taxa. In the PCRs, an initial 5 min denaturing step was followed by a touchdown profile, in which the annealing temperature decreased from 58 °C to 42 °C by 2 °C every third cycle, and the final 19 cycles had annealing at 42 °C. Thus, there was a total of forty-three cycles, followed by a final extension step of 5 min. During each cycle, denaturing and annealing lasted 1 min, but extension times were adjusted depending on the taxon (for nematines 2 min was enough, but extension times up to 3 min were used for some outgroup taxa having long introns). Because of primer–dimer formation in some samples, the PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen) and then sequenced as described above. Additional internal primers (Table 2) were used to sequence the exons in both directions, especially in cases in which heterozygosity (within-individual length variation) or excessive length in the middle intron prevented sequencing 'through' the intron.

Phylogenetic analyses

No insertions or deletions were observed in the 810-bp CoI sequences, and so the sequences could be aligned manually. Likewise, the two exon sequences (501 bp + 276 bp = 777 bp) of the EF-1α fragment were aligned by eye. Lacking a reliable alignment, EF-1α introns were excluded. In all, the final dataset included 1587 bases, 588–590 of which were parsimony informative depending on the transversion : transition (tv : ti) weights (CoI, 371–372 informative characters; EF-1α, 217–218 informative characters). All sequences have been deposited in GenBank under accession numbers DQ302166–DQ302408. The data matrix is available as an electronic supplement.

Maximum parsimony analyses in PAUP* version 4.01b10 (Swofford, 2002) were performed with three different tv : ti weights (1 : 1, 2 : 1, 3 : 1). Multistate (heterozygous) bases in EF-1α were treated as polymorphisms. Heuristic searches included 100 random addition sequences with tree bisection and reconnection (TBR) branch swapping, with searches starting from random trees, swapping on best trees only, and with no maxtrees limit. Branch support was estimated by bootstrapping (Felsenstein, 1985) 100 times over informative characters (heuristic searches with TBR branch swapping, twenty random addition sequences per pseudoreplicate). Possible incongruence between the genes was tested with the incongruence length difference (ILD)

Table 2. Primers used to amplify and sequence the F2 copy of the elongation factor-1α gene in the studied sawfly species. The primers M44-1 (Cho *et al*., 1995) and EF1a-r1200 were used for amplification and sequencing, other primers were used as internal sequencing primers as needed; f and r denote 'forward' and 'reverse' primers, respectively, and numbers indicate approximate annealing locations on the amplified fragments.

Primer	Sequence (5′−3′)
M44-1	GCT GAG CGY GAR CGT GGT ATC AC
EF1a-f50	AGA GAT TTY ATY AAG AAC ATG AT
EF1a-f400	ACC MAG CAG ACC MAC CGA CAA GG
EF1a-r405	AGA RCC TTG TCR GTG GGT CTG CT
EF1a-r700	ACC ACC RAT YTT GTA GAC RTC CT
EF1a-f705	TAC AAG ATY GGW GGT ATY GGA AC
EF1a-r800	GCG AAT GTY ACA ACR GTA CCT GG
EF1a-r990	GAG TCR CCR GCR ACG TAT CCA CG
EF1a-r1000	TTK GGY GGG TTG TTC TTY GAG TC
EF1a-r1200	CNG GRT GGT TCA RVA CRA TKA CCT

test (Farris *et al.*, 1994) in PAUP* (100 replicates excluding uninformative characters, twenty random addition sequences per replicate). Because the test indicated statistically significant incongruence with all weighting schemes (all $P < 0.01$), separate analyses were performed to identify possible areas of conflict between the two genes. It should be noted that the ILD test has been criticized as a poor indicator of data combinability, especially in cases in which evolutionary rates differ between data partitions (Barker & Lutzoni, 2002; Darlu & Lecointre, 2002). The sensitivity to rate heterogeneity is, of course, particularly relevant when mitochondrial and nuclear genes are involved (Kjer *et al.*, 2001; Johnson *et al.*, 2003; Lin & Danforth, 2004), and combining data partitions in spite of significant ILD test results can improve the accuracy of phylogenetic inference (Yoder *et al.*, 2001; Barker & Lutzoni, 2002; Cryan *et al.*, 2004; but see Hipp *et al.*, 2004). Consequently, the conclusions and discussion below are based mainly on analyses of the combined dataset.

For the combined data, the heuristic search results were checked using PAUP* in conjunction with the PAUPRat program (Sikes & Lewis, 2001), which implements the parsimony ratchet method (Nixon, 1999) for finding maximum parsimony trees in datasets with a large number of taxa. Because the optimal proportion of characters to be reweighted depends on the data and has to be determined empirically (Nixon, 1999; Sikes & Lewis, 2001), eleven consecutive ratchet searches were performed (each with 200 iterations), in which the proportion of reweighted characters was stepped up from 2% to 22% in 2% increments. Finally, the trees found in the separate searches were pooled and filtered in PAUP* to find the shortest trees.

Before performing the Bayesian phylogenetic analyses, the combined dataset and the two genes separately were analysed with Modeltest version 3.06, which uses hierarchical likelihood ratio tests (hLRTs) and the Akaike information criterion (AIC) to identify the simplest substitution model to which the addition of parameters does not result in a significant improvement (Posada & Crandall, 1998). For the combined dataset, both the likelihood ratio tests and the AIC indicated that a GTR + I + Γ model was optimal, but, when the genes were analysed separately, the tests gave conflicting results. However, a GTR + I + Γ model was indicated as optimal by either one of the tests for both CoI (hLRT) and EF-1α (AIC), and so all Bayesian analyses were made using a GTR + I + Γ_4 model of substitution.

Bayesian phylogenetic analyses were performed using MrBayes version 3.0b4 (Ronquist & Huelsenbeck, 2003), which permits the specification of complex substitution models that can be allowed to vary for different partitions of the data. Because CoI and EF-1α evolve with highly divergent rates in insects, and substitution rates vary with codon positions (Kjer *et al.*, 2001; Rokas *et al.*, 2002; Johnson *et al.*, 2003; Lin & Danforth, 2004), we used three different partition schemes: (1) one partition, i.e. a single GTR + I + Γ_4 model for the whole dataset; (2) two partitions, i.e. a separate model for the two genes; and (3) six partitions, i.e. a separate model for each codon position in both genes. Default priors were used in all analyses, and one cold and three incrementally heated ($t = 0.2$) chains were run for six million generations while sampling trees from the current cold chain every 100 generations. The first 10 001 trees were discarded as a burn-in, and the last 50 000 trees were used to calculate a majority-rule Bayesian consensus tree, in which the proportion of times that a clade was observed was an estimate of its posterior probability. Bayesian phylogenetic analyses also were performed separately for the two genes (as one- and three-partition analyses) to identify possible areas of conflict.

To estimate the ages of the various nematine groups, first we used PAUP* to test if the sequences have evolved in a clocklike fashion. The topologies obtained from the maximum parsimony and Bayesian analyses (Fig. 2) were used, and branch lengths were estimated using a GTR + I + Γ_4 maximum likelihood model (separately for CoI and EF-1α, and for the combined dataset), with and without enforcing a molecular clock (while using estimated substitution rates, empirical nucleotide frequencies and an estimated proportion of invariant sites and gamma shape parameter α). In all cases, the null hypothesis of clocklike evolution was rejected by a χ^2 likelihood ratio test (Felsenstein, 1981); therefore, we used instead TreeEdit version 1.0a10 (Rambaut & Charleston, 2002) to ultrametricize the two-partition Bayesian consensus tree (Fig. 3B) by nonparametric rate smoothing (NPRS; Sanderson, 1997). Rates were averaged across the root node formed by the deletion of *Sterictiphora*. Two different fossil calibration points were used to estimate absolute divergence times: the oldest known tenthredinid *Palaeathalia laiyangensis* Zhang from the late Jurassic or early Cretaceous in China (*c.* 145 million years ago; Darling & Sharkey, 1990; Labandeira, 1994), and the oldest known cimbicid *Eopachylosticta byrami* Cockerell from the middle Eocene in North America (*c.* fifty million years ago; Labandeira, 1994; Rasnitsyn, 2002).

Results

Maximum parsimony trees

Maximum parsimony analyses of the combined dataset yield different numbers of maximum parsimony trees depending on how much transversions are weighted over transitions. A 3 : 1 tv : ti ratio results in a single shortest tree (Figs 2A; 3A; TL = 10 981 steps, CI excluding uninformative characters = 0.168, RI = 0.454), a 2 : 1 ratio results in six maximum parsimony trees (TL = 8394, CI = 0.174, RI = 0.444) and a 1 : 1 ratio results in twenty-five maximum parsimony trees (TL = 5796, CI = 0.188, RI = 0.425). The parsimony ratchet searches found no shorter trees.

In general, the maximum parsimony trees are relatively similar regardless of the weighting scheme. The subfamily Nematinae remains monophyletic with all weighting schemes, although bootstrap support for ingroup monophyly is reasonably high only if transversions are given extra weight in the analyses (Fig. 2A). All parsimony

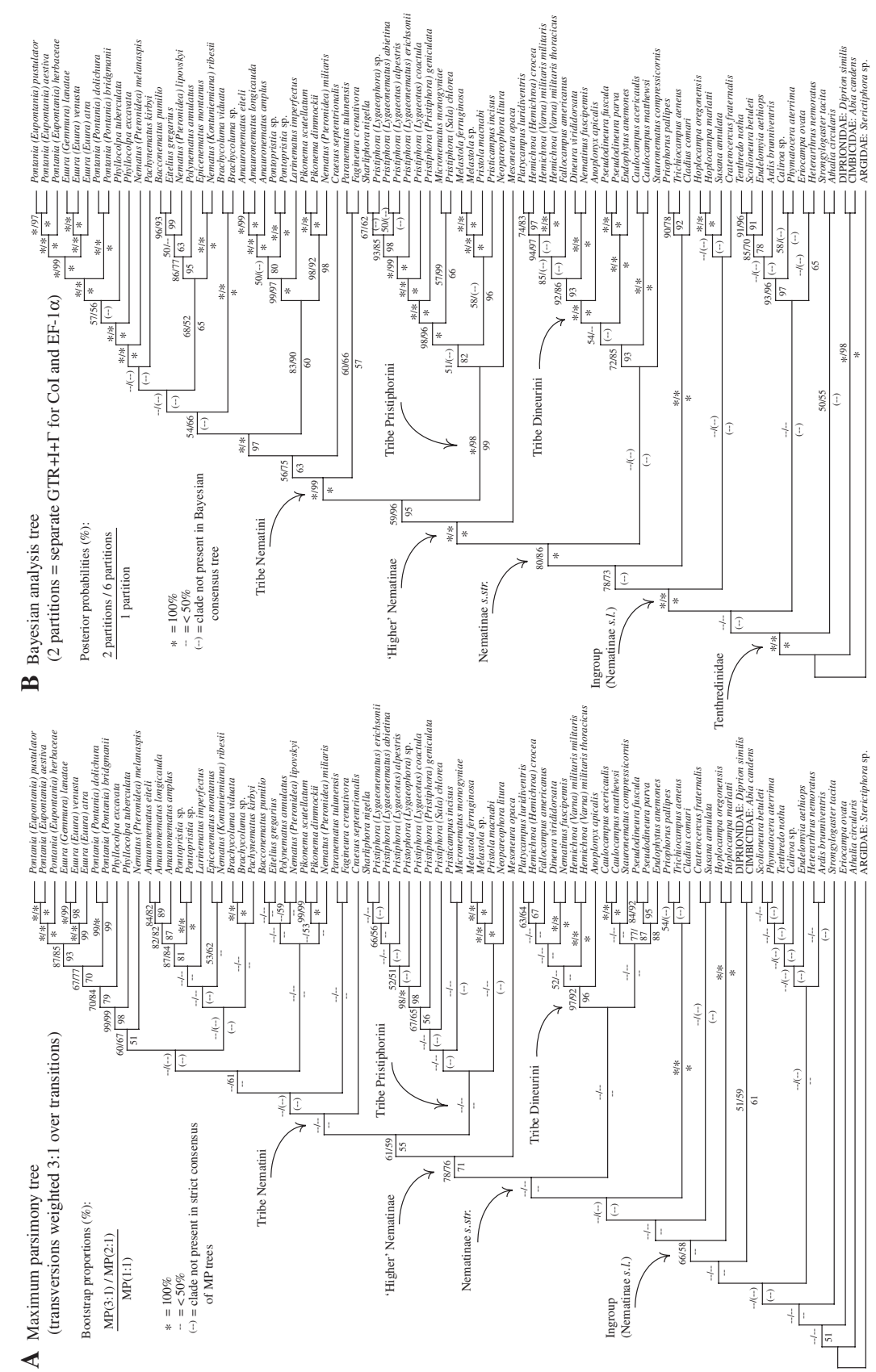

Fig. 2. Phylogeny of the subfamily Nematinae and selected outgroups from the Tenthredinoidea according to: (A) maximum parsimony analysis of the combined cytochrome oxidase I + elongation factor-1α (CoI + EF-1α) dataset with transversions weighted 3 : 1 over transitions; (B) two-partition Bayesian analysis of the combined dataset, which uses a separate GTR + I + Γ_4 model of substitution for the two genes. Numbers above and below the branches are bootstrap proportions in (A) and posterior probabilities in (B). In (A), bootstrap proportions are also shown for analyses in which transversion : transition weights were 2 : 1 and 1 : 1. In (B), posterior probabilities are also shown for six- and one-partition analyses.

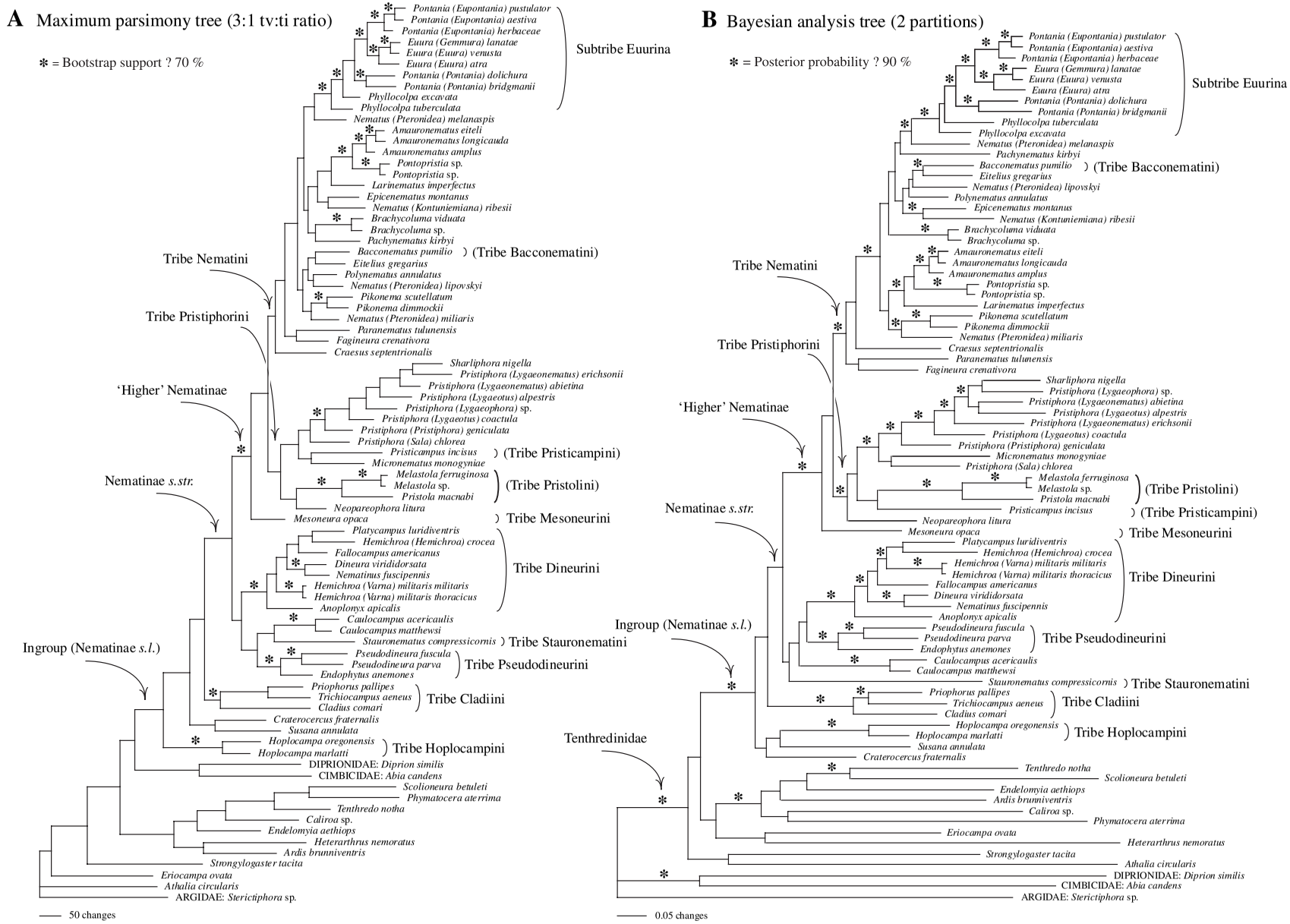

Fig. 3. Maximum parsimony (A) and Bayesian analysis (B) trees from Fig. 2 showing branch lengths. Selected nematine tribes and subtribes are indicated; tribes in parentheses should be lowered to a subtribal rank in order to make the large tribes Nematini and Pristiphorini monophyletic. Asterisks denote clades with ≥70% bootstrap support in (A) and ≥90% posterior probabilities in (B). tv : ti, transversion : transition.

analyses result in a paraphyletic Tenthredinidae, because the Diprionidae + Cimbicidae clade (= *Diprion* + *Abia*) is placed as the sister group of Nematinae, but the bootstrap supports for any groupings within the outgroup are low. Among-group relationships also are poorly resolved within the basal grade consisting of *Hoplocampa*, *Susana*, *Craterocercus* and the tribe Cladiini (= *Cladius* + *Priophorus* + *Trichiocampus*). In the monophyletic Nematinae *sensu stricto*, some larger groups are well supported, for example, the tribe Dineurini, the 'Higher' Nematinae (= *Mesoneura* and the tribes Pristiphorini and Nematini) and the subtribe Euurina (= *Phyllocolpa* + *Pontania* + *Euura*). Other strongly supported clades tend to be small and/or close to the tips of the trees (e.g. the tribes Pseudodineurini and Pristolini, and the large genus *Amauronematus*). The two largest nematine genera, *Nematus* and *Pristiphora*, come out as para- or polyphyletic in all analyses.

Separate analyses of the two genes result in trees that differ quite clearly from each other (results not shown). Clades present in all single-gene trees regardless of tv : ti weights include, in addition to several small clades close to the tips, the tribes Nematini and Pristolini, the subtribe Euurina, and the group formed by *Sharliphora* and all *Pristiphora* species except *P. geniculata* and *P. chlorea*. The ingroup is monophyletic only in the EF-1α trees, whereas in all CoI trees many outgroup taxa nest within the ingroup. Furthermore, *Hoplocampa* and *Caulocampus* group (in the outgroup) in the CoI trees, but *Caulocampus* tends to group with the Pseudodineurini species in the EF-1α trees. However, most differences between the trees are poorly supported, and some of the apparent conflict obviously results from saturation in the faster-evolving CoI gene. In general, EF-1α trees are more similar than CoI trees to the combined-data results.

Bayesian analysis trees

Trees from the Bayesian analyses of the combined data mostly are compatible with the parsimony results and with each other regardless of the partition scheme used (Figs 2B; 3B). Large clades present in both parsimony and Bayesian trees include Nematinae s. str, the 'Higher' Nematinae, and the tribes Dineurini, Pristiphorini and Nematini (Fig. 2). Strongly supported groups in the parsimony trees tend also to be strongly supported in the Bayesian trees, but some clades with weak bootstrap support have high posterior probabilities (e.g. the tribes Nematini and Pristiphorini). This is especially the case in relatively basal relationships, where posteriors are high, for example, for the ingroup and the Tenthredinidae. The posterior probabilities of branches produced by the three partition schemes are strongly correlated, but some clades exist for which the different analyses give contrasting results (e.g. the placement of *Pristicampus incisus*, and some groupings within the Dineurini).

The location of the root was tested by including honeybee (*Apis mellifera* L.) sequences (Crozier & Crozier, 1993; Danforth & Ji, 1998) in combined-data Bayesian analyses. With all partition schemes, *Apis* groups with non-tenthredinid exemplar species: in the one- and six-partition analyses with *Sterictiphora* (posterior probability = 96% in both analyses); in the two-partition analysis with *Abia* (posterior probability = 74%) (results not shown). This indicates that the root is correctly placed in the tree.

Separate one- and three-partition Bayesian analyses of EF-1α and CoI reveal that, in contrast with the parsimony results, the ingroup remains monophyletic in analyses based on EF-1α, but also in analyses based on CoI sequences only (results not shown). Other clades present in all separate analyses include the tribes Cladiini, Dineurini and Pristolini, the subtribe Euurina, the group formed by *Sharliphora* and all *Pristiphora* species except *P. chlorea*, and the large group consisting of all taxa in the tribe Nematini except *Craesus*, *Paranematus* and *Fagineura*. The 'Higher' Nematinae clade is present in all trees except the three-partition EF-1α tree. Strongly supported conflict between the two genes is evident, for example, within the aforementioned *Sharliphora* + *Pristiphora* clade, and within the Dineurini. Again, one main disagreement concerns the placement of *Caulocampus*: the CoI analyses group *Caulocampus* and *Hoplocampa* with a high (>94%) posterior probability, whereas the EF-1α analyses place *Caulocampus* as the sister of, or inside, the Pseudodineurini (but with low support).

The Bayesian tree with NPRS-corrected branch lengths provides estimates of the ages of various branching events, but the two different fossil calibration points result in over twofold differences in the inferred divergence times (Fig. 4). However, both estimates indicate that the Nematinae originated between 50 and 120 million years ago.

Discussion

Phylogenetic trees and correspondence with traditional classifications

The trees produced by the different phylogenetic analyses of the combined sequence data differ, but the general estimates are similar (Figs 2, 3). Both parsimony and Bayesian analyses support the monophyly of the Nematinae in a broad sense (*sensu lato*), and produce an apparently paraphyletic basal grade consisting of *Hoplocampa*, *Craterocercus*, *Susana* and the tribe Cladiini. The rest of the ingroup, Nematinae *sensu stricto*, is monophyletic in all results. Likewise, all analyses divide the 'Higher' Nematinae into one small (*Mesoneura*) and two large (Nematini and Pristiphorini) groups, and many smaller clades are present also in all phylogeny estimates.

As expected, combined-data analyses produced more strongly supported results, which were more congruent with morphological classifications (Ross, 1937; Benson, 1958; Smith, 1979; Taeger & Blank, 1998) and previous phylogenetic results (Schulmeister *et al.*, 2002; Schulmeister, 2003), than did separate analyses of each gene. This supports the view that combining data in spite

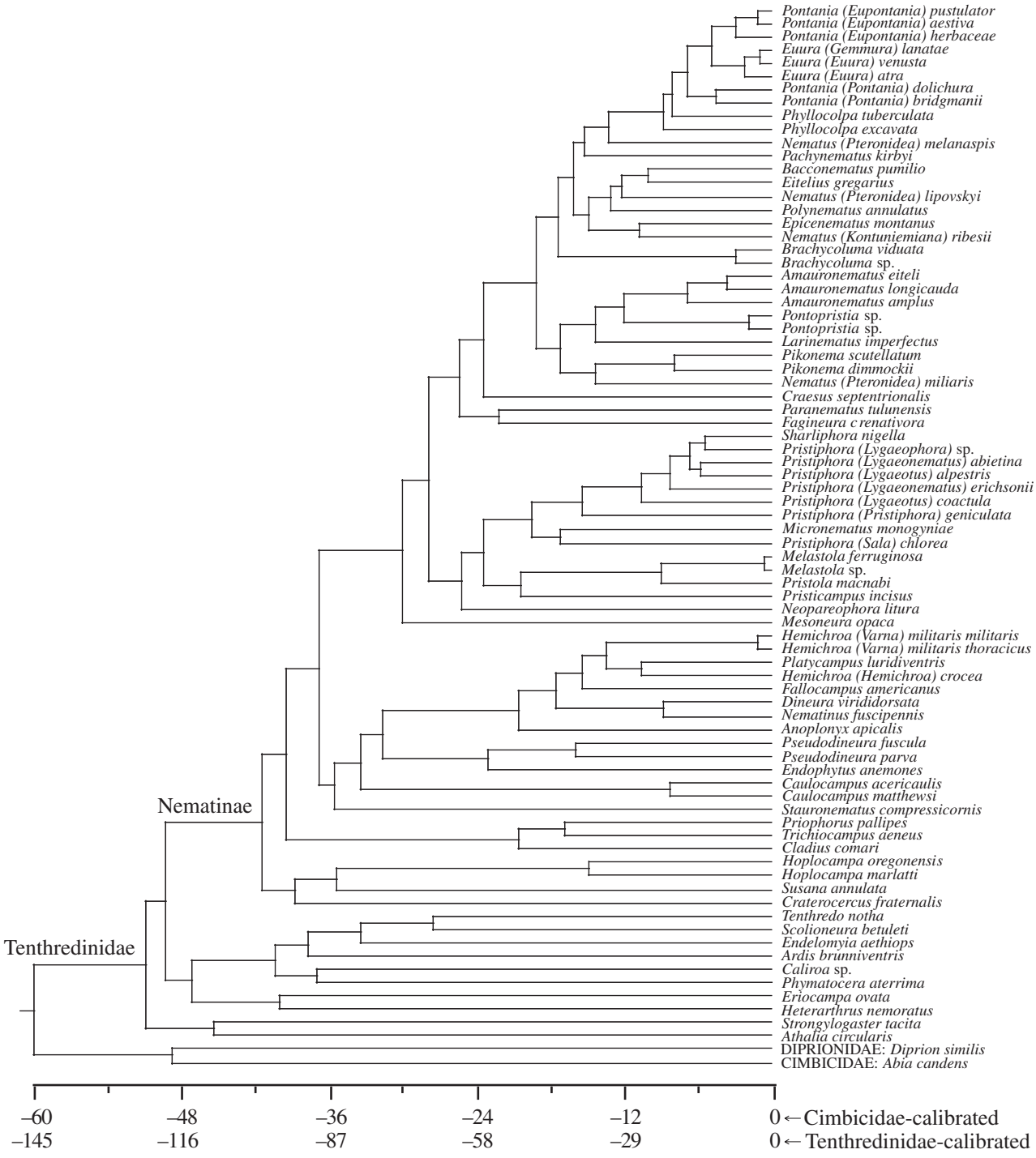

Fig. 4. Phylogeny of the Nematinae, with branch lengths ultrametricized using nonparametric rate smoothing (Sanderson, 1997; original branch lengths from the two-partition Bayesian analysis tree, Fig. 3B). The time scale has been calibrated with two different fossil calibration points (see 'Materials and methods').

of statistically significant incongruence can improve the accuracy of phylogenetic inference, particularly in datasets in which evolutionary rates differ between partitions (e.g. Yoder *et al.*, 2001; Barker & Lutzoni, 2002; Darlu & Lecointre, 2002; Cryan *et al.*, 2004). Interestingly, the clearly 'overparameterized' six-partition analysis (see

Nylander *et al.*, 2004) resulted in trees very similar to those from the one- and two-partition Bayesian analyses. This suggests that overparameterization may not, by itself, be a serious problem as long as at least some partitions within the dataset contain enough variation for parameter estimation, and especially if the partitioning allows more accurate parameter estimation in those 'informative' partitions.

The main disagreements between the parsimony and Bayesian results concern deep divergences within the outgroup, but the discordance appears to be due to a lack of resolution in the parsimony trees rather than true conflict (Fig. 2). Bayesian phylogenetic analyses are based on the likelihood function and an explicit model of nucleotide substitution, and thus are able to correct better for unequal base frequencies, rate heterogeneity and saturation (Huelsenbeck *et al.*, 2002; Ronquist & Huelsenbeck, 2003). By contrast to bootstrap values, posterior probabilities have a tendency to overestimate clade support (Huelsenbeck *et al.*, 2002; Suzuki *et al.*, 2002), but the 100% posterior probability of the ingroup branch with all partition schemes provides convincing evidence in favour of the monophyly of Nematinae s.l. All Bayesian analyses also show strong and consistent support for a monophyletic Tenthredinidae, which has been difficult to demonstrate in previous parsimony analyses of morphological and molecular data (Vilhelmsen, 2001; Schulmeister *et al.*, 2002; Schulmeister, 2003).

Our results are remarkably similar to Ross's (1937) morphology-based tree (Fig. 1), which was drawn with an approach that seems to have been philosophically parsimonious (Ross, 1937: 60). Although the monophyly of Nematinae s.l. is supported by morphological traits, the subfamily is defined by character state combinations rather than unambiguous and unreversed synapomorphies: most members of the Nematinae share a characteristic, comparably reduced wing venation, have penis valves that are divided into a lateral and a median flap, and have larvae that lack abdominal prolegs on their eighth segment (Marlatt, 1896; Yuasa, 1922; Ross, 1937; Maxwell, 1955; Lorenz & Kraus, 1957; Zinovjev, 1982). The most conspicuous morphological synapomorphy for the subfamily is the presence of eversible ventral glands between the abdominal prolegs of the larvae (Maxwell, 1955; Lorenz & Kraus, 1957; Zinovjev, 1982). The glands have a defensive function in some species (Smith, 1970; Boevé & Pasteels, 1985; Boevé *et al.*, 1997), but apparently they have been lost several times, especially in conifer-feeding groups such as *Susana* and *Pikonema* (Maxwell, 1955).

The interrelationships within the basal grade, which consists of *Hoplocampa*, *Craterocercus*, *Susana* and the tribe Cladiini, are weakly resolved in all analyses. It should be noted that, with the exception of *Craterocercus*, all these taxa have been excluded from the Nematinae at one time or another (e.g. Yuasa, 1922; Ross, 1937, 1951; Maxwell, 1955; Lacourt, 1998, 1999). In particular, *Susana* often has been classified as a separate subfamily, the Susaninae (Ross, 1951). The genus is enigmatic because, in many larval characteristics, it seems to be intermediate between nematines, other tenthredinids and diprionids. For example, the larvae have abdominal prolegs on segment 8, have diprionidlike oesophageal pouches and lack abdominal ventral glands (Maxwell, 1955; Smith, 1969). However, these characters are poorly known in many nematine taxa, ventral glands have been lost or reduced also in other nematine groups (Maxwell, 1955; Boevé & Pasteels, 1985) and small prolegs on segment 8 are present in at least five other nematine genera (Lorenz & Kraus, 1957; Zhelochovtsev, 1988, 1994; Zinovjev, 1992). Furthermore, the 'primitiveness' of *Susana* is relative because, in wing venation characteristics, *Hoplocampa* is arguably closer to other tenthredinids: the basal anal cell in *Hoplocampa* forewings has a central constriction, perhaps a remnant of the constriction found in the larger anal cell of most outgroup taxa (Ross, 1937).

The 'Higher' Nematinae is strongly supported by both parsimony and Bayesian analyses. The group corresponds quite well with the definition of Ross (1937), which included species that have the most reduced wing venation and have left mandibles with an apical bladelike portion. According to our results, however, the 'Higher' Nematinae should include some genera that Ross (1937) left out or did not know (e.g. *Mesoneura*, *Neopareophora*, *Pristicampus*). The monotypic genus *Stauronematus* is placed in many locations in the analyses (Figs 2, 3), but the species has also proven difficult to classify on the basis of morphology (Vikberg, 1982; Lacourt, 1998); as it possesses the bladelike mandible and has no basal anal cell in the forewings, it seems likely that its correct origin is at the base of the 'Higher' Nematinae, where it was placed in the one- and six-partition Bayesian analyses.

Within the 'Higher' Nematinae there are two major groups, the tribes Pristiphorini and Nematini, both of which correspond quite well with traditional classifications. For example, the former corresponds closely to the definitions of the (sub)tribe Pristiphorini *sensu* Vikberg (1982) and Lacourt (1998). However, as currently defined, both of these tribes clearly are paraphyletic (Fig. 3), and so it would be appropriate to lower the small tribes Bacconematini, Pristolini and Pristicampini to a subtribal rank. *Pristiphora* itself is paraphyletic because *Micronematus* and *Sharliphora* are grouped inside the genus, which is not surprising because both genera originally were included in *Pristiphora* (Benson, 1958; Wong, 1969). Intergeneric relationships are particularly complex within the Nematini, but it is evident that the traditional genus *Nematus* and its largest subgenus *Pteronidea* are paraphyletic groups defined by symplesiomorphies. Both CoI and EF-1α results, as well as the combined analyses, confirm the monophyly of the gall-inducing subtribe Euurina (Vikberg, 1982; Nyman *et al.*, 1998, 2000) which has been questioned recently (Zinovjev & Vikberg, 1999; see also Roininen *et al.*, 2005).

Conflicts between our phylogenetic results and traditional classifications are most evident in cases in which classifications have apparently been affected by similarities in host plant use. The most obvious example is the inclusion of

Mesoneura and *Craterocercus* in the polyphyletic tribe Mesoneurini: both taxa feed on *Quercus* and are superficially similar, robust sawflies, but, at the same time, they exhibit distinct differences in traits that are usually used for the classification of sawflies (wing venation, structure of mandibles, saw and sawsheath). *Mesoneura* species share many morphological characteristics with the 'Higher' Nematinae (e.g. no basal loop in the anal cell of forewings, left mandible with thin, bladelike apical portion), whereas *Craterocercus* species possess many traits that are plesiomorphic within Nematinae (e.g. basal loop in the anal cell of forewings, gradually tapering mandibles). Another example is *Pikonema sensu* Zhelochovtsev (1988, 1994), which is a compilation of morphologically diverse species from at least three separate conifer-feeding groups (*Pikonema sensu stricto*, *Epicenematus* and probably *Pristiphora*).

Our phylogenetic results do not solve the problem of the sister group of the Nematinae, but apparently the subfamily was a relatively early offshoot from the Tenthredinidae, as indicated by the Bayesian phylogenetic analyses (Figs 3B, 4; see also Ross, 1937). The two fossil calibration points result in over twofold differences in the inferred age of Nematinae (Fig. 4), but the Tenthredinidae-calibrated estimate of approximately 120 million years is likely to be more correct, as the discrepancy may result from an underestimation of the true age of Cimbicidae due to missing fossil evidence. However, even the higher age estimate indicates that the appearance of Nematinae happened well after the ancient supercontinent Pangaea started to split into a northern (Laurasia) and southern (Gondwana) half approximately 160 million years ago (Condie, 1997). Thus, the present reversed latitudinal gradient of species richness in the subfamily (Kouki *et al*., 1994; Kouki, 1999) may reflect a long history of temperate origins and diversification, followed by recent invasion of tropical areas, as suggested by Malaise (1942). Many fossil sawflies from Oligocene deposits (approximately 23–35 million years ago) in North America have been assigned to modern nematine genera (Cockerell, 1922; Carpenter, 1992), but the identifications are based mainly on wing venation characteristics and thus are uncertain (e.g. Cockerell, 1914). Nevertheless, the presence of several New World endemic taxa (*Craterocercus*, *Susana* and *Caulocampus*) close to the base of the ingroup suggests a North American origin for the subfamily.

Conclusions

Our phylogenetic analyses based on mitochondrial CoI and nuclear EF-1α gene sequences strongly support the monophyly of Nematinae in a broad sense, which includes *Susana*, *Hoplocampa* and the tribe Cladiini. In addition, the phylogenetic trees provide a relatively well-resolved overall view of relationships between tribes and genera within the subfamily. Although the results correspond generally to previous morphology-based inferences and classifications, some striking conflicts are also evident; in particular, some of the large tribes and genera are clearly para- or polyphyletic. The creation of a phylogenetically orthodox classification system for the Nematinae will be difficult, and such a classification could hide some relevant morphological and biological differences between nematine taxa.

The elucidation of among-group relationships close to the root of the Nematinae phylogeny will require further work, for example, by adding molecular and morphological data and by increasing species sampling in taxa belonging to the basal grade. Furthermore, analyses focusing on the diverse but relatively young tribes Nematini and Pristiphorini would help to clarify nematine systematics and could uncover factors that have contributed to the apparently rapid radiation of the subfamily in the Northern Hemisphere. Hopefully the results presented here can provide a sound basis for such future detailed analyses of various nematine subgroups.

Supplementary material

The data matrix is available at: http://www.blackwell-synergy.com under the DOI reference doi: 10.1111/j.1365-3113.2006.00336.x

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