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Molecular phylogeny and evolution of host-plant use in conifer seed chalcids in the genus *Megastigmus* (Hymenoptera: Torymidae)

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Abstract. Phylogenetic relationships amongst *Megastigmus* species (Chalcidoidea: Torymidae) associated with conifer seeds were inferred from DNA sequence data. Twenty-nine species of seed chalcids were analysed using two different genes, cytochrome *b* (mitochondrial DNA) and the D2 domain of the 28S ribosomal DNA. Maximum-parsimony and maximum-likelihood analyses showed that taxa formed two monophyletic groups, one clade comprising all species associated with Cupressaceae and Taxodiaceae hosts with the exception of *Chamaecyparis*, and the other clade composed of species associated with Pinaceae. Species infesting Cupressaceae and Taxodiaceae seemed to be specialized to particular host genera or even to be species specific, which was consistent with a taxonomic radiation following initial host adaptation. By contrast, *Megastigmus* species associated with Pinaceae appeared capable of shifting onto different congeneric species or even onto a new host genus, with their evolution apparently less constrained by plant association. We hypothesized that the *Megastigmus* group associated with Pinaceae may have a much higher invasive potential than that related to Cupressaceae. The study also confirmed the presence of invasive Nearctic species in the Palaearctic, and demonstrated the existence of a cryptic species complex.

Introduction

Most insects infesting cones and seeds of conifers are specialists incapable of developing on other substrates (Turgeon *et al.*, 1994). An important group is the seed chalcid wasp genus *Megastigmus* Dalman (Hymenoptera: Chalcidoidea: Torymidae: Megastigminae). More than 125 species of *Megastigmus* have been described, of which fifty-nine are seed feeders (Grissell, 1999). Other species, most of which occur in Australia, are presumed to be parasitoids, gall-makers or have unknown hosts. Within the obligate seed-feeding group, forty-one species are associated with conifers (Pinaceae, Cupressaceae and Taxodiaceae), some being considered as economic pests, whereas the others develop in seeds of angiosperms, especially Rosaceae and Anacardiaceae (Roques & Skrzypczynska, 2003). The species associated with conifers are considered to be highly specialized, being either species specific (even if several potential host species coexist in the same place) or restricted to a conifer genus. However, the degree of host specialization remains unclear for some species. For most phytophagous insects, long-term association with a particular host eventually results in the loss of genetic variation for the ability to use alternative hosts. Specialists thus might become constrained irreversibly on a restricted set of host plant species

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considered to be chemically similar (Futuyma & Moreno, 1988; Becerra, 1997; Kelley & Farrell, 1998). Nevertheless, recent studies have suggested that not only secondary plant chemistry, but also a set of other parameters (biogeographical, genetic and ecological constraints), might explain host shifts better than plant phylogeny and plant geographical distribution (Termonia *et al*., 2001). A few documented cases on *Megastigmus* species have suggested that shifts to new hosts of different genera may occur (Grissell, 1999; M.-A. Auger-Rozenberg, pers. obs.). However, wasp misidentifications and the possible occurrence of cryptic species may have confounded these observations.

Megastigmus has a nearly worldwide distribution, but conifer seed-feeding species seem to be restricted to the Holarctic region. Most species have been described from the West Palaearctic region and from North America, but the discovery of an increasing number of species from China is likely (Roques *et al*., 1995; A. Roques, pers. obs.) and cryptic species probably exist in Central Asia (Grissell, 1999). Moreover, these species exhibit a large invasive potential facilitated by the globalization of seed trade. Some of their life cycle features tend to facilitate insect introduction (e.g. entire development concealed within the same seed) and establishment in exotic countries (e.g. parthenogenesis and prolonged diapause, allowing them to cope with the heterogeneity in space and time of host abundance; for reviews, see Turgeon *et al*., 1994; Roques *et al*., 2003). Here again, as many morphological characters can be misleading in chalcidoid taxonomy, the possibility remains that an invasive *Megastigmus* species actually is a cryptic native species that would have been misidentified.

Despite their taxonomic, morphological and ecological diversity, our knowledge of the phylogenetic relationships within Megastigminae remains limited, and the patterns of host-associated radiations have been largely unexplored. The evolutionary relationships amongst *Megastigmus* species have been questioned previously with allozyme markers (Roux & Roques, 1996), but using a limited data set and with low phylogenetic resolution. A reconstructed evolutionary history would provide a crucial framework for understanding the origins and evolution of the highly specialized association between conifers and seed chalcids. Specifically, it could test if *Megastigmus* species occurring on the same host genus or family share a recent common ancestor; moreover, it could estimate the degree of biological constraint due to host use and, furthermore, help test hypotheses for the geographical distribution and radiation of the genus *Megastigmus* worldwide. Moreover, molecular data would assist in investigating cryptic species, both in the case of apparent host shifts and supposed invasive species.

Here, we present the first phylogenetic reconstruction of *Megastigmus* associated with conifers, based on DNA sequencing of twenty-nine species, twenty-six of which are conifer seed feeders. Partial sequences of the cytochrome *b* gene (mitochondrial DNA, mtDNA) and of the D2 region of the 28S ribosomal subunit (rDNA) were used to reconstruct the phylogenetic history of host plant use and associated genetic variation. Cytochrome *b* (cyt. *b*) is a mitochondrial protein-coding gene used in molecular systematics (Gimeno *et al*., 1997; Simmons & Weller, 2001). The few studies that have used cyt. *b* to resolve systematic relationships within Chalcidoidea indicate that it is appropriate for the resolution of intrageneric and intraspecific relationships (Kerdelhué *et al*., 1999; Lopez-Vaamonde *et al*., 2001). The nuclear 28S rDNA transcript evolves more slowly than cyt. *b* and may be appropriate in Hymenoptera for divergences ranging from closely related species to family level divergences (Belshaw *et al*., 1998; Rasplus *et al*., 1998; Gibson *et al*., 1999; Mardulyn & Whitfield, 1999; Campbell *et al*., 2000; Babcock *et al*., 2001; Lopez-Vaamonde *et al*., 2001; Rokas *et al*., 2002).

Materials and methods

Insect collection

From 1994 to 2004, an extensive survey of seed chalcids was undertaken on different native and exotic species of conifers across the Northern Hemisphere (Table 1). The sampled seed lots were all radiographed using a Faxitron-43855® apparatus (15 kV, 3 mA, 3′30′ to 4′30′ depending on seed species) and X-ray-sensitive films (Kodak® 'Industrex M'). The insect-infested seeds were placed in individual rearing boxes stored in an outdoor insectary located at INRA, Orléans, France [107 m above sea-level (a.s.l.)]. Adult emergence was recorded over the 3 years following seed maturation because of a possible prolonged diapause (Roques & Skrzypczynska, 2003). After emergence and identification, wasps were preserved in 100% alcohol at −20 °C. For one species (*M. strobilobius* Ratzeburg), we failed to collect infested seeds or emerging adults; consequently, dry museum specimens were used. All individuals (both native and introduced) sampled from the Western Palaearctic region were identified following Roques & Skrzypczynska (2003). The Chinese species (*M. cryptomeriae* Yano, *M. likiangensis* Roques & Sun and *M. pingii* Roques & Sun) were identified by A. Roques, and Nearctic species (*M. hoffmeyeri* Walley, *M. thyoides* Kamijo, *M. tsugae* Crosby) by J. Turgeon. The descriptions of two new species are included in this paper (Appendix: *M. thuriferana* Roques and El Alaoui on *Juniperus thurifera* and *M. formosana* Roques and Pan on *J. formosana*). In addition to the *Megastigmus* species reared from conifers, we also included a seed feeder from Rosaceae (*M. rosae* Bouček) and two European endoparasitoids of cynipid gall wasps (*M. dorsalis* Fabricius and *M. stigmatizans* Fabricius). Three species of *Torymus* (Torymidae: Toryminae) were used as outgroup (Grissell, 1999). The species used in the study, their collecting locality and distribution are summarized in Table 1. Voucher material of specimen remnants and associated complete specimens from the same original series are kept in ethanol in the collection of the National Institute of Agronomical Research, Orléans, France.

Table 1. Collection data for the specimens of *Megastigmus* used in this study.

Species name	Host plant	Collection site	Natural range	Code
M. amicorum Bouček	*Juniperus phoenicea*	Ericeira, Portugal	Palaearctic	Mami.POR
M. amicorum Bouček	*Juniperus phoenicea*	Luberon Mt, France	Palaearctic	Mami.FRA
M. atedius Walker	*Picea* sp.	Vernon, Canada	Nearctic	Mate.CAN
M. atedius Walker	*Picea sitchensis*	Roldskov, Denmark	Nearctic	Mate.DEN
M. atlanticus Roques & Skrzypczynska	*Cupressus atlantica*	Idni, Morocco	Palaearctic	Matl.MAR
M. bipunctatus Swederus	*Juniperus communis*	Briançon, France	Palaearctic	Mbip.FRA
M. borriesi Crosby	*Abies koreana*	Skovhaven, Denmark	Palaearctic	Mbor.DEN
M. cryptomeriae Yano	*Cryptomeria japonica*	Honshu, Japan	East-Asia	Mcry.JPN
M. cryptomeriae Yano	*Cryptomeria fortunei*	Zheijang, China	East-Asia	Mcry.CHI
M. dorsalis Fabricius	*Quercus faginea*	Buçaco, Portugal	Palaearctic	Mdor.POR
M. formosana Roques and Pan	*Juniperus formosana*	Dongchuan, China	East-Asia	Mfor.CHI
M. hoffmeyeri Walley	*Tsuga canadensis*	Ontario, Canada	Nearctic	Mhof.CAN
M. lasiocarpae Crosby	*Abies amabilis*	Boston Bar Creek, Canada	Nearctic	Mlas.CAN
M. likiangensis Roques & Sun	*Picea likiangensis*	Lijiang, China	East-Asia	Mlik.CHI
M. milleri Milliron	*Abies grandis*	Gesves, Belgium	Nearctic	Mmil.BEL
M. pictus Förster	*Larix decidua*	Krynica, Poland	Palaearctic	Mpic.POL
M. pingii Roques & Sun	*Juniperus pingii*	Zhongdian, China	East-Asia	Mping.CHI
M. pinsapinis Hoffmeyer	*Cedrus atlantica*	Tala-guilef Djurdura, Algeria	Palaearctic	Mpins.ALG
M. pinsapinis Hoffmeyer	*Cedrus atlantica*	Veraza, France	Palaearctic	Mpins.FRA
M. pinus Parfitt	*Abies magnifica*	Placerville, U.S.A.	Nearctic	Mpin.USA
M. pinus Parfitt	*Abies procera*	Skovhaven, Denmark	Nearctic	Mpin.DEN
M. pinus Parfitt	*Abies grandis*	Vernon, Canada	Nearctic	Mpin.CAN
M. pinus Parfitt	*Abies alba*	Mt Ventoux, France	Nearctic	Mpin.FRA
M. rafni Hoffmeyer	*Abies nordmanniana*	Nogent/Vernisson, France	Nearctic	Mraf.FRA
M. rafni Hoffmeyer	*Abies magnifica*	Placerville, U.S.A.	Nearctic	Mraf.USA
M. rosae Bouèek	*Rosa tomentosa*	Briançon, France	Palaearctic	Mros.FRA
M. schimitscheki Novitzky	*Cedrus libani*	Kapidag, Turkey	Palaearctic	Msch.TUR
M. schimitscheki Novitzky	*Cedrus atlantica*	Mt Ventoux, France	Palaearctic	Msch.FRA
M. specularis Walley	*Abies fraseri*	North Carolina, U.S.A.	Nearctic	Mspec.USA
M. specularis Walley	*Abies sibirica*	Krasnoyarsk, Russia	Nearctic	Mspec.RUS
M. spermotrophus Wachtl	*Pseudotsuga menziesii*	Cowichan Lake, Canada	Nearctic	Msper.CAN
M. spermotrophus Wachtl	*Pseudotsuga menziesii*	Rangiora, New Zealand	Nearctic	Msper.NZL
M. spermotrophus nigrodorsatus Milliron	*Pseudotsuga macrocarpa*	Los Angeles Nat. For., U.S.A.	Nearctic	MspeN.USA
M. stigmatizans Fabricius	*Quercus faginea*	Buçaco, Portugal	Palaearctic	Msti.POR
M. strobilobius Ratzeburg	*Picea* sp.	Riimaru, Estonia	Palaearctic	Mstr.EST
M. suspectus Borries	*Abies alba*	Piwniczna, Poland	Palaearctic	Msus.POL
M. suspectus Borries	*Abies pinsapo*	Sierra del pinar, Spain	Palaearctic	Msus.ESP
M. thyoides Kamijo	*Chamaecyparis thyoides*	North Carolina, U.S.A.	Nearctic	Mthy.USA
M. thuriferana Roques & El Alaoui	*Juniperus thurifera*	Tizrag, Morocco	Palaearctic	Mthu.MAR
M. thuriferana Roques & El Alaoui	*Juniperus thurifera*	Rié, France	Palaearctic	Mthu.FRA
M. tsugae Crosby	*Tsuga heterophylla*	Mt Newton, Canada	Nearctic	Mtsu.CAN
M. wachtli Seitner	*Cupressus sempervirens*	Aghios Ioannis, Crete	Palaearctic	Mwa.GRE
Torymus azureus Boheman	*Picea abies*	Suchora, Poland	Palaearctic	TorP.POL
Torymus sp.	*Sorbus* sp.	Peyrau, France	Palaearctic	TorS.FRA
Torymus sp.	*Juniperus sabina*	Pallon, France	Palaearctic	TorJ.FRA

DNA isolation, polymerase chain reaction (PCR) amplification and sequencing

DNA was extracted from the entire body of adult females. Total DNA was isolated and purified following procedures from the DNeasy Tissue Kit (Qiagen) and eluted in 200 μL of elution buffer. We used the Promega Taq package or the Sigma RedTaq for PCR amplifications. The forward and reverse primers used were: CP1 (5′-GAT GAT GAA ATT TTG GAT C-3′; Harry *et al.*, 1998) and CB2 (5′-ATT ACA CCT CCT AAT TTA TTA GGA AT-3′; Jermiin & Crozier, 1994) for the cyt. *b* gene and D1F (5′-ACC CGC TGA ATT TAA GCA TAT-3′; Harry *et al.*, 1996) and D3R (5′-TAG TTC ACC ATC TTT CGG GTC-3′; Lopez-Vaamonde *et al.*, 2001) for the 28S gene. For some species, we used CP2 (5′-CTA ATG CAA TAA CTC CTC C-3′; Harry *et al.*, 1998) instead of CB2 because of amplification failures. To amplify the species for which

we only had dry specimens (*M. strobilobius*), we used CB1 (5′-TAT GTA CTA CCA TGA GGA CAA ATA TC-3′; Jermiin & Crozier, 1994) as an internal forward primer, in conjunction with CB2 or CP2, and we designed a new internal reverse primer, called MCBR (5′-CGA TTT AAA GTT GCA TTA TC-3′), that was used in conjunction with CP1.

The cycling programme was the same for both fragments: denaturation step at 94 °C for 1 min, annealing for 1 min at 48 °C for cyt. *b* and at 57 °C for 28S, and extension at 72 °C for 1 min, with 30–35 cycles being performed. All PCR products were then purified with the QIAquick PCR Purification Kit (Qiagen) or Genelute PCR Clean-Up Kit (Sigma). Purified PCR products were directly sequenced with the amplification primers. Sequencing was performed using the Big-Dye Terminator Sequencing Kit (PE Applied Biosystems) and carried out with an ABI 3100 automatic sequencer.

The two gene fragments were sequenced in two to four individuals for each species, except for cryptic and rare species because of the small number of specimens available. When individuals were identified as originating from a different biogeographical region (i.e. when invasive specimens were found), additional specimens from native areas were sequenced in order to allow genetic comparison between native and introduced individuals.

Each unique haplotype sequence is available from GenBank (Accession Nos. AY898662 to AY898706 and AY900452 to AY900492).

Sequence alignment and phylogenetic analyses

Sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) as implemented in BIOEDIT. The cyt. *b* contained no indels and was aligned unambiguously. For 28S sequences, final alignment was obtained manually and gaps were treated as a fifth character. For both genes, we performed a chi-squared test of homogeneity of base frequencies across taxa in PAUP 4*b10 (Swofford, 2003).

As a first step, each gene (cyt. *b* and 28S) was treated separately. Sequence data were analysed using MODELTEST 3.06 (Posada & Crandall, 1998) to ascertain the substitution model that best described our data. This program allows comparison of different models of DNA substitution to be tested in a hierarchical hypothesis testing framework. The models were determined via likelihood ratio tests. Statistics for nucleotide variation and complete genetic distances were computed with MEGA 2.0 (Kumar *et al*., 2001) and PAUP 4*b10 based on the model of evolution selected by MODELTEST. Phylogenetic trees were reconstructed for each gene independently with PAUP 4*b10 using both the maximum-parsimony (MP) and maximum-likelihood (ML) methods. For ML trees, we used the model of evolution selected by MODELTEST; for MP analyses, heuristic searches were conducted with fifty random addition replicates using tree bisection-reconnection (TBR) branch-swapping options. Evaluation of statistical confidence in nodes was based on 500 and 200 bootstrap replicates in MP and ML analyses, respectively.

To test whether the phylogenetic signal between the nuclear and mitochondrial genes was in significant conflict, we performed a partition homogeneity test as implemented in PAUP 4*b10 with 1000 replicates (Farris *et al*., 1995). The combined data set was then subjected to MP analyses, with the same options as described above.

Results

Nucleotide alignments

We obtained sequences 716 base pair (bp) long for cyt. *b* from twenty-nine species of *Megastigmus* and the three outgroup taxa (*Torymus* sp.), and sequences 975–999 bp long for 28S from twenty-five species of *Megastigmus* and the three outgroups. We were unable to amplify 28S from *M. atlanticus*, *M. formosana*, *M. hoffmeyeri* and *M. strobilobius*.

cyt. b sequences. A total of 327 variable sites was detected; of the 276 phylogenetically informative sites, 25.3% were at first, 9.5% were at second and 65.2% were at third codon positions. Aligned cyt. *b* sequences appeared to be of mitochondrial origin, rather than nuclear copies, as we found no evidence for pseudogenes (Bensasson *et al*., 2001). Sequences were of the correct length and reading frame, contained no stop codons, overlapping fragments contained no conflicts, and base composition was homogeneous across taxa as revealed by the chi-squared test of base composition homogeneity ($P > 0.05$). As usual in mtDNA (Jermiin & Crozier, 1994; Simmons & Weller, 2001), a high A + T content (77.4%) was observed in the cyt. *b* fragment. Comparison of nucleotide compositions amongst positions of codons showed the highest average A + T content in the third position, at which 94.2% of all nucleotides were either A or T. The first and second positions showed relatively lower A + T contents (70.3% and 67.8%, respectively).

28S sequences. Of the 999 aligned bases, 191 were variable and, of these, 130 were informative. Aligned 28S sequences contained several inferred insertions or deletions (indels), the largest being a 20 bp deletion in an outgroup taxon as compared to the ingroup taxa. The placement of indels was, in general, unambiguous and easily identifiable because indels were sufficiently infrequent that they generally did not overlap. Proportions of each base were more homogeneous than in cyt. *b* (T: 21.4%; C: 26.6%; A: 20.5%; G: 31.5%), as noted in other Hymenoptera (e.g. Lopez-Vaamonde *et al*., 2001). Moreover, base composition was homogeneous across taxa (chi-squared test of homogeneity of base frequencies not significant).

Genetic distances

cyt. b sequences. The model of molecular evolution selected by MODELTEST to fit the mtDNA sequence data

was the general time reversible (GTR) model (base frequencies: freqA = 0.3519; freqC = 0.0913; freqG = 0.1040; and freqT = 0.4527) including a proportion of invariable sites (I = 0.425) and gamma-distributed rate variation amongst sites (G = 0.965). Sequence divergences were calculated hereafter using this model of evolution. Sequence divergence between entomophagous species and seminiphagous species ranged from 22.5% to 35.7%. Within species related to conifers, sequence divergence ranged from 0.01% to 21.4%. After grouping of *Megastigmus* species depending on conifer family attacked (Pinaceae/Cupressaceae/Taxodiaceae), we calculated within- and between-group mean distances (Table 2). Lower values were observed within groups, with similar values within Cupressaceae and within Pinaceae. Within conifer-associated *Megastigmus*, similar values were obtained between groups. The values were also homogeneous between the three conifer groups. Most intraspecific pairwise sequence divergence ranged from 0% to 1.5% for the wasps collected in their native range as compared with specimens from the introduced range (*M. pinus* Parfitt from California and Denmark; *M. pinus* from Canada and France; *M. rafni* Hoffmeyer from the U.S.A. and France; *M. specularis* Walley from the U.S.A. and Russia; *M. atedius* Walker from Canada and Denmark; *M. spermotrophus* Wachtl from Canada and New Zealand; *M. pinsapinis* Hoffmeyer from Algeria and France; *M. schimitscheki* Novitsky from Turkey and France). The two specimens identified as *M. cryptomeriae* collected on *Cryptomeria japonica* in Japan and *C. fortunei* in China diverged by 0.4%.

28S sequences. For the 28S sequence data, the best model of DNA substitution selected by MODELTEST was the Tamura-Nei model (1993) with among-site rate heterogeneity (TrN + G). This was used to calculate the distance matrix ($\alpha = 0.2883$; base frequencies: freqA = 0.2199; freqC = 0.2508; freqG = 0.2967; and freqT = 0.2325). The distances were much lower than for the mtDNA data set. The mean distances between groups are presented in Table 3. Sequence divergence between ingroup and outgroup species ranged from 7.8% to 8.3%. Distances between any *Megastigmus* associated with conifer ranged from 0% to 2.2%, whereas within-host family distances never exceeded 0.6%.

Phylogenetic analysis

cyt. b sequences. MP analysis of the mtDNA sequences found 307 most parsimonious trees (1024 steps, consistency index (CI) = 0.45, retention index (RI) = 0.63). Fig. 1 shows the phylogenetic trees inferred by the MP and ML methods with bootstrap resampling, which have broadly similar topologies (see below for details).

The *Megastigmus* species attacking conifers formed a monophyletic group, and the entomophagous species *M. dorsalis* and *M. stigmatizans* were placed as the sister group to the remainder. However, the position of *M. rosae* remained unclear from mtDNA analyses, as it was placed as the sister taxon of conifer-related species in MP analyses, but fell within this group in ML analyses (exact position unresolved).

The conifer-related *Megastigmus* species were then grouped according to conifer families. All species developing on Pinaceae formed a strongly supported monophyletic clade, and the same was true for all species associated with Cupressaceae, except for *M. thyoides*. This latter species develops on the genus *Chamaecyparis* in the Nearctic region and was placed as the sister species of the monophyletic group associated with Pinaceae. *Megastigmus* developing on Taxodiaceae are represented by a species associated with *Cryptomeria*, whose position remained unresolved within the conifer-associated *Megastigmus*, but was separated clearly from species on Pinaceae and Cupressaceae.

For all introduced species (*M. specularis*, *M. pinus*, *M. rafni*, *M. atedius*, *M. pinsapinis*, *M. schimitscheki* and *M. spermotrophus*), specimens collected in native and introduced regions always clustered with a very strong support (between 94% and 100%).

Within the Pinaceae group, some species formed monophyletic subgroups. With the exception of *M. rafni*, all species related to the Nearctic *Abies* species (*M. lasiocarpae* Crosby, *M. milleri* Milliron, *M. specularis* and *M. pinus*) formed a strongly supported monophyletic clade. However, the data suggested that the individuals identified as *M. pinus* actually fell into two subclades, independent of the 'native' (from North America) or 'introduced' (from Europe) status of the collected sample, as one clade grouped individuals from Denmark and the U.S.A., whereas the other group comprised specimens from France and Canada.

Table 2. Mean genetic distances within and between groups of *Megastigmus* species (cytochrome *b*).

	Cupressaceae	Pinaceae	Taxodiaceae	Rosaceae	Entomophagous	Outgroup
Cupressaceae	0.083					
Pinaceae	0.141	0.083				
Taxodiaceae	0.147	0.142	0.004			
Rosaceae	0.171	0.157	0.143	–		
Entomophagous	0.281	0.277	0.283	0.276	0.208	
Outgroup	0.479	0.464	0.510	0.444	0.573	0.334

Table 3. Mean genetic distances within and between groups of *Megastigmus* species (28S).

	Cupressaceae	Pinaceae	Taxodiaceae	Rosaceae	Entomophagous	Outgroup
Cupressaceae	0.006					
Pinaceae	0.013	0.003				
Taxodiaceae	0.016	0.012	0.000			
Rosaceae	0.018	0.012	0.017	–		
Entomophagous	0.033	0.027	0.032	0.026	0.010	
Outgroup	0.083	0.078	0.083	0.080	0.083	0.035

Two clades grouped species associated with two genera of Pinaceae: except for *M. borriesi* Crosby, the *Megastigmus* associated with the Palaearctic *Abies* and *Cedrus* (*M. schimitscheki*, *M. pinsapinis* and *M. suspectus* Borries) formed a strongly supported clade. Similarly, the two Nearctic species developing on *Picea* and *Tsuga* (*M. atedius* and *M. tsugae*, respectively) were clustered together with significant support.

For the monophyletic group of *Megastigmus* associated with Cupressaceae, the exact relationships amongst species were poorly resolved, differing slightly depending on the analysis (in particular, the relationships between *M. formosana* and *M. amicorum* Bouček were unclear).

28S sequences. The MP heuristic search produced three trees of equal length (240 steps, CI = 0.917, RI = 0.948; data not shown). The ML bootstrap tree (Fig. 2), generated using the TrN + G model, had the same topology as the MP bootstrap tree. Strong bootstrap values (93%) supported the monophyly of *Megastigmus* developing on conifers. The monophyly of the clades associated with Cupressaceae (except for *M. thyoides*) and with Pinaceae was also well supported (100% and 76%, respectively). Whatever the method, the nuclear bootstrap trees were less resolved than the mtDNA trees, but the deepest nodes (which grouped the clades according to the host families) were well supported. The main difference from cyt. *b* was the position of the *Megastigmus* species on Taxodiaceae, namely *M. cryptomeriae*, which was placed as the sister taxon to all *Megastigmus* associated with Cupressaceae, whereas the relationship was unresolved with cyt. *b* alone.

Combined data set. The partition homogeneity test revealed no significant conflict between the phylogenetic signals of the cyt. *b* and 28S sequence data sets. Therefore, we used a combined data set for subsequent phylogenetic reconstruction (1733 characters, including 515 variable and 398 parsimony informative sites). MP analysis of the combined data set resulted in fifty equally most parsimonious trees of 1213 steps (CI = 0.55, RI = 0.69). The bootstrap consensus is shown in Fig. 3. Combined sequences indicated that the entomophagous species were the sister group to the rest. The Rosaceae seed feeder *M. rosae* was placed as the sister species of the conifer group. All species developing on Pinaceae were significantly clustered, with *M. thyoides* (Cupressaceae) as the sister species of this group. The species on *Juniperus* and *Cupressus* were also clustered, together with that on Taxodiaceae. Analyses of the combined data set resolved the position of species developing on *Pseudotsuga* as the sister group of all other species associated with Pinaceae.

Discussion

Molecular systematics of Megastigmus: evidence for complexes of cryptic species

Our data clarify the taxonomic status of some species or species groups, which have been described mainly on the basis of morphological and biological criteria. Owing to their economic importance, *Megastigmus* species are substantially discussed in the literature, yet most species remain poorly known or even undescribed. Several previous studies advocated the use of mtDNA sequences as a tool for identifying closely related species, and many studies have stated explicitly the appropriateness of mtDNA in resolving the relationships amongst closely related species (e.g. Crozier *et al*., 1995; Sperling & Hickey, 1995; Davison *et al*., 2001; Wahlberg *et al*., 2003). Genetic distance measures only the degree of genetic divergence between taxa and is not related explicitly to reproductive isolation and the reality of separate species (Ferguson, 2002). In some cases, it is difficult to define species boundaries and to decide if they belong to a species complex (with specialized taxa), or if they are fully differentiated sister species that diverged recently (Johns & Avise, 1998; Clark *et al*., 2001; Wahlberg *et al*., 2003).

Our study demonstrated that well-known differentiated species diverged by more than 4.0% for the mitochondrial gene, with the lowest value (4%) observed between *M. wachtli* and *M. atlanticus* on *Cupressus* and between *M. pictus* and *M. hoffmeyeri* on *Larix* and *Tsuga*. However, two species, recently separated by Pintureau *et al*. (1991) on morphological criteria, namely *M. pinsapinis* associated with *Cedrus* and *M. suspectus* associated with *Abies*, showed low differentiation, with interspecific distances of 1.8–3.6% for cyt. *b* and no sequence divergence for 28S. Although *M. suspectus* infests different *Abies* species all over Europe, it has also been reported from *Cedrus* seeds (Roques & Skrzypczynska, 2003; Fabre *et al*., 2004). Moreover, specimens identified as *M. suspectus* showed an

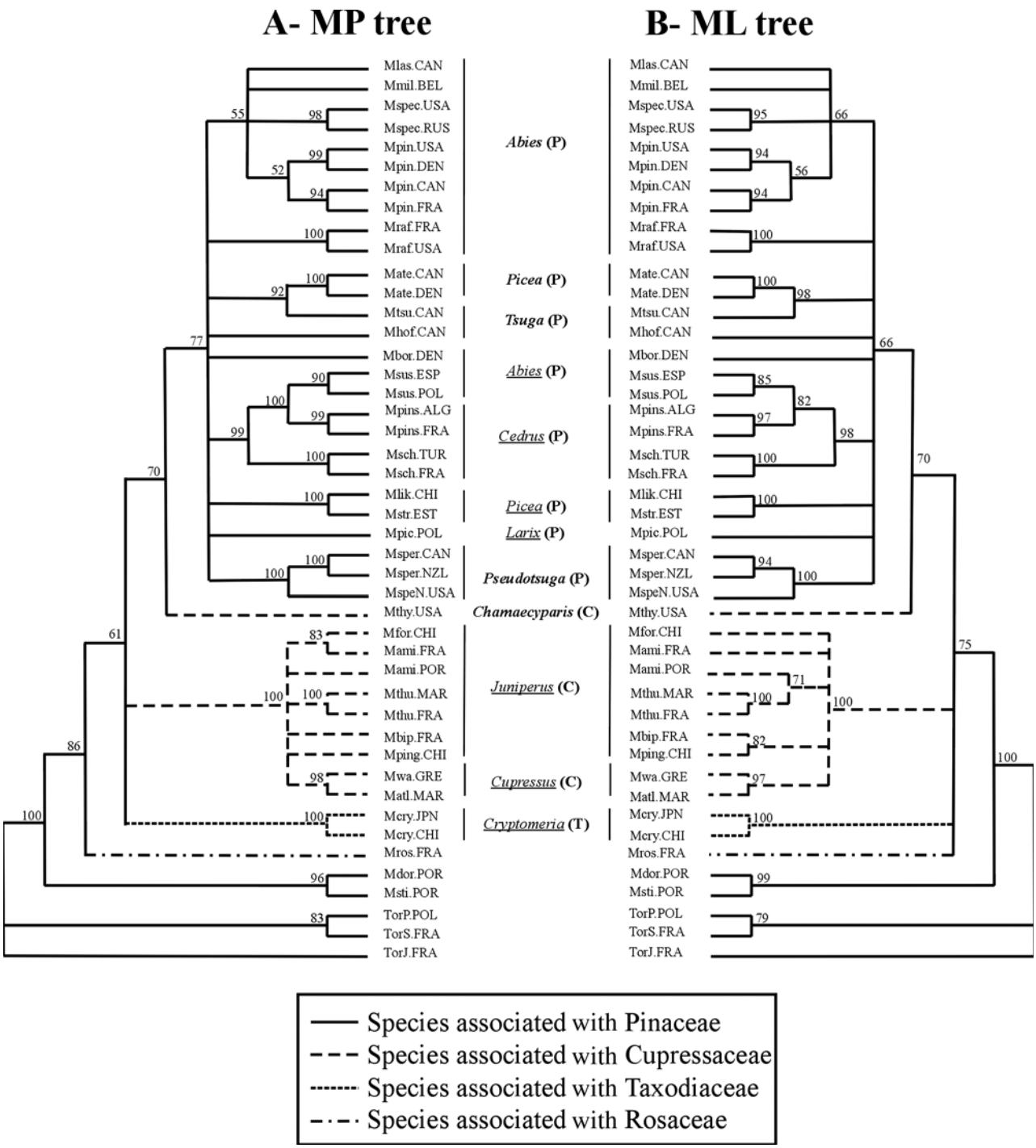

Fig. 1. (A) Maximum-parsimony (MP) tree of *Megastigmus* cytochrome *b* (cyt. *b*) mitochondrial DNA (mtDNA) sequences. Numbers above branches indicate bootstrap support from 500 bootstrap replicates. (B) Maximum-likelihood (ML) tree of *Megastigmus* cyt. *b* mtDNA sequences found under the GTR + I + G model. Numbers above branches indicate bootstrap support from 200 bootstrap replicates. Unnumbered nodes received < 50% bootstrap support. In the Pinaceae group, the species originating from the Nearctic region are in bold and the species originating from the Palaearctic region are underlined.

intermediate morphology in sympatric populations (M.-A. Auger-Rozenberg, pers. obs.) and the two specimens of *M. suspectus* analysed in this study differed by 2%. The weak morphological differences (essentially in colour patterns) observed between the two species and the low distances noted in cyt. *b* could indicate a species complex

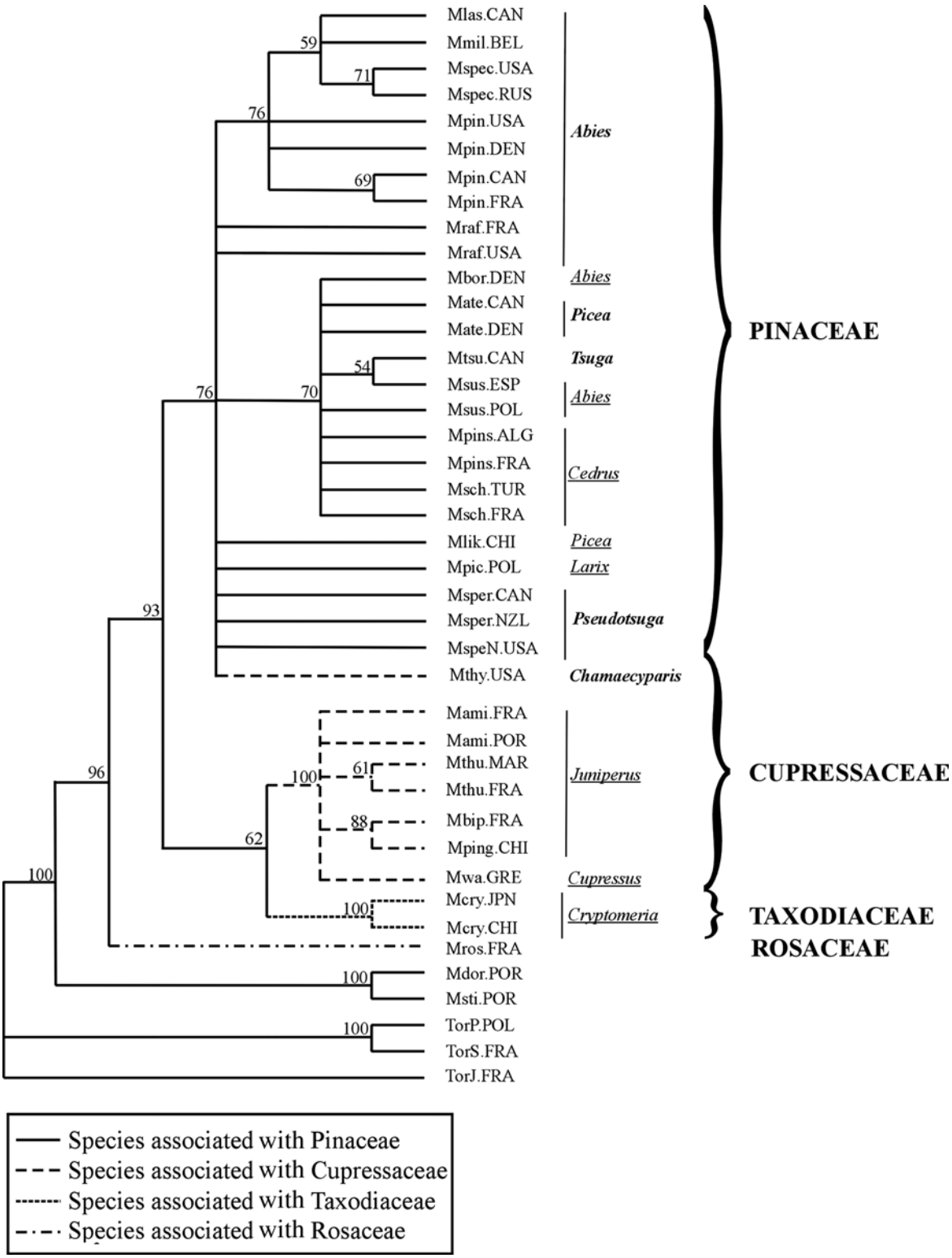

Fig. 2. Maximum-likelihood (ML) tree of *Megastigmus* 28S ribosomal DNA (rDNA) sequences found under the TrN + G model. Numbers above branches indicate bootstrap support from 200 bootstrap replicates. Unnumbered nodes received < 50% bootstrap support. In the Pinaceae group, the species originating from the Nearctic region are in bold and the species originating from the Palaearctic region are underlined.

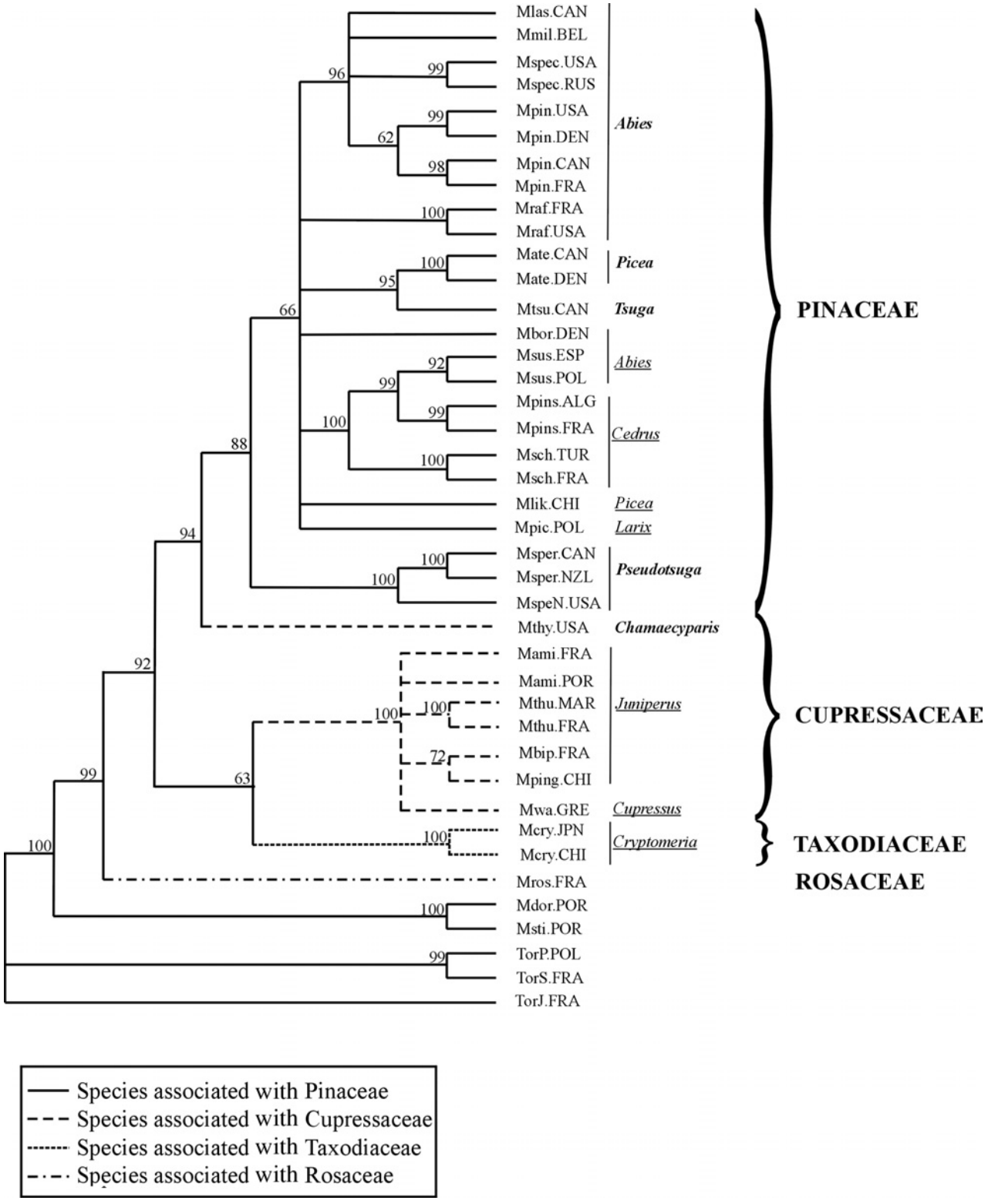

Fig. 3. Bootstrap consensus tree of most parsimonious trees found by combining both mitochondrial and nuclear sequence data. Numbers above branches indicate bootstrap support from 500 bootstrap replicates. Unnumbered nodes received < 50% bootstrap support. In the Pinaceae group, the species originating from the Nearctic region are in bold and the species originating from the Palaearctic region are underlined.

structured by host genus, rather than fully differentiated species. Moreover, both *M. suspectus* and *M. pinsapinis* exhibit thelytokous parthenogenesis, whereas nearly all other seed chalcids attacking conifers are arrhenotokous (except for *M. pictus* Förster, Roques & Skrzypczynska, 2003). The genetic distances observed thus could be explained by the slow accumulation of point mutations between different groups of females, rather than by speciating taxa. The taxonomic status of *M. suspectus* and *M. pinsapinis* cannot be unravelled at this stage, and will require further analysis with different molecular markers.

Another doubtful taxonomic position involves *M. spermotrophus nigrodorsatus*, which has been considered until now as a subspecies of *M. spermotrophus*. Milliron (1949) noted no morphological differences between females, but found distinct colour patterns between males. *M. spermotrophus nigrodorsatus* infests only the seeds of the big-cone Douglas-fir, *Pseudotsuga macrocarpa*, which is restricted to a small area in southern California. It has never been observed to attack seeds of Douglas-fir, *P. menziesii*, which is infested by *M. spermotrophus* larvae in both its native North American range and the areas of introduction (Europe and New Zealand). Our congruent molecular results (mean distances of 3.3% in cyt. *b* and 0.3% in 28S) suggest that *M. spermotrophus nigrodorsatus* actually could be a distinct species rather than a subspecies of *M. spermotrophus*. A study of gene flow with microsatellite markers would test this result.

For some conspecific populations also (*M. amicorum* from France and Portugal, and *M. pinus* from Canada and France, vs. from the U.S.A. and Denmark), we measured unexpectedly high levels of genetic distances (from 3.0% to 4.7% for cyt. *b*). Scheffer & Grissell (2003) have already observed high pairwise distances, reaching 4.0% in cytochrome c oxydase subunit I (COI) between populations of the same species, *M. transvaalensis* associated with Anacardiaceae, and suggested the presence of more than one species. Likewise, our results pose questions about the boundaries of the two species *M. amicorum* and *M. pinus*. Specimens identified as *M. amicorum* collected on *J. phoenicea* in Portugal diverged from the population sampled on the same host in France by 4.2% in cyt. *b*. Roques & Skrzypczynska (2003) noted that *M. amicorum* is distributed widely in the Mediterranean basin, and reported that some populations, especially from Portugal, differed morphologically from the type material. Considerable infraspecific genetic variation also exists within the host *J. phoenicea* itself, the Portuguese and Spanish populations being distinct from the other Mediterranean populations (Adams *et al.*, 2002). We hypothesize that *M. amicorum* comprises several specialized or divergent populations or subspecies, requiring species sampling over all its distribution range and testing of all known host species.

Concerning *M. pinus*, the studied specimens are clearly separated into two groups, the first with the individuals from Denmark and the U.S.A. and the second with the individuals from France and Canada. Between-group divergence reaches 4.7% between the Danish and the French specimens. Each specimen was collected on one different *Abies* species without clear relationships between insect groups and host species. These groups also need further investigation in order to assess whether they represent a species complex or differentiated populations. Cryptic species are often revealed to be diagnosable by consistent differences in morphology, once identified initially using genetic data. Indeed, further work should focus on intensive morphological examination of individuals of the putative species or subspecies.

Finally, the specimens collected on *J. thurifera* in Morocco and southern France clearly belong to the same species (genetic distances for cyt. *b* as low as 0.7%), whereas they diverge from *M. amicorum* by interspecific distance values (4.4–5.8%). These results confirm that the *Megastigmus* developing on *J. thurifera* belong to a distinct species, described in the Appendix, that differs from *M. amicorum*.

Evolution of host use

Evolutionary history of Megastigmus on Cupressaceae. Amongst the conifer group, all species infesting Cupressaceae (except for *M. thyoides*) and *M. cryptomeriae* associated with Taxodiaceae are well differentiated from the species developing on Pinaceae, and form a sister group to the remaining taxa. Taxodiaceae and Cupressaceae together form a single monophyletic lineage (Stefanovic *et al.,* 1998). The establishment of Taxodiaceae is estimated to have occurred by the middle Jurassic (Cheng *et al.,* 2000). The modern genera of Taxodiaceae were present during the Cretaceous period, and the Cupressaceae sensu stricto are considered to have proliferated later during the Late Cretaceous from a taxodiaceous ancestor (Brunsfeld *et al.,* 1994; Tsumura *et al.,* 1995). Within Taxodiaceae, *Cryptomeria* is the only genus to be attacked by a seed chalcid. There were very little differences (0.4%, cyt. *b*) between populations from the two Asiatic countries. The exact position of *M. cryptomeriae* with respect to the Cupressaceae and Pinaceae groups remains unresolved.

The *Megastigmus* on Cupressaceae sensu stricto formed a distinct clade. They all originate from the Palaearctic region, even though potential Cupressaceae hosts exist in the Nearctic region and in the Southern Hemisphere. According to Gadek *et al.* (2000), the Cupressaceae are clearly monophyletic and the family is split into two clades, from the Northern Hemisphere and the Southern Hemisphere, respectively. The geographical distribution of the plants may correlate with the separation of Gondwana from Laurasia about 100 Myr ago in the middle Cretaceous (Brunsfeld *et al.*, 1994; Kusumi *et al.*, 2002), before the appearance of modern chalcidoid families (Rasnitsyn, 1975), which could explain the lack of *Megastigmus* on Cupressaceae in the Southern Hemisphere. The apparent lack of Megastigmus species on *Cupressus* and *Juniperus* in North America is more intriguing, because a number of

species of *Juniperus* are distributed across North America, whereas *Cupressus* is known from Oregon to Mexico. Two hypotheses can be proposed to explain this distribution: (1) seed feeders related to Nearctic Cupressaceae ancestors existed but disappeared after colonization, or (2) the common ancestor of *Megastigmus* colonized the conifer families' ancestors in the Eurasian continent after the separation of North America and Europe which occurred during the Cretaceous (Brown & Lomolino, 1998). However, exit holes putatively belonging to *Megastigmus* were observed recently on fruits of Utah juniper, *J. osteosperma*, in Colorado, U.S.A. (A. Roques, pers. obs.). Therefore, Nearctic *Megastigmus* species could possibly exist on Cupressaceae, but are yet to be discovered and described. Concerning the species observed on European *Juniperus*, *M. amicorum* was collected on *J. phoenicea* and has also been reported on *J. oxycedrus* and *J. excelsa* in the eastern Mediterranean basin (Roques & Skrzypczynska, 2003). The new species *M. thuriferana* was collected only on *J. thurifera*, and the third described species on Juniperus in Europe, *M. bipunctatus* Swederus, has been reported from seeds of J. communis and J. sabina but never from *J. phoenicea* or *J. thurifera*, even though these four host species can be found in sympatry in southern France (A. Roques, pers. obs.). All these results suggest that the *Megastigmus* related to *Juniperus* hosts exhibit a high level of host specificity, even though rare host shifts have been observed for *M. amicorum* from native *Juniperus* to the introduced *Cupressus arizonica* and *C. goveniana* (Roques & Skrzypczynska, 2003). Thus, a more exhaustive study on the *Megastigmus* associated with the c. 60 species of *Juniperus* occurring in the Northern Hemisphere may allow the discovery of even more cryptic species in this group.

In our study, the Cupressaceae/Taxodiaceae group appears as the sister group of the clade including both the *Megastigmus* on Pinaceae and *M. thyoides*. Interestingly, this species develops in a species of Cupressaceae. This peculiar phylogenetic position suggests a radiation and a specialization on *Chamaecyparis* concomitant with the split of Cupressaceae- and Pinaceae-associated *Megastigmus*. No seed chalcid other than *M. thyoides* has been found on Nearctic species of Cupressaceae, and always in seeds of *Chamaecyparis thyoides*, despite our large sampling efforts in the last few years, especially on the other *Chamaecyparis* species (A. Roques and J. Turgeon, pers. obs.). The genus *Chamaecyparis*, closely related to *Cupressus*, includes three species in North America and four species in Eastern Asia. Another *Megastigmus* species (*M. chamaecyparidis*) exists on *Chamaecyparis obtusa* in Japan (Grissell, 1999), but we failed to collect it. Further sampling clearly is needed in North America in order to understand the potential constraints on *Chamaecyparis* and the lack of attacks on other Nearctic Cupressaceae.

Evolutionary history of Megastigmus on Pinaceae. Our study included most *Megastigmus* species associated with Pinaceae. Molecular analyses consistently recovered a topology in which species attacking Pinaceae were distinguished from other host conifers. Pinaceae was the first conifer family to diverge, and is supposed to be the sister group of extant Coniferales (Stefanovic *et al.*, 1998; Gugerli *et al.*, 2001). No infestation of native *Pinus* by *Megastigmus* has been recorded in the Palaearctic region, even though more than half of the potential Pinaceae hosts belong to that genus. *M. albifrons* Walker has been reported from *Pinus* in the southern United States and Mexico (Grissell, 1999), but it was not included in our study. Another species, *M. atedius*, primarily associated with *Picea spp.* in the Nearctic region, is also reported on *Pinus*, which most probably is due to a horizontal transfer. To permit the development of *Megastigmus* in a host, the timing of oviposition and larval development must match the phenology of the host seed. In *Megastigmus* that exploit Pinaceae, oviposition occurs before ovule fertilization, which takes place just after pollination. Species of the genus *Pinus* differ from other Pinaceae by having a period of cone and seed dormancy preceding fertilization, which requires the presence of pollen for normal gametophyte development (Rouault *et al.*, 2004). The difference in development of the host gametophyte could explain the lack of *Megastigmus* on *Pinus*.

Amongst Pinaceae, unlike Cupressaceae, there is no clear pattern of host specialization, with the exception of species related to *Pseudotsuga*. *M. atedius*, a Nearctic species associated with *Picea*, is clustered with *M. tsugae* strictly infesting *Tsuga* spp., whereas the two Eurasiatic species associated with *Picea spp.* are clustered together. The Nearctic and Palaearctic species associated with *Abies* do not form a monophyletic group. The Palaearctic species on *Abies* cluster with the species associated with *Cedrus*. Low mtDNA divergence within the complex '*Abies*/*Cedrus*' suggest that this group, including the different individuals of *M. suspectus*, *M. pinsapinis* and *M. schimitscheki*, is the result of a recent colonization and diversification. In the Nearctic region, the species *M. pinus* is known to attack the different sympatric *Abies* species occurring in the North American West coast and several *Abies* species in the introduction range. There seems to be no host differentiation between the different specimens of *M. pinus* that were collected on four different *Abies* species.

Moreover, in their native areas, *Megastigmus* species can shift onto most of the introduced tree species congeneric to the original host. Reciprocally, in their introduction range, the invasive species attack trees if they belong to the same genus as the native hosts (Roques & Skrzypczynska, 2003). These results may suggest that the shift from one host genus to another was probably not constrained, and that the data reflect local geographical evolution rather than strict host radiation. It also indicates either an important plasticity of the *Megastigmus* species or a lack of biological barriers amongst the different host species.

Evidence of exotic invasive species

Our systematic sampling scheme on various potential hosts worldwide showed that many *Megastigmus* species

were found outside of their native range. The pairwise mtDNA sequence divergence between conspecific individuals collected in the native range and in the area of introduction never exceeded 1.4% for cyt. *b*. Indeed, the introductions appear to be more frequent than reported in the literature (Grissell, 1999). For instance, almost all the North American species related to the genus *Abies* are now present in Europe: *M. pinus* (individuals of the two groups, see above); *M. rafni* (widespread in France, which was not previously known); *M. milleri* (found in France for the first time); and *M. specularis* (occurrence in Europe and Siberia confirmed). For native Mediterranean species, we can confirm the presence of *M. pinsapinis* in southern France on *Cedrus* (probably introduced from North Africa to France in the middle of the 20th century), and the recent introduction of *M. schimitscheki* from Turkey to France (less than 20 years ago, Fabre *et al*., 2004).

The *Megastigmus* species developing in Pinaceae seeds seem to shift hosts more easily than the species associated with Cupressaceae, and their invasive potential is clearly much higher as they are more likely to adapt to new hosts in the introduction area. Successful installation of Nearctic species on European *Abies* species has already been observed. Moreover, these species are easily introduced to new regions because of the present expansion in international trade of conifer seeds. Thus, the *Megastigmus* species infesting seeds of Pinaceae should be considered as dangerous potentially invading pests.

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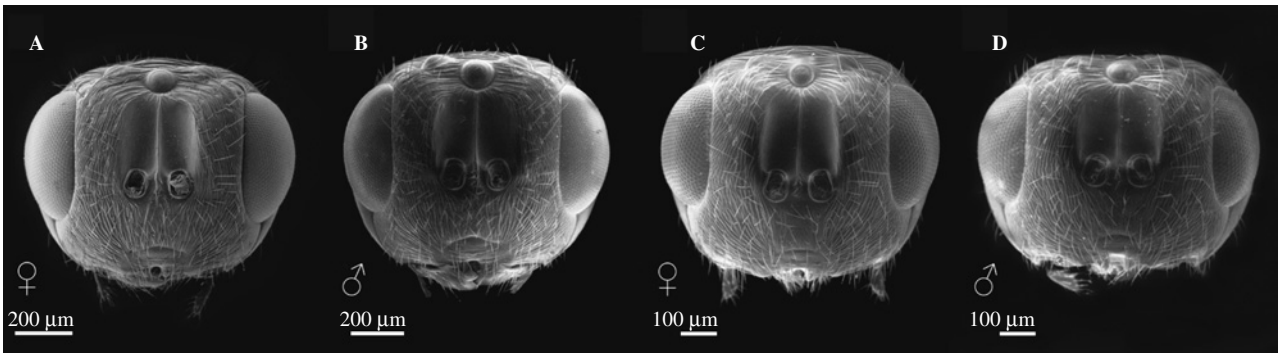

Fig. 4. Electroscan front view of head of *Megastigmus thuriferana* (A, female; B, male) and *M. formosana* (C, female; D, male).

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Appendix
Megastigmus thuriferana Roques & El Alaoui[1], sp.n. (Figs 4A,B; 5A,B; 6A,B; 7A,B)

Description

Holotype female. Body length (without ovipositor) 3.8 mm; length of exserted part of ovipositor 2.1 mm. Body colour entirely dark orange–yellow. Head dark orange–yellow except brown occelli. Pilosity pale on face, dark on dorsum of head with bristles protruding from conspicuous reddish dots. Antenna dark brown except yellowish scape and pedicel. Pronotum pale yellow; remainder of thorax dark orange–yellow except blackish notaulus and anterior suture of mid-lobe of mesoscutum. Pilosity on thorax black. Scutellum with 7 lateral bristles. Legs orange except claws brown. Wings subhyaline; forewing stigma light brown without any infuscation; basal cell including 4 setae, closed by a basal setal line with 6 setae and a costal setal line with 4 setae. Propodeum dark orange–yellow with conspicuous bristles on callus. Gaster predominantly orange–yellow with a dark brown patch on dorsum of terga III–V. Ovipositor sheaths black. Face rounded in outline, ratio width : height about 1.4; clypeus convex; torulus oval, c. 1.2× as long as wide; interantennal area as broad as torulus width; scrobe elongate, c. 2.4× as long as wide; eyes little protruding. Scape rather small, only 0.7× as long as combined length of pedicel, anellus, 1st and 2nd funicular segments; pedicel elongate, 2.2× as long as wide; anellus subquadrate; 1st funicular segment elongate, 1.2× as long as pedicel, 2.7× as long as wide; 2nd funicular segment less elongate, 2.1× as long as wide; following funicular segments progressively tending to subquadrate, with 7th funicular segment only 1.8× as long as wide. Pronotum, mid- and lateral lobes of mesoscutum, and axilla with strong cross-striae. Mid-lobe of mesoscutum 0.7× as long as scutellum. Scutellum 0.9× as long as wide, with weak transverse carinae on the anterior part; frenum 0.4× length of scutellum, smooth with a few longitudinal carinae on the lateral parts. Stigma oval elongate, about 1.7× as long as wide; upper part of stigmal vein elongate, c. 0.3× as long as stigma length; uncus short, 0.5× as long upper part of stigmal vein; marginal vein 0.6× as long as postmarginal vein. Propodeum roughly quadrate, with cross-striae tending to reticulate in the anterior part and a very weak median carina on the posterior part. Ovipositor sheaths 0.6× as long as body but 1.2× longer than gaster.

[1]El Alaoui El Fels M.A., Museum d'Histoire Naturelle de Marrakech, Laboratoire d'Ecologie Vegetale FSSM, Université Cadi Ayyad, Maroc.

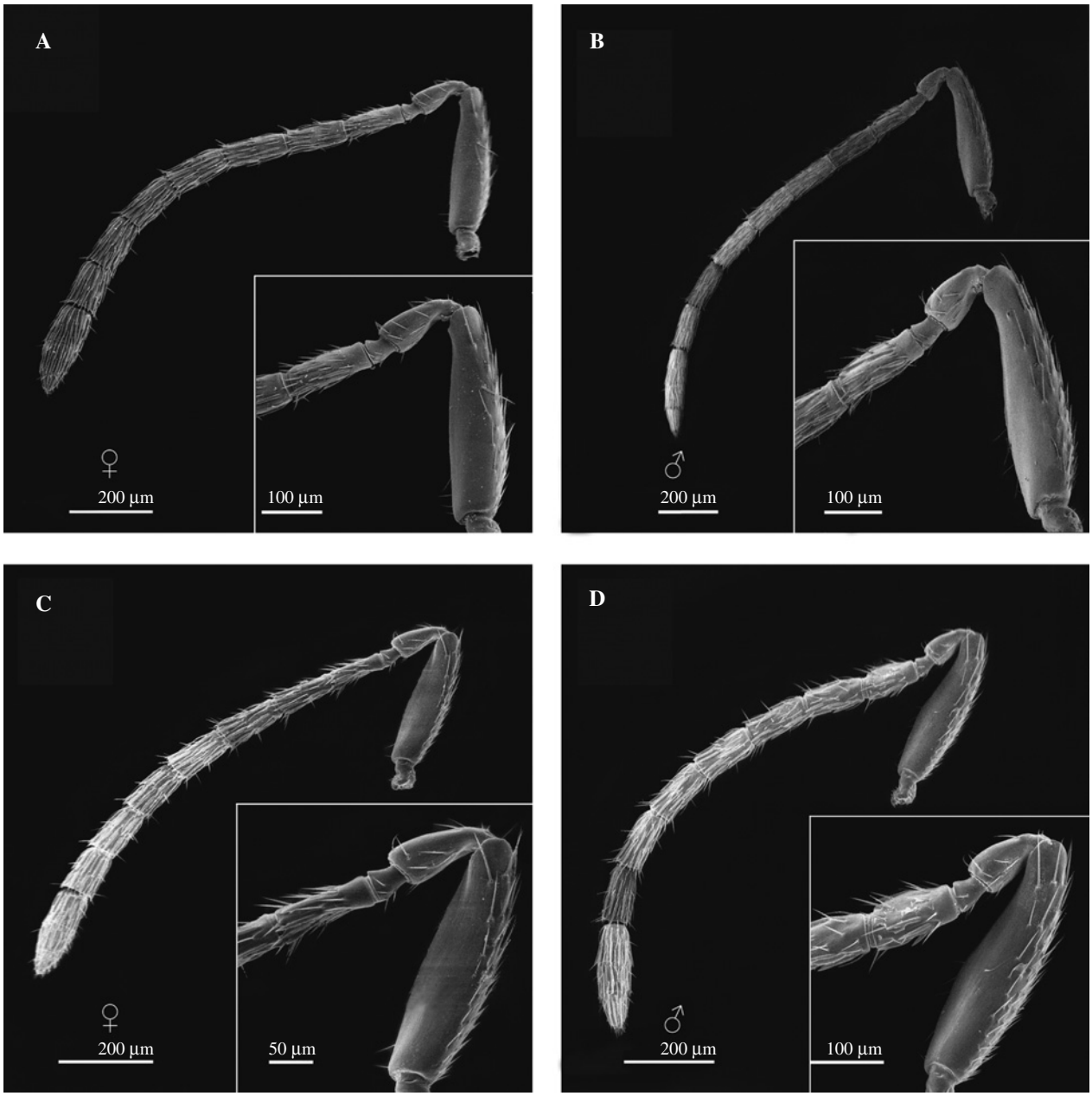

Fig. 5. Electroscan view of antenna of *Megastigmus thuriferana* (A, female; B, male) and *M. formosana* (C, female; D, male).

Allotype male. Length 4.2 mm. Differs from female as follows: thorax dark yellow with notauli and sutures of prepectus, axillae and callus black; gaster dark yellow with a conspicuous dark brown petiole, the four following segments with a transverse dark brown band on anterior part of tergum which extends laterally on sides, last segment yellow. Face more rounded in outline, ratio width : height about 1.2×; clypeus sinuose; interantennal area 0.8× as broad as torulus width. Scape more elongate, 0.8× as long as combined length of pedicel, anellus, 1st and 2nd funicular segments; 1st funicular segment elongate, 1.3× as long as pedicel, 2.5× as long as wide; 2nd funicular segment 2.4× as long as wide; following funicular segments similarly elongate, with 7th funicular segment 2.4× as long as wide. Mid-lobe of mesoscutum proportionally more elongate than in female, about as long as scutellum. Scutellum nearly as long as wide, with weak transverse carinae on the anterior part; frenum 0.4× length of scutellum, smooth with a few longitudinal carinae on the lateral parts. Stigma oval elongate, about 1.3× as long as wide; upper part of stigmal vein elongate, *c*. 0.2× as long as stigma length; uncus long, 0.6× as long as upper

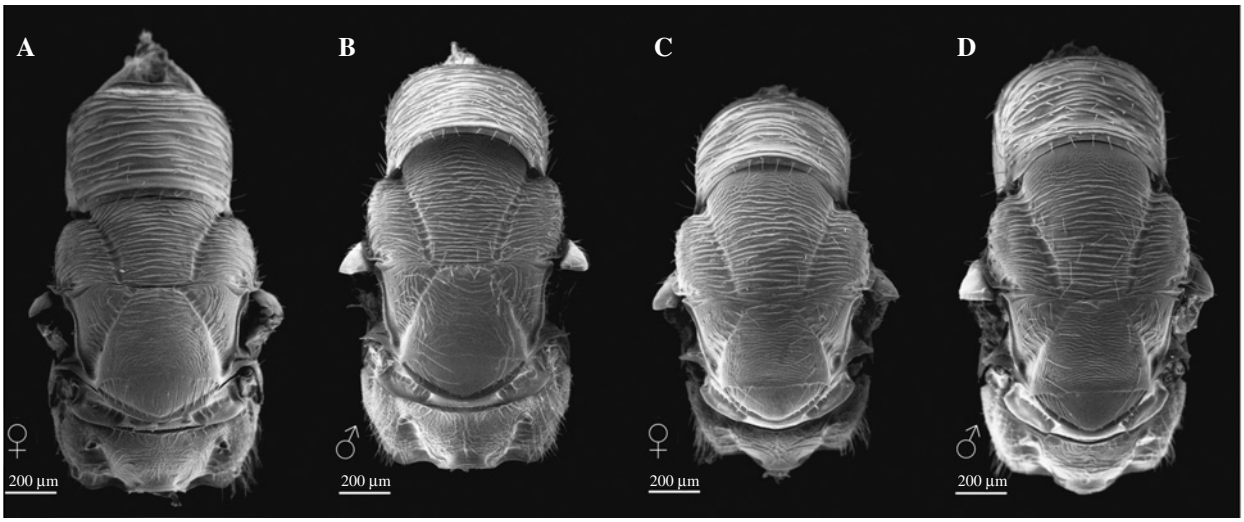

Fig. 6. Electroscan dorsal view of thorax of *Megastigmus thuriferana* (A, female; B, male) and *M. formosana* (C, female; D, male).

part of stigmal vein; basal cell including 8 setae, closed by a row of 6 setae on basal setal line and a row of 9 setae on costal setal line; postmarginal vein 2.3× as long as stigmal vein; marginal vein 0.6× as long as postmarginal vein. Propodeum with a weak median carina on middle and posterior part. Aedeagus elongate, with a 4-teeth digitus.

Variation. Females range in length from 3.5 to 4.3 mm, males from 3.6 to 4.4 mm. Body colour varies little in females except gaster. Two females from Tizrag show prepectus and sutures of lateral lobes of mesoscutum densely coloured in black, whilst callus is black laterally in one female of Saint-Crépin. At Rié, most females differ in gaster colour, with first apparent tergum black and the four following terga with broad, transverse, triangular black band. The colour pattern varies more in males. Half of the specimens from Rié and Saint-Crépin are darker than those of Tizrag, with several pieces entirely black: face, collar of pronotum, ventrum, lateral parts of thorax except tegula, acropleuron, callus and part of prepectus dark orange–yellow, propodeum except longitudinal yellow spot along median line, fore-coxa except apex, sides of mid- and hind coxa, elongated spot on middle of fore and hind femur, gaster except dark orange–yellow spots on lateral sides and extremity. In these specimens, forewing stigma is dark brown and roughly ovoid, about 1.1× as long as wide, with very short stigmal vein (0.1× as long as stigma length), and 8–12 setae in basal cell of forewing.

Material examined. Holotype, ♀, Tizrag, 2500 m elevation, High Atlas Mountains, Morocco, emerged from seed of *Juniperus thurifera*, 18.vii.1999, M. A. El Alaoui El Fels, deposited at Museum National d'Histoire Naturelle (MNHN), Paris. Paratypes, 7♀, 9♂ as follows (all MNHN unless stated otherwise). Morocco: 3♀, 5♂, same data as holotype. France: 4♀, 4♂, Saint-Crépin, Hautes-Alpes, emerged from seed of *J. thurifera*, 11.viii.2002, A. Roques [2♂, 2♀ in personal collection of A. Roques, INRA, Orléans, France (AR)]; 6♀, 6♂, Rié Mt., Haute-Garonne, emerged from seed of J. thurifera, 22.vii.2002, A. Roques (3♂, 3♀ in AR collection).

Hosts. Develops specifically in seeds of incense juniper, *Juniperus thurifera* (Cupressaceae).

Distribution. Widely distributed all over the patchy distribution range of *J. thurifera*: Morocco, Spain, France (Pyrénées, southern French Alps, Corsica).

Comments. Two other species, *M. amicorum* Bouček and *M. bipunctatus* Swederus, attack juniper seeds in Europe and North Africa (Roques & Skrzypczynska, 2003). The relative length of the exserted part of female ovipositor allows an easy differentiation of females of the three species. In *M. bipunctatus*, ovipositor is slightly shorter (0.9×) than gaster, whilst that of *M. amicorum* is 1.4× longer than gaster. Males of *M. bipunctatus* have thoracic dorsum mostly brownish to olive-brown, whereas that of the two other species is mostly yellowish to orange–yellow. *M. amicorum* differs by the presence of longitudinal carinae on the middle part of frenum. In addition, these two species have only three teeth on the digitus of the aedagus.

Megastigmus formosana Roques & Pan[2], sp.n. (Figs 4C,D; 5C,D; 6C,D; 7C,D)

Description

Holotype female. Body length (without ovipositor) 2.9 mm; length of exserted part of ovipositor 1.8 mm.

[2]Pan Y., South-west Forest College, White Dragon Temple, Kunming, CN-650224, China.

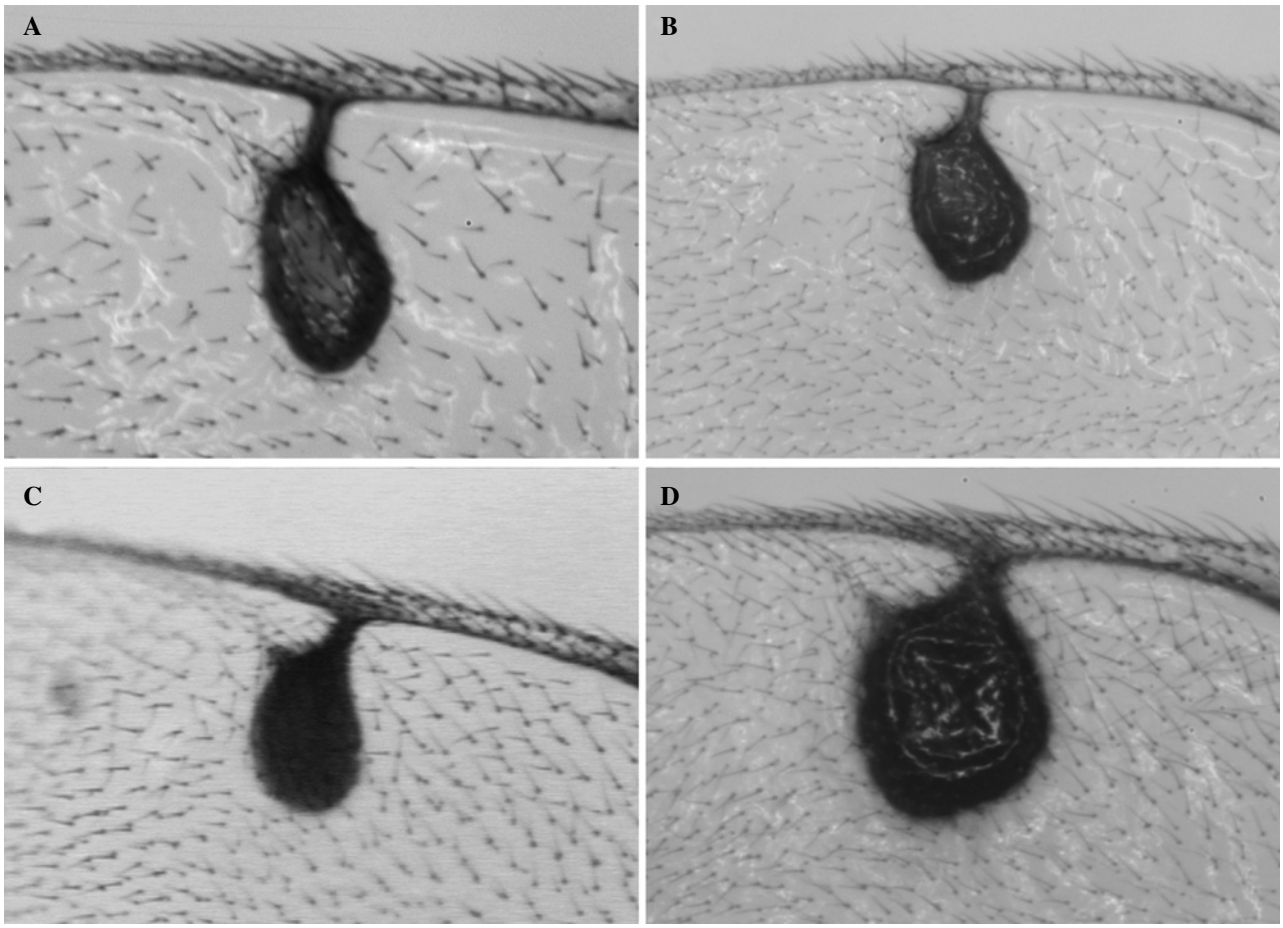

Fig. 7. Stigma of *Megastigmus thuriferana* (A, female; B, male) and *M. formosana* (C, female; D, male).

Body colour entirely orange–yellow except ocelli, antenna, mid-lobe of mesoscutum and gaster. Ocelli black, antenna dark brown except scape and pedicel yellowish. Pilosity pale on face, dark on dorsum of head. Pronotum yellowish; remainder of thorax orange–yellow except narrow brown band along anterior suture of mid-lobe of mesoscutum. Pilosity on thorax black. Scutellum with 5 lateral bristles. Legs orange, claws brown. Wings subhyaline; forewing stigma light brown without infuscation; basal cell with 6 setae, closed by row of 6 setae on basal setal line and row of 5 setae on costal setal line. Gaster predominantly orange–yellow with three first terga (III–V) brown. Ovipositor sheaths black. Face subquadrate in outline, ratio width : height about 1.3; clypeus convex; torulus oval, *c*. 1.2× as long as wide; interantennal area 0.8× as broad as torulus width; scrobe elongate, *c*. 2.7× as long as wide; eyes little protruding. Scape 0.7× as long as combined length of pedicel, anellus, 1st and 2nd funicular segments; 1st funicular segment elongate, 1.1× as long as pedicel, 3.8× as long as wide; 2nd funicular segment less elongate, 2.8× as long as wide; following funicular segments progressively tending to subquadrate, with 7th funicular segment only 1.4× as long as wide. Pronotum with strong cross-striae. Mid-lobe of mesoscutum reticulate in the upper third, then sculptured with strong cross-striae. Mid-lobe of mesoscutum 1.2× longer than scutellum. Scutellum as long as wide, with irregular, sinuose, transverse carinae; frenum 0.3× length of scutellum, smooth with 4 longitudinal carinae on the lateral parts. Stigma oval elongate, about 1.7× as long as wide; upper part of stigmal vein elongate, *c*. 0.3× as long as stigma length; uncus elongate, 0.7× as long as upper part of stigmal vein. Propodeum roughly triangular, with cross-striae tending to reticulate; median carina irregular. Ovipositor sheaths 0.6× as long as body, 0.7× as long as thorax and gaster combined but 1.3× longer than gaster.

Allotype male. Length 3.7 mm. Body colour orange–yellow and black. Face and occiput black, remainder of head orange–yellow. Pronotum yellowish; remainder of thorax dorsum orange–yellow except anterior suture of mid-lobe of mesoscutum brownish, infuscated. Pilosity on thoracic dorsum black. Lateral parts of thorax entirely brownish. Dorsellum yellowish, lateral panel of metanotum yellowish with median longitudinal, brownish stripe.

Scutellum with 5 lateral bristles. Fore coxa brownish except apex yellowish, mid- and hind coxa brownish; remainder of legs yellowish except claws brownish. Forewing stigma light brown without infuscation; basal cell with 16 setae, closed by row of 6 setae on basal setal line and row of 10 setae on costal setal line. Propodeum mostly blackish except upper part of callus and a spot at base yellowish. Gaster blackish except extremity pale yellow. Face subquadrate in outline, ratio width : height about 1.3; clypeus convex; torulus oval, *c*. 1.2× as long as wide; interantennal area 0.6× as broad as torulus width; scrobe less elongate than in female, *c*. 2.3× as long as wide; eyes little protruding. Scape elongate, 0.8× as long as combined length of pedicel, anellus, 1st and 2nd funicular segments; 1st funicular segment 1.5× as long as pedicel, 1.9× as long as wide; 2nd funicular segment more elongate, 2.3× as long as wide; following funicular segments progressively enlarging, with 7th funicular segment only 1.9× as long as wide. Pronotum with strong cross-striae. Mid-lobe of mesoscutum reticulate in upper third, then sculptured with strong cross-striae. Mid-lobe of mesoscutum 1.3× longer than scutellum. Scutellum as long as wide, with irregular, sinuose, transverse striae tending to reticulate on anterior part; frenum 0.3× length of scutellum, smooth with 4 longitudinal carinae on the lateral parts. Stigma rounded, 1.1× as long as wide; upper part of stigmal vein very short, *c*. 0.1× as long as stigma length; uncus nearly as long (0.9×) as upper part of stigmal vein; postmarginal vein 1.8× as long as stigmal vein; marginal vein 0.6× as long as postmarginal vein. Propodeum with broken median carina. Aedeagus elongate, with 3-tooth digitus.

Variation. Females range in length from 2.8 to 3.9 mm, males from 3.5 to 4.2 mm. Body colour is little variable in female. In males, brownish parts often turn to black.

Material examined. Holotype, ♀, Dongchuan, Yunnan, China, emerged from seed of *Juniperus formosana*, 14.iv.1997, Y.Z. Pan, deposited at Museum National d'Histoire Naturelle (MNHN), Paris. Paratypes, 8♀, 7♂ as follows. China: 6♀, 5♂, same data as holotype [3♀, 3♂ (MNHN); 3♀, 2♂ at South-west Forestry College, Kunming, Yunnan, China]; Lijiang, Yunnan: 2♀, 2♂, emerged from seed of *J. formosana*, 11.iv.2002, Y.Z. Pan (MNHN).

Hosts. Develops specifically in seeds of *Juniperus formosana* Hayata var. *mairei* (Lemée and H. Lev.) (Cupressaceae).

Distribution. Widely distributed all over Yunnan (China).

Comments. Three other species, *M. pingii* Roques and Sun, *M. rigidae* Xu and He and *M. sabinae* Xu and He, attack juniper seeds in China (Xu & He, 1989; Roques *et al*., 1995; Xu *et al*., 1998; Roques & Skrzypczynska, 2003). The length of the exserted part of female ovipositor is proportionally smaller in *M. formosana* than in both M. *pingii*, where it equals the combined length of thorax and gaster (0.7× in *M. formosana*), and *M. sabinae*, where it is 1.6× longer than gaster (1.3× in *M. formosana*). The female habitus largely resembles that of *M. rigidae* from which it differs by its overall colour (orange–yellow vs. yellowish white), the presence of a narrow brown band on the anterior margin of the mid-lobe of mesoscutum, a smaller antenna pedicel (0.9× vs. 1.1× as long as 1st funicular segment), and a more elongate 1st funicular segment (3.8× vs. 3× as long as wide). The rounded shape of the stigma and the short, thick upper part of stigmal vein allow males of *M. formosana* to be distinguished from those of *M. pingii* (stigma oval-elongate, 1.5× as long as wide) and *M. sabinae* (upper part of stigmal vein elongate). Male stigma is largely similar but a bit more rounded in *M. rigidae* (1.2× vs. 1.1× as long as wide). *M. rigidae* differs by its overall colour (dark brown) and the two first funicular segments of antenna, the 1st segment being more elongate than the 2nd, whereas the converse is observed in *M. formosana*.