Synchronous intercontinental splits between assemblages of woodpeckers suggested by molecular data

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The woodpeckers (Piciformes: Picinae) comprise a widely distributed and species-rich clade of birds that is strongly associated with trees for feeding, nesting, or both. Because of this association, woodpeckers provide a useful model for evaluating the impact of climatic and tectonic events on the diversification of forest birds during the Tertiary. In order to resolve the biogeographical history of the woodpeckers, we have analysed sequences from two nuclear introns and one mitochondrial gene using likelihood and Bayesian approaches. Our analyses favour a tropical Eurasian origin; divergences between African, Indo-Malayan and New World clades with subsequent colonizations of Africa and the New World occurred synchronously during the Middle Miocene, a period corresponding to the expansion of the C4 grasses and the uplift of the Himalayan-Tibetan plateau. The taxonomic diversification of woodpeckers at this time may be attributed to the fragmentation of forests in response to the drier climate, which in turn prevented gene flow between tropical stocks in Africa, Indo-Malaya and the New World. Our estimates of colonization times of South America predate the closure of the Panama Isthmus and support the hypothesis of a short-lived, terrestrial corridor at the end of the Miocene, 5.7 Myr BP.

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Introduction

The woodpeckers (Piciformes: Picinae) comprise a strongly supported monophyletic group of 25 genera and 180 species. They are well known for their capacity to climb tree trunks and peck at wood to extract insect larvae. With the exception of the species of a few genera (e.g. *Geocolaptes*), the life history of these birds is closely associated with trees for feeding, nesting, or for both. The Picinae has a nearly worldwide distribution, with species present in all major biogeographical units except Australasia and Madagascar. Monophyly of the woodpeckers has never been doubted and is supported by several morphological and molecular data sets (e.g. Simpson & Cracraft 1981; Swierczewski & Raikow 1981; Webb & Moore 2005; Benz *et al*. 2006).

Neither the traditional classifications nor the current phylogenetic hypotheses for woodpecker relationships recognize continental groupings as monophyletic (Short 1970; Webb & Moore 2005; Benz *et al*. 2006), suggesting the occurrence of several intercontinental dispersal events during their evolutionary history. This situation contrasts considerably with that of the closely related barbets and toucans, where the African, Indo-Malayan and South American stocks each represent monophyletic assemblages (Prum 1988; Moyle 2004).

The absence of congruence between the currently recognized biogeographical patterns for the barbets plus allies and the woodpeckers may be explained by the fact that some woodpecker genera (e.g. *Picoides*) are also adapted to more temperate zones and thus would have been able to cross the Bering Strait during their evolutionary history. No data indicate whether the temperate-adapted species form a monophyletic group, or whether they repeatedly and independently arose from tropical stocks. Relationships of tropical taxa at the intercontinental level were for long among the most debated issues in woodpecker systematics (e.g. Short 1970; Goodge 1972; Wolters 1975–1982). This is particularly highlighted by the cases of *Celeus* (one species in Asia and ten in South America) and the African woodpeckers (*Campethera*, *Dendrocopos*, *Geocolaptes*).

As traditionally defined, the genus *Celeus* occurs in two disjunctive areas: Indo-Malaya and South America. Morphological distinctness of the Asian *Celeus* has sometimes led authors to retain for it its own monotypic genus, *Micropternus* (Wolters 1975–1982), and even to consider that *Micropternus* could be more closely related to the Eurasian genus *Picus* than to the South American members of *Celeus* (Winkler & Christie 2002). The relationships of the three African genera have long been puzzling. African woodpeckers have been considered to be the result of one (Goodwin 1968; Short 1970), two (Goodge 1972; Benz *et al.* 2006), or even three independent colonization events (Webb & Moore 2005). None of the hypotheses concerning the relationships of the African genera have received strong support and their biogeographical affinities are still in need of clarification.

Other lineages of birds, such as the barbets (Piciformes: Ramphastidae *sensu* Dickinson 2003) and woodcreepers (Passeriformes: Dendrocolaptinae) also forage by gleaning and probing tree trunks and branches; some of them (e.g. *Megalaima*, *Pogoniulus*, *Tricholaema*) are even able to peck wood to extract insects (Short 1973). Most Ganbets genera occur in Africa, to which seven out of the total of 19 genera are restricted. The remaining genera occur either in Indo-Malaya (three genera) or South America (nine, of which six are the toucans). Short (1970) suggested that the diversification of woodpeckers in Africa may have been limited by competition with the African barbets for nest sites. In South America, the interaction between woodpeckers and woodcreepers has recently been seen as providing a possible explanation of the observed shift in morphology and foraging behaviour in the latter (Fjeldså *et al.* 2005). The timing of woodpecker evolution is thus of particular interest in order to understand their interaction with other wood-gleaning and probing lineages.

To better understand how, where and when the woodpeckers evolved, we have analysed phylogenetic relationships in the group using 2655 bp of mitochondrial and nuclearDNA obtained for 22 of the 25 recognized genera.

Materials and methods

Taxonomic sampling

We obtained tissue samples from 46 of the 180 recognized Picinae species, representing 22 (88%) of the 25 genera (Dickinson 2003). Several species per genus were included in the case of highly diversified genera (e.g. *Celeus*, *Dendrocopos*, *Dendropicos*, *Melanerpes*), with the main objective being to cover the morphological diversity of each group. We lacked tissues of the monotypic and island endemics *Sapheopipo* (Okinawa), *Xiphidiopicus* (Cuba), as well as of the monotypic Asian genus *Hypopicus*. *Sapheopipo* and *Hypopicus* have now proved to be nested within *Dendrocopos* and closely related to *D. major* and *D. leucotos* (Winkler *et al.* 2005; Benz *et al.* 2006). Members of the subfamilies Jynginae and Picumninae were included as proximate outgroups (Benz *et al.* 2006; Fuchs *et al.* 2006). Trees were rooted with a representative of the Indicatoridae, the sister group of the Picidae (e.g. Swierczewski & Raikow 1981; Johansson & Ericson 2003; Webb & Moore 2005; Benz *et al.* 2006). In total, 52 taxa were included in the analyses. Sample origins and GenBank accession numbers are listed in Table 1.

Laboratory procedures

We obtained nucleotide sequences for two nuclear introns (myoglobin intron-2 and β-fibrinogen intron-7) and a mitochondrial protein-coding gene (ND2), representing a total of 2655 bp. We extracted DNA from frozen or alcohol-preserved tissues (blood, liver, muscle) using a CTAB-based protocol (Winnepenninckx *et al.* 1993) with an overnight Proteinase K (0.1 mg/mL) digestion. Myoglobin intron-2 was amplified with primers Myo2 or Myo2 Pi-F (5′-CCT GTC AAA TAT CTG GAG GTA TG-3′ (Fuchs *et al.* 2006) and Myo3F (Slade *et al.* 1993; Heslewood *et al.* 1998). The whole ND2 gene was amplified with primers L5219-Met and H6313-Trp for most of the samples (Sorenson *et al.* 1999). The ND2 gene (1041 bp) was sequenced for all taxa but *Piculus rivolii*. We were not able to amplify and sequence the second half of the gene for this species despite the use of additional primers pairs (L2258, H6681) (Sorenson *et al.* 1999). These primers were also used for some samples that were difficult to amplify or sequence with L5219-Met and H6313-Trp. β-fibrinogen intron-7 was amplified with primers FIB7U and FIB7L (Prychitko & Moore 1997). The thermocycling conditions followed standard procedures for these genes (Prychitko & Moore 1997; Fuchs *et al.* 2004). Three microliters of the amplification products were electrophoresed on 1.5% agarose gel and visualized under UV light with ethidium bromide to check for the correct fragment size and to control for the specificity of the amplifications. PCR products were purified directly using the QiaQuick PCR Purification Kit (Qiagen, Holden, Germany) and cycle-sequenced using the CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter Inc, Fullerton, CA, USA)

Table 1 List of taxa studied (following Dickinson 2003 for genera and Winkler & Christie 2002 for tribes), tissue or voucher number and GenBank accession numbers. Asterisks indicate that a voucher specimen has been deposited.

Species	Tribe	Voucher or tissue number	Origin	Myoglobin	Fibrinogen	ND2
Ingroup						
Picinae						
Blythipicus pyrrhotis	Picini	MNHN 15-62	China	DQ352418	DQ352397	DQ361295
Campephilus haematogaster	Campephilini	ZMUC 114730	Ecuador	DQ188143	AF240016	DQ188169
Campephilus leucopogon	Campephilini	USNM 609524*	Ecuador	DQ352423	DQ352403	DQ361279
Campethera caroli	Campetherini	MNHN 03-04	Cameroon	DQ188157	DQ188131	DQ188183
Campethera nivosa	Campetherini	MNHN 01–28	Cameroon	DQ352447	AY489408	DQ361306
Celeus brachyurus	Colaptini	USNM 620445*	Myanmar	DQ352417	DQ352398	DQ361282
Celeus grammicus	Colaptini	ZMUC 114122	Ecuador	DQ352420	DQ352399	DQ361307
Celeus lugubris	Colaptini	NRM 947231*	Paraguay	DQ352441	DQ352396	DQ361299
Chrysocolaptes lucidus	Picini	MNHN 4-2D	Thailand	DQ352432	DQ352414	DQ361301
Colaptes auratus	Colaptini	AMNH PAC820*	USA	DQ188152	AY082398	DQ188178
Colaptes melanochloros	Colaptini	NRM 947052*	Paraguay	DQ352436	DQ352390	DQ361298
Dendrocopos canicapillus	Campetherini	MNHN 14-42	China	DQ352419	DQ352404	DQ361303
Dendrocopos leucotos	Campetherini	NRM 996095*	Sweden	DQ188142	DQ188116	DQ188168
Dendrocopos macei	Campetherini	LSUMZ B-23827*	Captive	DQ352438	DQ352395	DQ361296
Dendrocopos mahrattensis	Campetherini	USNM 586658*	Myanmar	DQ352426	DQ352405	DQ361280
Dendrocopos major	Campetherini	MNHN C29*	France	DQ188153	DQ188127	DQ188179
Dendrocopos medius	Campetherini	MNHN K15	France	DQ352422	DQ352388	DQ361294
Dendrocopos minor	Campetherini	NRM 986593*	Sweden	DQ188154	AF394321	DQ188180
Dendropicos elliotii	Campetherini	FMNH 484843*	Uganda	DQ352428	DQ352389	DQ361292
Dendropicos fuscescens	Campetherini	FMNH 384481*	Uganda	DQ352450	AF394334	DQ361288
Dendropicos griseocephalus	Campetherini	ZMUC 115454	Tanzania	DQ352429	DQ352400	DQ361302
Dendropicos pyrrhogaster	Campetherini	FMNH 396512*	Uganda	DQ352449	DQ352387	DQ361308
Dinopium javanense	Picini	NRM 20026532*	Captive	DQ352421	DQ352406	DQ361305
Dryocopus lineatus	Campephilini	NRM 967106*	Paraguay	DQ352439	DQ352394	DQ361291
Dryocopus martius	Campephilini	MNHN C30*	France	DQ188140	DQ188114	DQ188166
Gecinulus grantia	Picini	MNHN 5-16	Lao RDP	DQ352444	DQ352407	DQ361300
Geocolaptes olivaceus	Campetherini	UWBM 53192*	South Africa	DQ352440	DQ352408	DQ361281
Hemicircus canente	Meiglyptini	MNHN JF317	Cambodia	DQ352416	DQ352415	DQ361312
Meiglyptes tristis	Meiglyptini	LSUMZ B-36352*	Malaysia	DQ352425	DQ352386	DQ361310
Melanerpes carolinus	Melanerpini	USNM 626309*	USA	DQ352424	DQ352401	DQ361284
Melanerpes flavifrons	Melanerpini	NRM 967085*	Paraguay	DQ352437	DQ352393	DQ361286
Melanerpes herminieri	Melanerpini	MNHN K14*	France	DQ352445	DQ352385	DQ361304
Mulleripicus funebris	Meiglyptini	ZMUC 114105	Philippines	DQ352433	DQ352409	DQ361311
Picoides mixtus	Campetherini	NRM 976765*	Paraguay	DQ188151	AF394324	DQ188177
Picoides tridactylus	Campetherini	ZMUC 115007	Poland	DQ188138	AF394332	DQ188164
Picoides villosus	Campetherini	FMNH 428786*	USA	DQ352431	DQ352391	DQ361290
Piculus chrysochloros	Colaptini	NRM 966938*	Paraguay	DQ352442	DQ352392	DQ361309
Piculus rivolii	Colaptini	ZMUC 114108	Peru	DQ352435	AF240015	DQ361278
Picus canus	Picini	MNHN 05–09	Lao RDP	DQ188156	DQ188130	DQ188182
Picus chlorolophus	Picini	USNM 620432*	Myanmar	DQ352448	DQ352410	DQ361293
Picus flavinucha	Picini	USNM 620313*	Myanmar	DQ352427	DQ352411	DQ361289
Picus mentalis	Picini	LSUMZ B-36478*	Malaysia	DQ352446	AY279221	DQ361297
Picus viridis	Picini	MNHN C38*	France	DQ188155	DQ188129	DQ188181
Reinwardtipicus validus	Picini	LSUMZ B-38653*	Malaysia	DQ352443	DQ352412	DQ361283
Sphyrapicus ruber	Melanerpini	USNM 621107*	USA	DQ352434	DQ352413	DQ361285
Veniliornis nigriceps	Colaptini	ZMUC 115548	Bolivia	DQ352430	DQ352402	DQ361287
Picumninae						
Picumnus cirratus	Picumnini	NRM 996693*	Paraguay	AY816219	DQ188124	AY816227
Picumnus innominatus	Picumnini	MNHN 4-2H	Thailand	DQ188145	DQ188119	DQ188171
Sasia africana	Picumnini	MNHN 03-05	Cameroon	DQ188149	DQ188123	DQ188175
Sasia ochracea	Picumnini	NRM 947313	Vietnam	DQ188136	DQ188110	DQ188162
Jynginae						
Jynx torquilla	Jynginae	MNHN 15-03	China	DQ188146	DQ188120	DQ188172
Outgroup						
Indicator minor	Indicatoridae	ZMUC 115456	Tanzania	DQ188132	DQ188106	DQ188158

Abbreviations: AMNH, American Museum of Natural History, New York; FMNH, Field Museum of Natural History, Chicago; LSUMZ, Museum of Natural Science, Louisiana State University, Baton Rouge; MNHN, Muséum National d'Histoire Naturelle, Paris; NRM, Swedish Museum of Natural History, Stockholm; USNM, United States National Museum, Washington; UWBM, University of Washington, Burke Museum, Seattle; ZMUC, Zoological Museum University of Copenhagen.

in both forward and reverse directions with the same primers used for PCR amplifications and finally run on an automated CEQ2000 DNA Analysis System sequencer (Beckman Coulter). We obtained sequences from both strands of DNA for all taxa.

No length variations between alleles were detected for myoglobin intron-2 and β-fibrinogen intron-7. The occurrence of single nucleotide polymorphism (SNP) in the myoglobin intron-2 and β-fibrinogen intron-7 sequences was suggested by the presence of double peaks. Those double peaks were coded using the appropriate IUPAC code. Absence of insertions, deletions and stop-codon in the reading frame of the protein-coding ND2 gene suggest that we had not amplified nuclear pseudogenes (Sorenson & Quinn 1998). Multiple alignments of intron sequences were accomplished by hand using SE-AL V1.0AL (Sequence Alignment Editor Version 1.0 alpha 1; Rambaut 1996) after an initial alignment by Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Gaps were treated as missing data. Alignment of the two introns were deposited in the EMBL database (Accession numbers ALIGN_000963 and ALIGN_000964 for myoglobin intron-2 and β-fibrinogen intron-7, respectively).

Phylogenetic inferences

We used model-based approaches (maximum likelihood [ML] and Bayesian inference [BI]), as implemented in PHYML v. 2.1b (Guindon & Gascuel 2003) and MrBayes v. 3.1 (Ronquist & Huelsenbeck 2003), to reconstruct the phylogenies. Likelihood models were estimated with MrModeltest v. 2.0 (Nylander 2004) using the Akaike Information Criterion. Bootstrapping in ML was performed in PHYML v. 2.1b (100 replicates with the same model as for the tree search) (Guindon & Gascuel 2003). Bayesian analyses for the concatenated data set were only performed allowing different parameters (base frequencies, rate matrix, shape parameter, proportion of invariable sites) to vary between the three or five partitions (ND2 or each codon position of ND2, myoglobin intron-2, β-fibrinogen intron-7), using the *prset* and *unlink* options.

Models selected were: GTR + γ + I for ND2, GTR + γ + I for 1st codon position, GTR + γ + I for 2nd codon position, GTR + γ for 3rd codon, K80 + γ for myoglobin intron-2 and GTR + γ for β-fibrinogen intron-7. Three incrementally heated and one cold Metropolis-coupled MCMC chains were run for $3*10^6$ generations with trees sampled every 100 generations. The first $2.5*10^5$ generations were discarded ('burn-in' phase) and the posterior probabilities were estimated for the trees saved during the remaining generations. Four independent Bayesian runs initiated from random starting trees were performed for each data set and the log-likelihood values and posterior probabilities were checked to ascertain that the chains reached stationarity.

We checked for significant incongruence between the individual gene trees by comparing the topologies and nodal support obtained under different analytical methods (ML, BI). Criteria for incongruence were set at 70% for the bootstrap values and at 0.95 for posterior probabilities.

Biogeography

To infer ancestral areas and habitats of the Picinae, we categorized biogeographical areas in two (Old World, New World) or four discrete characters (Africa, Eurasia, Nearctic and Neotropics) and habitat in two (tropical, temperate) discrete characters. As we did not sample all woodpecker species, we had to assign a biogeographical area to each operational taxonomic unit that represents a species group (or genus) in our study. *Dendrocopos minor* and *Picoides tridactylus* were both assigned to the New World despite the fact that they are also present in (*P. tridactylus*) or endemic to (*D. minor*) the Old World. *D. minor* is member of a clade of four species of which three (*Picoides pubsecens*, *P. scalaris*, *P. nuttallii*) are Nearctic endemics (Weibel & Moore 2002a, 2002b). *P. tridactylus* is present in both the Palearctic and the Nearctic biomes but a second, sister, species (*P. arcticus*) is endemic to the Nearctic. Further references for area assignments are listed in the Supplementary Material. The biogeographical characters were not included in the phylogenetic analyses, and were added to the matrix only for the biogeographical analyses. Proximate outgroups (Jynginae, Picumninae and Indicatoridae) were also coded to have reliable estimates of the ancestral states in the basal nodes. We used MrBayes v. 3.1 to reconstruct the ancestral areas and habitats of the woodpeckers. This method takes account of both phylogenetic and mapping uncertainty (see Ronquist 2004 for a review). The model specified for the biogeographical and habitat partitions was the Mkv model (Lewis 2001). In addition, we also mapped the biogeographical and habitat characters onto the tree using a parsimony algorithm, with the assistance of MacClade 4.0 (Maddison & Maddison 2000).

Divergence times

Bayesian approaches for the divergence time estimates were performed using the MULTIDISTRIBUTE package (Thorne *et al*. 1998; Thorne & Kishino 2002). We estimated different branch length variance-covariance matrices for our five partitions (three codon position of ND2, myoglobin, fibrinogen) using ESTBRANCHES. Outputs were then analysed simultaneously by MULTIDIVTIME. The Markov Chain Monte Carlo analyses followed the default settings of the software and as priors we set the distance between the tip and the root to 45 Myr (± 22.5 Myr). This date corresponds to the estimation of the split between the the Indicatoridae and the Picidae by Sibley & Ahlquist (1990), assuming that ΔT_{50} H 1.0 corresponds to 4.5 Myr (Sibley & Ahlquist 1990).

The prior for the substitution rate per site per Myr at the root node was empirically set to 0.0185 (± 0.0185). The

Bayesian topology obtained with the concatenated data set partitioned by gene and codon position was specified for the dating analyses. The calibration points used were the splits between *Sasia africana* and *S. ochracea* (7.8–8 Myr BP) and between *Picumnus innominatus* and *P. cirratus* (7.8–8 Myr BP). These two dates were obtained by a previous study which specifically focused on piculets (Fuchs *et al*. 2006) and which used a calibration point of 4.5–5.5 Myr BP for the split between *S. ochracea* and *S. abnormis*. At that period, two important seaways (nearly 100 km wide) separated continental Eurasia (range of *S. ochracea*) from Borneo and the Thai-Malay peninsula south of the Isthmus of Kra (range of *S. abnormis*) (Woodruff 2003). We previously hypothesized that this seaway promoted the differentiation between *S. abnormis* and *S. ochracea*, two species with poor inferred dispersal capacities (tiny size, short and rounded wings, short tail). We acknowledge that the primary calibration point may still be open to debate, but its use should not alter one of our major points of interest, the synchrony of splits between continental stocks.

Results

We obtained between 584 (*Dendropicos fuscescens*) and 682 bp (*Picoides tridactylus*) for the myoglobin intron-2, resulting in alignment of 687 bp, of which 102 were informative. No bias in nucleotide composition was detected ($\chi^2 = 11.66$, d.f. = 153, $P = 1.0$). ML analysis yielded one topology (–ln = 3279.24). The four Bayesian runs (–ln = 3325.71 ± 1.82) converged toward the same 50% majority consensus rule tree. The ML and BI trees were very similar (differences concern poorly supported or polytomized nodes) (Fig. 1).

We obtained between 694 (*Reinwardtipicus validus*) and 860 bp (*Dendropicos elliotii*, *D. fuscescens*, *D. pyrrhogaster*, *Picoides mixtus*) for the β-fibrinogen intron-7, resulting in alignment of 951 bp. We excluded nucleotides 24–35 and 450–461 in our deposited alignment due to uncertainties in primary homology assessment. After exclusion of these regions, the alignment was 927 bp long, of which 161 bp were informative. No bias in nucleotide composition was detected ($\chi^2 = 17.10$, d.f. = 153, $P = 1.0$). ML analysis yielded one tree (–ln = 4693.15). All Bayesian analyses converged toward the same 50% majority consensus rule tree (–ln = 4737.30 ± 1.99) (Fig. 2).

Of the 1041 bp of the ND2 gene, 558 were parsimony informative. No bias in nucleotide composition was detected for the whole gene ($\chi^2 = 79.01$, d.f. = 153, $P = 1.0$) or for first and second codon positions ($\chi^2 = 34.78$, d.f. = 153, $P = 1.0$ and $\chi^2 = 10.2$, d.f. = 153, $P = 1.0$). However, significant bias was detected for the third codon position ($\chi^2 = 212.41$, d.f. = 153, $P < 0.002$). ML analyses yielded one tree (–ln = –17067.74) topologically very similar to the non-partitioned ND2 Bayesian analyses (–ln = 17039.62 ± 1.34). Partitioned Bayesian analyses with the best fit model assigned to each codon position yielded a 50% majority consensus tree that only differed at a few nodes from the other ND2 analyses (–ln = 16472.81 ± 1.10) (Fig. 3).

The analyses of the concatenated data set recovered a well supported phylogeny of the woodpeckers where 41 out of the 46 (89%) nodes received posterior probabilities greater than 0.95 (partitioned by gene and codon position analyses; –ln = 24662.21 ± 1.26, Fig. 4). The Picinae were recovered as monophyletic in all analyses with posterior probabilities of 0.95 or larger (Figs 1–4). The results differ from the traditional taxonomy in that five (*Celeus*, *Colaptes*, *Dendrocopos*, *Picoides*, *Piculus*) out of 22 studied genera (24%) are strongly recovered as para- or polyphyletic. The monophyly of two further genera (*Picus* and *Dryocopus*) could not be clearly confirmed.

Our analyses revealed that the Asian genus *Hemicircus* is the sister group of all other woodpecker genera (PP = 1.0 and a one base pair deletion at position 853 of our fibrinogen alignment). Within the Picinae tree, the relationships of the New World genus *Campephilus* provided the only conflict observed between well-supported nodes in the gene trees. *Campephilus* clustered with the Indo-Malayan genera *Chrysocolaptes* and *Reinwardtipicus* in the analyses of the mitochondrial gene (PP = 1.0), with *Blythipicus* being the closest relative of the latter clade (PP = 0.60).

In contrast, the analyses of the fibrinogen sequences yielded support for a relationship of *Campephilus* with Clade A (PP = 1.0 and a one G insertion in position 598 of our fibrinogen alignment), a relationship also favoured by myoglobin, albeit not significantly supported (PP = 0.86). The relationships of *Blythipicus* were unresolved at the base of the tree in the fibrinogen analyses, while myoglobin provided support for a relationship of *Blythipicus* with the *Chrysocolaptes–Reinwardtipicus* clade (PP = 0.95). The concatenated analyses favoured the same relationships as the myoglobin, i.e. *Campephilus* is the sister group of Clade A (PP = 0.98) and *Blythipicus* as the sister group of *Chrysocolaptes–Reinwardtipicus* (PP = 0.98).

The remaining genera clustered in two main clades (Clades A and B). All the relationships within Clade A received posterior probabilities of 1.0. Clade A consists of two lineages: one (Clade C) includes two genera (*Sphyrapicus* and *Melanerpes*) with a New World distribution, while the other (Clade D) consists of the genera *Dendrocopos*, *Dendropicos*, *Picoides* and *Veniliornis*. Within Clade D, *Dendrocopos* and *Picoides* were recovered as polyphyletic. In contrast to Clade A, relationships between biogeographical assemblages within Clade B did not receive strong support. The genus *Celeus* was polyphyletic as the Asian species (*C. brachyurus*) clustered with other Asian genera (*Dinopium*, *Gecinulus* and *Meiglyptes*) while the two South American species grouped with the New World genera *Colaptes* and *Piculus*, the widespread genus *Dryocopus* and the Indo-Malayan genus *Mulleripicus* (Fig. 4). The monotypic *Geocolaptes* was the sister group of *Campethera* (PP = 1.0).

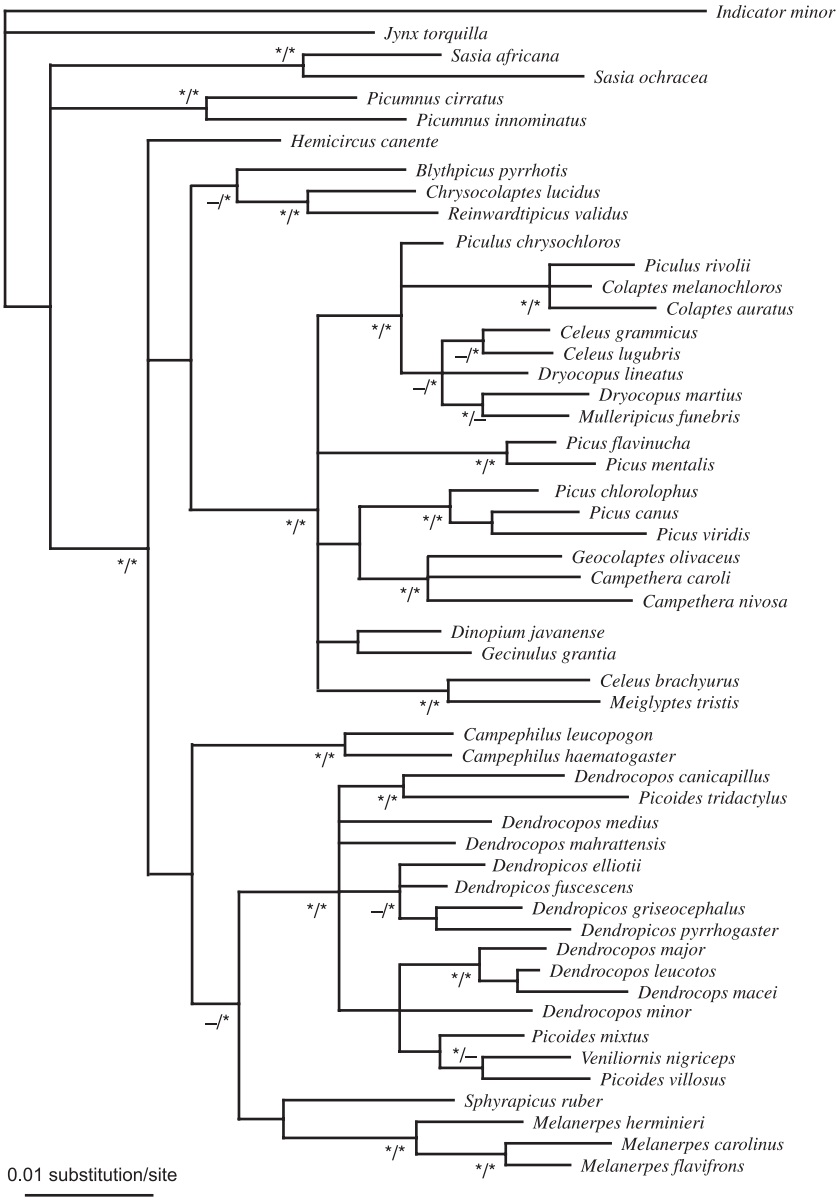

Fig. 1 Bayesian tree (mean of the four runs: –ln = 3325.71 ± 1.82) obtained from myoglobin intron-2. *Represents bootstrap values/posterior probabilities greater than 70/0.95.

Reconstruction of the ancestral habitats of the woodpeckers indicates that the group most likely originated in a Eurasian tropical environment (Fig. 5). We detected slight differences in inferring the ancestral area of Clade A and *Campephilus*. The two-state analyses indicated that Clade A and *Campephilus* originated through a single colonization event of the New World while the four-state analyses indicated one pantropical dispersal from Eurasia to South America for *Campephilus* and one subsequent dispersal from Eurasia to North America by the ancestor of Clade C. The second scenario is less likely and may be attributable to the fact that we could not include any of the two North American species of *Campephilus* (*C. principalis* and extinct *C. imperialis*).

Up to nine lineages, mostly terminal, independently changed to temperate habitats during their evolutionary history (*Colaptes auratus*, *Dryocopus martius*, *Geocolaptes olivaceus*, *Picus canus–P. viridis*, *Sphyrapicus ruber*, *Melanerpes carolinus*, *Picoides tridactylus*, *Dendrocopos medius*, *Dendrocopos leucotos–D. major*; Fig. 5 and Supplementary Material), while only one lineage switched back from a temperate to a tropical habitat (*Picoides mixtus–Veniliornis nigriceps*). The main results from the dating analyses are presented in Table 2 and Fig. 6.

Discussion

Our analyses revealed a well supported phylogeny of the woodpeckers, with nearly 90% of the nodes receiving posterior

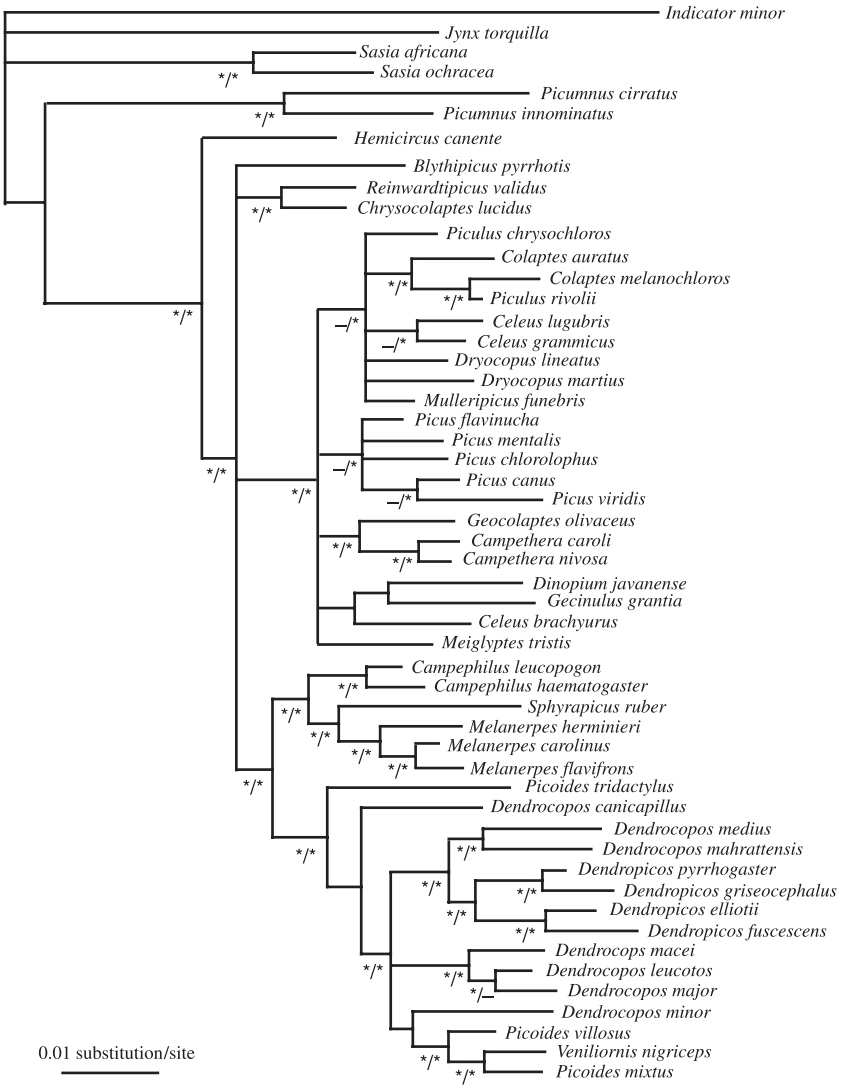

Fig. 2 Bayesian tree (mean of the four runs: –ln = 4737.30 ± 1.99) obtained from β-fibrinogn intron-7. *Represents bootstrap values/posterior probabilities greater than 70/0.95.

Table 2 Estimation of divergence times (in Myr) inferred from our analyses. Node names refer to Fig. 4.

Split	Date estimate	Biogeography
Hemicircus-other Picinae	13.4 ± 1.7	
Campephilus-Clade A	12.0 ± 1.5	Split Eurasia/New World
Clade B	8.4 ± 1.2	Splits Eurasia/Africa and Eurasia/New World
Clade E	5.3 ± 0.9	Split Eurasia/New World
Clade F	8.7 ± 1.4	Split Eurasia/New World
Clade G	6.9 ± 1.2	Splits Eurasia/Africa
Clade H	7.5 ± 1.2	Split Eurasia/New World
Clade I	5.0 ± 0.9	Split North-America/South-America

probabilities of 0.95 or above. Below, we discuss (1) the points of congruence and conflict between our hypotheses and two recent surveys of woodpecker molecular systematics (Webb & Moore 2005; Benz *et al*. 2006) and (2) the biogeographical history of the woodpeckers as inferred from our data.

Phylogeny and evolution of morphology

Our results are in agreement with several aspects of woodpecker systematics suggested in these recent studies. Among the groupings supported by Webb & Moore (2005), Benz *et al*. (2006) and our study are:

(1) The monophyly of our clades A and B, which, correspond, respectively, to the Dendropicini and Malarpicini *sensu* Webb & Moore (2005) and Benz *et al*. (2006);

(2) The sister-group relationships between our clades C and D;

(3) The monophyly of an assemblage containing the genera *Celeus*, *Mulleripicus*, *Dryocopus*, *Colaptes* and *Piculus*. Within this clade, we confirmed, using a taxonomic sampling that partially overlapped with those of Webb & Moore (2005) and Benz *et al*. (2006), that the New World genera *Colaptes* and *Piculus* are mutually paraphyletic in a complex manner. However, definitive taxonomic and biogeographical conclusions await an exhaustive sampling at the species level within these two genera.

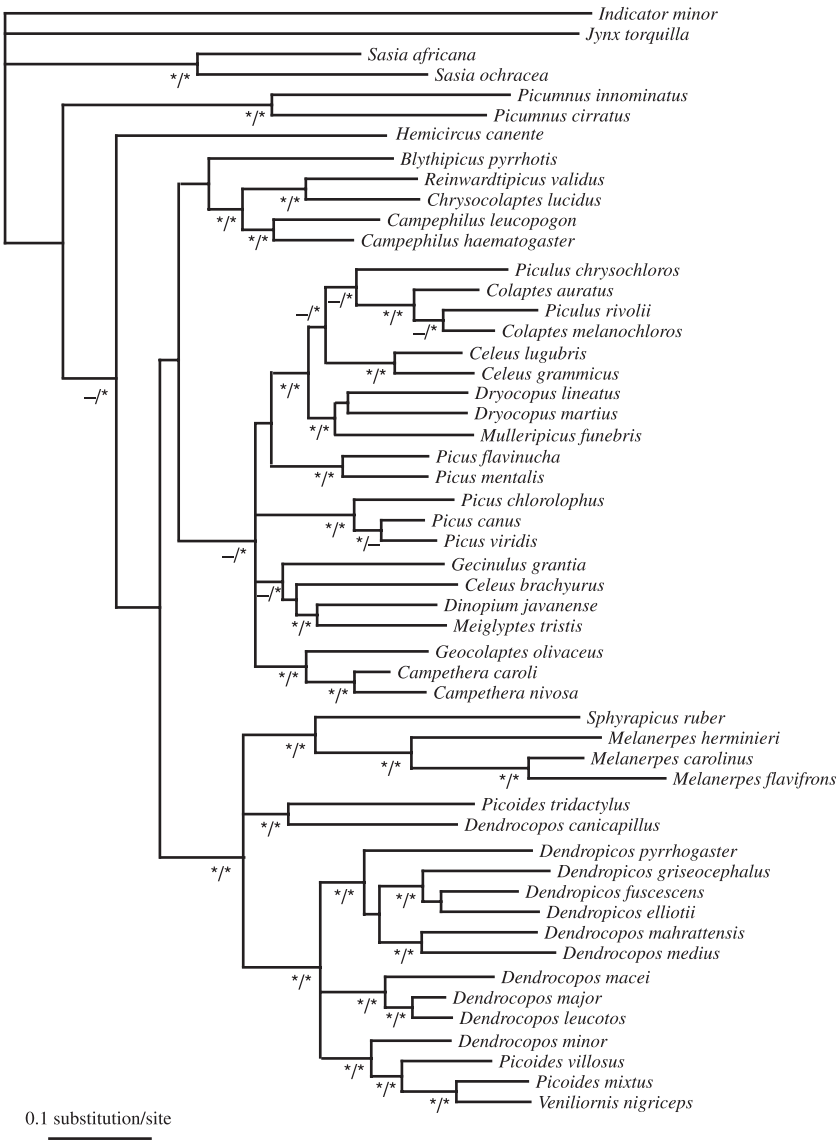

Fig. 3 Bayesian tree (mean of the four runs: –ln = 16472.81 ± 1.10) obtained from ND2 with the best-fitting model assigned to each codon position (partitioned by codon position analysis). *Represents bootstrap values/posterior probabilities greater than 70/0.95.

Celeus has for long been regarded as atypical among woodpeckers in that it is the only pantropically distributed genus, with one species endemic to Indo-Malaya (subgenus *Micropternus*) and ten to South America (subgenus *Celeus*). The Indo-Malayan species *C. brachyurus* clustered with three Asian genera (*Meiglyptes*, *Dinopium* and *Gecinulus*), while the South American species (*C. lugubris* and *C. grammicus*) are only distantly related to this clade. This result was also recovered by Benz *et al*. (2006) with the sampling of two other species of South American *Celeus* (*C. loricatus* and *C. flavescens*). The four Indo-Malayan taxa (*Celeus brachyurus*, *Meiglyptes*, *Dinopium* and *Gecinulus*) vary in their plumage and foraging behaviour; *Dinopium* and *Gecinulus* excavate holes in trees or bamboos to search for insect larvae, while *Meiglyptes* and the Asian *Celeus* (hereafter called *Micropternus*) feed on ants and termites on branch tips (Styring 2002). The different foraging behaviours are reflected in differences in bill shape, proportions of toes, tarsus and tails. We estimated the basal diversification of this Indo-Malayan clade at 7.6 ± 1.2 Myr BP. The uplift of the Himalayan-Tibetan plateau 8–10 Myr BP led to a gradually more humid climate in response to the intensified Asian monsoons (Zisheng *et al*. 2001). This would have favoured the formation of more complex ecological niches and thus the diversification in morphology and foraging behaviour observed in this Asian clade. This statement is further supported by the studies of Styring (2002) and Styring & bin Hussin (2004), who showed that tropical rainforests in Malaysia offer a higher number of microhabitats and

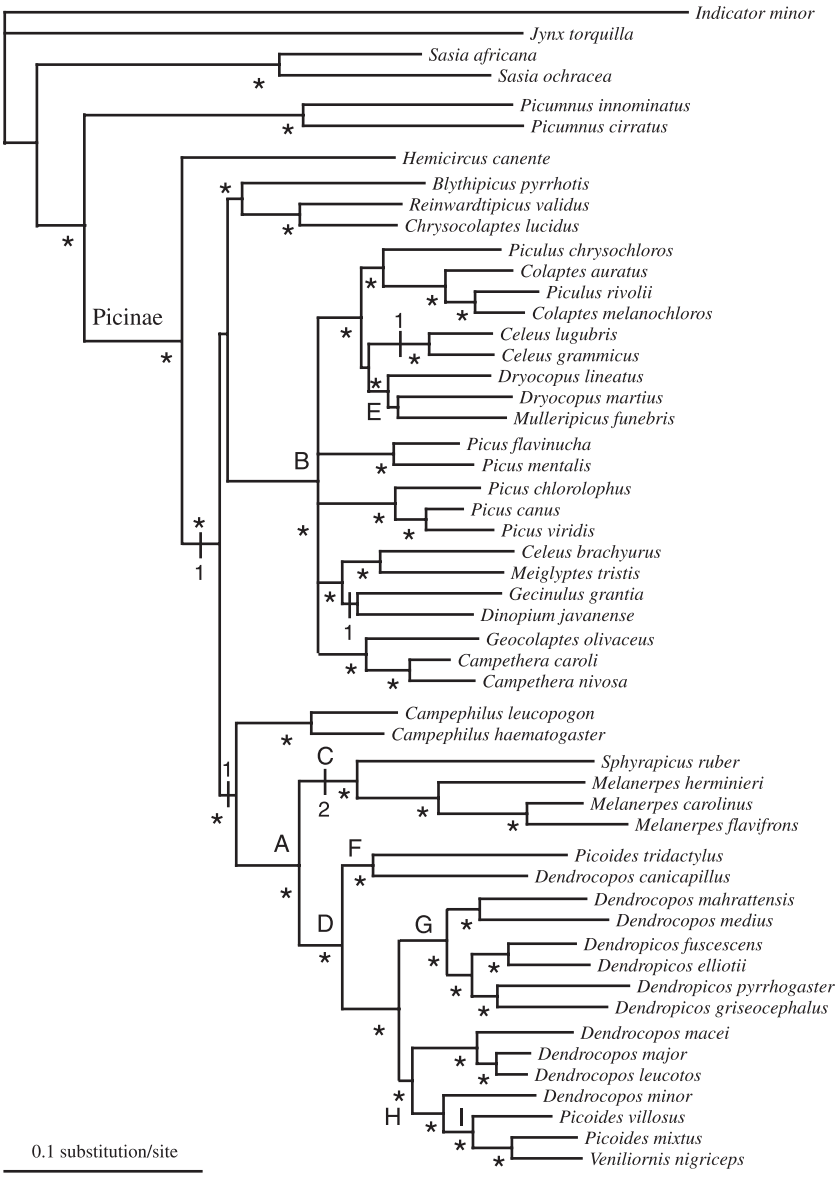

Fig. 4 Bayesian tree (mean of the four runs: –ln = 24662.21 ± 1.26) obtained from the concatenated data set partitioned by gene and codon position. The optimal model parameterization as estimated by MrModeltest was assumed for each partition. *Represents posterior probabilities greater than 0.95. Vertical bars and numbers (corresponding to indel length) on branches indicate synapomorphic insertion/deletion events for the Picinae. A one G insertion in a G rich region (at position 361 in our myoglobin alignment) supports both the *Dinopium/Gecinulus* and New World *Celeus* clades. Autapomorphic indels are not indicated.

ecological niches available to woodpeckers than do forests in temperate regions.

Further comparison of the present work with the molecular studies of Webb & Moore (2005) and Benz *et al*. (2006) is difficult due to their use of a composite sequence for *Geocolaptes*, and differences in sampling (genes and taxa) and analytical strategies.

Use of a composite sequence

Our ongoing work on the phylogeny and biogeographical history of the Old World genus *Picus* (Fuchs *et al*. in prep.) revealed that the cyt *b* sequence of *Geocolaptes olivaceus* (AY940801) published by Webb & Moore (2005) and subsequently used by Benz *et al*. (2006) is very different from the one we produced (52 substitutions on a 415 bp fragment, 12.5% divergence). Two hypotheses may explain this result: (1) amplification and sequencing of a nuclear pseudogene, or (2) sample mix-up and contamination. The affinities of the cyt *b* sequence of *Geocolaptes* we produced are identical to those obtained for all other mitochondrial and nuclear loci we sequenced and analysed from here, i.e. *Geocolaptes* is closely related to *Campethera* (Fuchs *et al*. in prep.). A BLAST analysis of the *Geocolaptes* AY94080 sequence revealed that its closest sequence is a cyt *b* sequence of *Dendrocops minor* (AF389318) that differs by only five transitions. Thus, the cyt *b* sequence of *Geocolaptes* (AY940801) produced by Webb & Moore (2005)

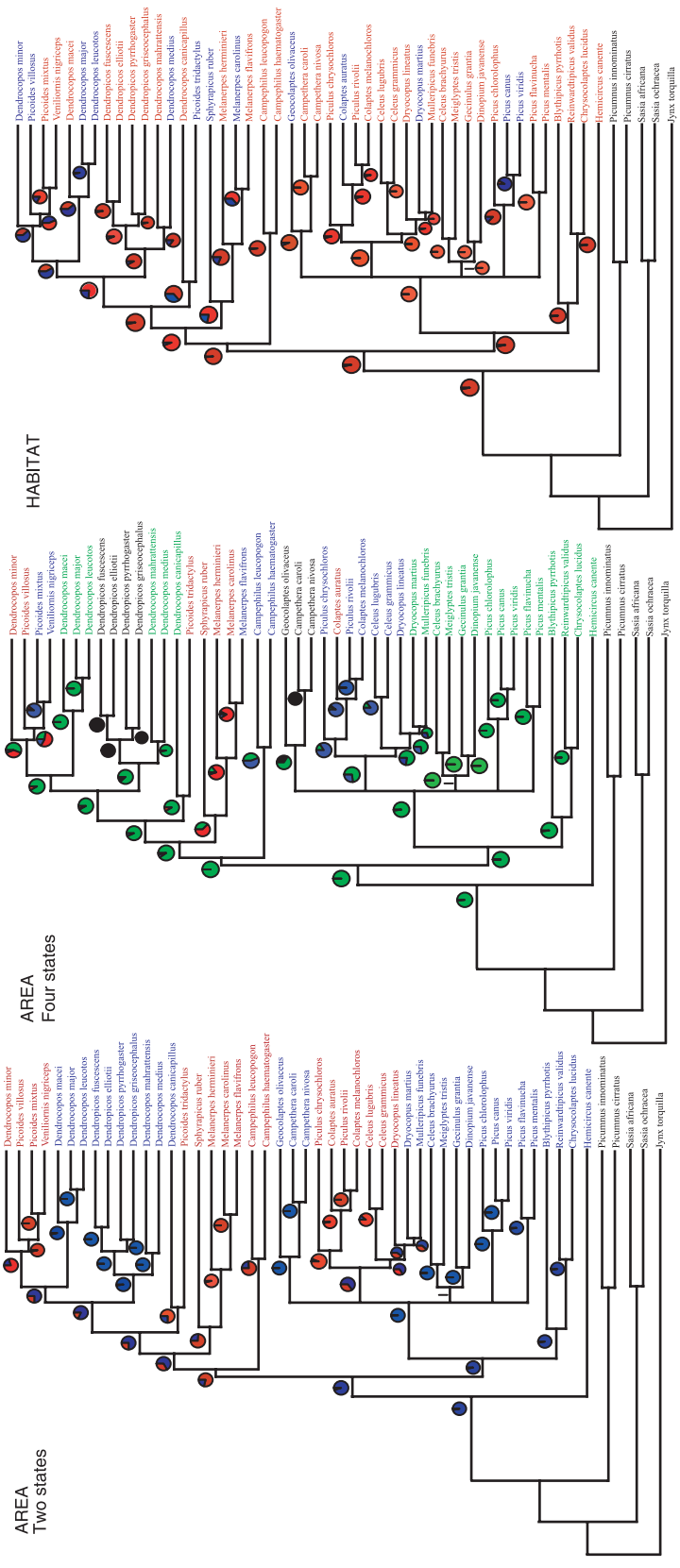

Fig. 5 Ancestral areas (left and centre) and habitats (right) inferred using the Bayesian reconstruction method of ancestral states. Pie charts on the left tree indicate the probabilities that the common ancestor was distributed in the Old World (blue) or New World (red). The charts on the centre tree indicate the probabilities that the common ancestor was Eurasian (green), African (black), North American (red) or South American (blue). The charts on the right tree indicate the probabilities that the common ancestor was a tropical (red) or a temperate (blue) species. Coding states are indicated by the colour of the taxon name. Pie charts are only indicated for the Picinae.

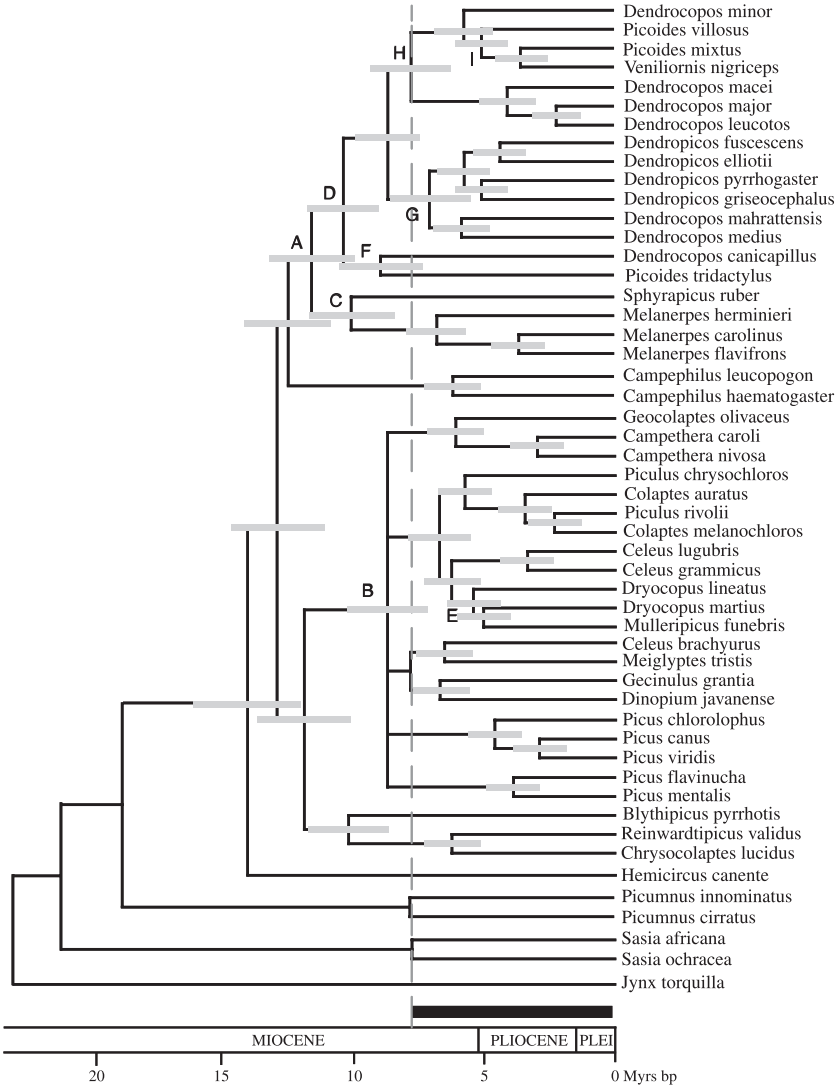

Fig. 6 Chronogram based on the Bayesian tree. Grey bars represent the standard deviations around mean. The black line above the time scale indicates the period of the formation of the Northern ice sheets, the expansion of the C4 grasses, and the uplift of the Tibetan plateau (Zachos *et al*. 2001; Zisheng *et al*. 2001). Letters A-G refer to the clades defined in Fig. 4 and Table 2.

likely represents a sample mix-up, contamination or an error in sequence file processing involving *D. minor*, a species already sequenced in their laboratory (Weibel & Moore 2002a,b). As a consequence, interpretation of the phylogenetic relationships within Clade B (Malarpicini) in the studies of Webb & Moore (2005) and Benz *et al*. (2006) should be tentative since it is not known to what extent the use of this incorrect sequence altered the phylogenetic reconstruction.

Differences in genes and sampling strategies

Webb & Moore (2005) used sequences from three mitochondrial loci (cyt *b*, CO1 and 12S) for 26 species representing 17 genera; Benz *et al*. (2006) analysed three loci (nuclear β-fibrinogen intron-7 and mitochondrial cyt *b* and ND2) for 35 species and 21 genera. We gathered sequences from two nuclear introns (β-fibrinogen intron-7 and myoglobin intron-2) that are situated on different chromosomes in the chicken genome (myoglobin is on chromosome 1 and β-fibrinogen is on chromosome 4) and one mitochondrial gene (ND2) for 46 species and 22 genera. Unlike Webb & Moore (2005) and Benz *et al*. (2006), we also analysed each gene individually to detect incongruences between molecular markers. As a by-product of these different strategies, our analyses revealed the existence of five major and strongly supported lineages within the Picinae (*Hemicircus*, *Campephilus*, *Blythipicus–Reinwardtipicus–Chrysocolaptes*, Clade A, Clade B), instead of the three previously recognized (Malarpicini, Dendropicini, and Megapicini) (Webb & Moore 2005; Benz *et al*. 2006).

In addition, we also detected a conflict between the nuclear and mitochondrial genomes concerning the relationships of the New World genus *Campephilus*. All the mitochondrial genes analysed favoured a relationship of *Campephilus* as the

sister group of the Indo-Malayan *Chrysocolaptes–Reinwardtipicus* clade (Webb & Moore 2005; this study). In contrast, the two nuclear introns we analysed favoured a relationship of *Campephilus* as sister group to Clade A (myoglobin intron-2) or even nested within it (fibrinogen intron-7).

A conflict between results based on the maternally inherited mitochondrial genome vs. bi-parentally inherited nuclear genome may be explained by the occurrence of ancestral hybridization in the early evolution of the woodpeckers followed by random lineage sorting. If so, it would have involved an ancestor of the genus *Campephilus* and an ancestor of the *Chrysocolaptes–Reinwardtipicus* clade (assuming that *Campephilus* is closer to Clade A) or an ancestor of Clade B (assuming that *Campephilus* is the sister group of *Chrysocolaptes–Reinwardtipicus*). As our two nuclear introns, situated on different chromosomes in the chicken genome, favoured the relationship of *Campephilus* with the Clade A, we regard this hypothesis as the most likely. Analyses of additional nuclear genes and taxa, including genes situated on the sex-linked Z-chromosome, will help to resolve this issue and is currently the focus of our work.

Another result that is in conflict with that of Benz *et al.* (2006) concerns the monophyly of the genus *Picus*. We sampled five species, the same three as Benz *et al.* (2006), as well as *P. chlorolophus* and *P. flavinucha*. Our analyses revealed that the two main lineages of *Picus* might not form a monophyletic assemblage. Indeed, myoglobin suggests that a subset of the genus *Picus* (*P. chlorolophus*, *P. canus and P. viridis*) is related to the African genera *Campethera* and *Geocolaptes* and this is further supported by an eight nucleotide deletion in the myoglobin sequences (positions 399–406 in our alignment, but an alternative alignment with no consequences on the inferred phylogenetic relationships exists). The ND2 gene does not support the monophyly of the genus *Picus*, albeit posterior probabilities for polyphyly were below the 0.95 threshold (Fig. 3).

On the other hand, fibrinogen strongly suggests that *Picus* is monophyletic (PP = 1.0 and replacement of a 128 bp fragment by an 18 bp fragment in the fibrinogen sequences). Monophyly of *Picus* was strongly recovered in Benz *et al.* (2006). However, given that these authors did not perform a preliminary analysis of each gene independently, it is not possible to evaluate the contribution of each gene to the monophyly. From our data on ND2 and fibrinogen, it is likely that almost all the signal for the monophyly of *Picus* in Benz *et al.* (2006) is attributable to the fibrinogen sequences. The pattern of molecular evolution within *Picus* is currently under consideration using an exhaustive taxonomic sampling at the species level and sequencing of eight loci (Fuchs *et al.* in prep.).

Biogeography

Benz *et al.* (2006) identified the monotypic Antillean Piculet (*Nesoctites micromegas*) as the sister group of the whole Picinae radiation. *Nesoctites* does not possess the synapomorphic stiffened tail feathers as well as the several specializations of the Picinae for excavating cavities in hard substrates (Swierczewski & Raikow 1981). We downloaded the ND2 and β-fibrinogen intron-7 sequences from GenBank (DQ479163 and DQ479231; myoglobin was not sequenced by Benz *et al.* 2006), published during the course of the present study, and performed complementary analyses to estimate the impact of *Nesoctites* on the reconstruction of the biogeographical history of the Picinae.

Bayesian analyses performed on the concatenated data set indicate that *Nesoctites* is the sister group of the Picinae (–ln = 25188.74, PP = 0.75, tree not shown). The addition of *Nesoctites* to the data set had no impact on the reconstruction of the biogeographical history within the Picinae (data not shown), and the ancestral area was still inferred to be Old Word in both the two-state (PP = 0.91) and four-state (PP = 0.94) biogeographical analyses. Thus, a possible explanation is that *Nesoctites* represents a relictual lineage, isolated from the Eurasian Picinae at the end of Mid-Miocene Climatic Optimum (Zachos *et al.* 2001), that only persists in the Caribbean and whose closest relatives disappeared when the more competitive Picinae colonized the New World from Eurasia.

Our molecular dating and biogeographical analyses revealed that the modern Picinae started to diversify 13.4 ± 1.7 Myr BP in Eurasia (Figs 5 and 6), at a period following the Mid-Miocene Climatic Optimum (Zachos *et al.* 2001). Two main diversification bursts subsequently occurred within the woodpeckers, a first one that led to the four remaining major clades (clades A and B, *Campephilus* and *Blythipicus–Chrysocolaptes–Reinwardtipicus*) estimated at 12 Myr BP, and a second one that led to the modern genera, *c.* 8 Myr BP.

The African stock of woodpeckers is the result of two independent colonizations from Eurasian ancestors (nodes B and G, Fig. 5), the first involving the *Dendropicos* lineage, and the second the genera *Geocolaptes* and *Campethera* (Fig. 4). The estimates of the splits between *Geocolaptes–Campethera* and *Dendropicos* with their respective closest relatives are 8.4 ± 1.2 Myr and 6.9 ± 1.2 Myr, respectively. Thus, taking standard deviations into account, we cannot exclude simultaneous colonizations of Africa by the two Picinae lineages. A similar date was obtained for the split between the African and Indo-Malayan *Sasia* piculets (Fuchs *et al.* 2006). Three out of the six splits between Eurasian and New World clades also occurred at the same period (nodes B: 8.4 ± 1.2 Myr BP, F: 8.7 ± 1.4 Myr BP and H: 7.5 ± 1.2 Myr BP on Fig. 4). These dates are contemporary with the estimated split between the Asian and South American *Picumnus* piculets (7.9 ± 0.1 Myr BP) (here used as a calibration point; see also Fuchs *et al.* 2006).

As all these lineages have similar habitat requirements, simultaneous splits from their respective closest relatives are not unlikely. Thus, our data suggest that African, Indo-Malayan and New World assemblages were isolated at the same time

from each other and that further diversification has mainly occurred *in situ*. Our estimates for the divergences between continental lineages are close to the period of formation of the Northern ice sheets, which resulted in a global increase in aridity and seasonality that favoured the spreading of the C4 grasses throughout the world *c.* 8 Myr BP (Flower & Kennett 1994; Morgan *et al*. 1994), as well as the onset of recurrent desert conditions in the Sahara desert *c.* 7 Myr BP (Schuster *et al*. 2006). The expansion of grasslands may in turn have resulted in large, nonforested areas in Eurasia and desert conditions in Africa that prevented gene flow between woodpeckers adapted to tropical conditions in Africa, Indo-Malaya and the New World.

Our estimates for the colonization of South America by the *Picoides mixtus–Veniliornis* lineage (node E) and *Celeus–Colaptes–Piculus* lineage (node A) are 5.0 ± 0.9 Myr BP and 8.4 ± 1.2 Myr BP, respectively. Thus, our data suggest that the colonization events of South America were not synchronous and probably occurred before the closure of the Panama Isthmus (around 3.5 Myr BP) (Keigwin 1978). Studies of terrestrial organisms have suggested that faunistic exchanges between North and South America were common also before the closure of the Panama Isthmus (Zeh *et al*. 2003; Garcia-Moreno *et al*. 2006). Early dispersals between North and South America suggest the existence of a rather short-lived, terrestrial corridor at the end of the Miocene, 5.7 Myr BP (Bermingham & Martin 1998). Our time estimates for two of the four lineages involving South and North American taxa corroborate the hypothesis of a terrestrial corridor in the Miocene. For the two remaining New World lineages (*Campephilus* and *Melanerpes*), our sparse taxonomic sampling prevents us from drawing any conclusions regarding dispersal times between North and South American lineages.

The colonizations of temperate habitats occurred more or less synchronously for the Eurasian lineages, as most lineages with a West Palearctic distribution (*D. martius*, *D. medius*, *D. leucotos-D. major*, *P. viridis-P. canus*) split from their tropical sister taxa at *c.* 5 Myr BP, close to the Miocene–Pliocene boundary (*Dryocopus*: 5.0 ± 0.9 Myr BP, *D. medius* 5.7 ± 1.1 Myr BP, *D. leucotos–D. major* 3.9 ± 0.9 Myr BP, *P. viridis–P. canus* 4.5 ± 0.8 Myr BP, Fig. 5). In contrast, the temperate habitats in the New World seems to have been colonized multiple times (for example, *Sphyrapicus* split from their closest relatives at 9.9 ± 1.5 Myr BP, while *Colaptes auratus* split at 3.4 ± 0.7 Myr BP).

We acknowledge the following points. First, that the precise timing of the events we have highlighted throughout this study may be open to question given the hypotheses proposed for our primary calibration point and the lack of clearly reliable fossil information. Second, that our primary calibration point (split *Sasia ochracea/S. abnormis*) should be cross-validated using an independent calibration point. Nevertheless, our conclusions on the relative time divergences and synchrony of splits between continental lineages are not sensitive to the calibration point and should thus be interpreted as indicating that the same external factor promoted the differentiation between continental stocks of woodpeckers.

Conclusion

The Picinae represents a recent and species-rich clade, the diversification and radiation of which was probably initiated by the exploitation of a new ecological niche, the excavation of cavities to search for insect larvae. We suggest that most modern genera of woodpeckers evolved *c.* 8 Myr BP. The taxonomic diversification of woodpeckers at this time may be attributed to the fragmentation of forests in response to the drier climate, which in turn synchronously prevented gene flow between tropical stocks in Africa, Indo-Malaya and the New World. The phylogeny and relative time frame proposed here may serve to resolve other evolutionary questions involving, for example, competition with groups of gleaning and probing organisms.

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