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Molecular support for a rapid cladogenesis of the woodpecker clade Malarpicini, with further insights into the genus *Picus* (Piciformes: Picinae)

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ABSTRACT

Previous studies have suggested that the woodpecker genus *Picus* (Aves: Picidae) may not be monophyletic. In order to evaluate this hypothesis, we analyzed DNA sequences from all but two species of *Picus*, as well as from representatives of all genera in the tribe Malarpicini, within which *Picus* is nested. We sequenced seven loci (four autosomal, one Z-linked and two mitochondrial) with different evolutionary dynamics. The species currently placed in *Picus* fall into two subclades that may not form a monophyletic assemblage. Consequently, we propose to place *miniaceus* Pennant 1769, *flavinucha* Gould 1834 and *mentalis* Temminck 1825 in the genus *Chrysophlegma* Gould, 1850, while the remaining species are retained in *Picus*. The inclusion in our study of representatives of all genera included in the tribe Malarpicini, a group of woodpeckers which has proven difficult to resolve in several previous molecular studies, also allowed us to determine the earliest divergences within this clade. The results suggest that the low level of basal resolution in Malarpicini is attributable to multiple cladogenetic events in a short period of time rather than insufficient character sampling. This conclusion is supported by the observation of nucleotide insertion–deletions that support mutually exclusive phylogenetic hypotheses in different gene trees. We attribute this pattern of incongruent indels, together with short internodes in the tree, to incomplete lineage sorting.

1. Introduction

The higher-level systematics of woodpeckers (Piciformes: Picinae) has received much attention in recent years (Weibel and Moore, 2002a,b, 2005; Benz et al., 2006; Fuchs et al. 2006, 2007). A comparison of the topologies recovered in these studies reveals that the overall pattern is highly consistent and that several traditionally recognized genera are not monophyletic (e.g. *Celeus*, *Colaptes*, *Dendrocopos*, *Picoides*, *Piculus*). Nevertheless, several aspects of the woodpecker radiation remain to be clarified using an expanded sampling of both taxa and loci.

One case that requires further phylogenetic study is the absence of resolution within the clade Malarpicini coupled with the uncertainties surrounding the monophyly of the genus *Picus* (Webb and Moore, 2005; Benz et al., 2006; Fuchs et al., 2007). The genus *Picus* (15 species), endemic to the Palearctic and Indo-Malayan bio-regions, is homogeneous in overall morphology and proportions, albeit highly variable in plumage patterns. Based on plumage, two species groups could be intuitively recognized within *Picus*: a 'yellow-naped' group that contains species that are endemic to the tropical forests of the Indo-Malayan region, and a 'red-crowned' group occurring in both tropical and temperate forests of Eurasia (Palearctic and Indo-Malayan regions). Despite large variation in plumage patterns, monophyly of *Picus* has not been questioned, although some authors have recognized subgeneric divisions (e.g. Wolters, 1975–1982). Recent molecular studies have challenged this view; using sequences from three mitochondrial genes (12S, cytochrome *b* and CO1), Webb and Moore (2005) suggested that at least one species from the yellow-naped assemblage (*Picus miniaceus*) is closer to the Indo-Malayan genus *Meiglyptes*, whereas the sampled representative of the red-crowned species group (*P. canus*) clustered with the African endemic *Campethera*, a genus in which several species are green in plumage and have a red-crown. However, neither of these hypotheses received strong

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support. Fuchs et al. (2007) included five species of *Picus* in their study of woodpecker relationships based on one mitochondrial gene (ND2) and two nuclear introns (myoglobin intron 2 and β-fibrinogen intron 7). Neither the genus *Picus* nor the yellow-naped species group was recovered as monophyletic; the lesser yellow-nape (*P. chlorolophus*) being closer to the sampled members of the red-crowned species group (*P. canus* and *P. viridis*) than to the greater and checker-throated woodpeckers (*P. flavinucha* and *P. mentalis*). Contrary to previous molecular studies, Benz et al. (2006), who sampled three species (*P. mentalis*, *P. canus* and *P. viridis*) that were recovered as polyphyletic by Webb and Moore (2005) and Fuchs et al. (2007), recovered strong support for monophyly of *Picus* when analyzing sequences from two mitochondrial genes (ND2 and cytochrome *b*) and one nuclear intron (β-fibrinogen intron 7). By analyzing the loci individually, Fuchs et al. (2007) found that only one gene, β-fibrinogen intron 7, supported monophyly of *Picus*, while the myoglobin intron 2 data favoured polyphyly of *Picus* (although with low posterior probabilities). The data from the mitochondrial loci were inconclusive on this point. The conflicting results between these studies concerning the monophyly of *Picus* may be due to both differences in the sampling strategies of genes and taxa, and differences in analytical approach. Resolving the evolutionary history of the genus *Picus* thus requires both better taxonomic sampling and more sequence data.

In this paper we address the question of the monophyly of *Picus* by analyzing DNA sequences from seven loci for all but two species of the genus. We have also included representatives of eleven genera thought to be close to *Picus* (Fuchs et al., 2007), all placed in the tribe Malarpicini (*sensu* Webb and Moore, 2005). Malarpicini has been recovered with strong support in several studies (Webb and Moore, 2005; Benz et al., 2006; Fuchs et al., 2007), and there is also support for further divisions within Malarpicini that match the geographic distribution of taxa: an African *Geocolaptes–Campethera* clade, an Indo-Malayan *Micropternus–Dinopium–Meiglyptes–Gecinulus* clade, and an essentially New World *Colaptes–Piculus–Celeus–Dryocopus–Mulleripicus* clade. The more precise internal relationships of Malarpicini were left largely unresolved, however, and our taxonomic sampling may also provide more information about this.

2. Materials and methods

2.1. Taxonomic sampling

We included samples from all but two *Picus* species, *P. squamatus* and *P. vaillantii*. The latter taxon is endemic to northern Africa and is sometimes considered a subspecies of *P. viridis* (Winkler and Christie, 2002). *P. squamatus* is restricted to Afghanistan, Pakistan and Darjeeling (India). When possible, we sampled two individuals per species, choosing samples from the most distant parts of the breeding range. We also included representatives of all genera from the tribe Malarpicini, within which *Picus* is nested (Webb and Moore, 2005; Benz et al., 2006; Fuchs et al., 2007). As outgroups we used two species outside the Malarpicini, *Campephilus haematogaster* and *Picoides mixtus* (Webb and Moore, 2005; Benz et al., 2006; Fuchs et al., 2007). Taxonomic sampling and GenBank Accession Nos. are given in Table 1.

2.2. Laboratory procedures

We gathered sequence data from two mitochondrial protein-coding genes (cytochrome *b*, ATP6), four autosomal nuclear introns (myoglobin intron 2, β-fibrinogen intron 7, GAPDH intron 11, transforming growth factor β-2 intron 5) and one Z-linked nuclear intron (BRM intron 15) for nearly all taxa included in this study; for *P. miniaceus* we used only a cytochrome *b* sequence available from GenBank (AY940811). The species identity of this sequence was confirmed by sequencing a fragment of cytochrome *b* from a museum study skin at MNHN (CG 1950-767). Extraction, PCR-amplifications made use of standard protocol and are identical to those reported in Fuchs et al. (2007). All primers used for amplification and sequencing are reported in Table 2. Cloning of some PCRs products were performed using the Qiagen PCR cloning kit (QIAGEN SA, Courtaboeuf), following the manufacturer's protocol. Between five and eight clones per PCR product were cycle-sequenced.

2.3. Alignment and phylogenetic analyses

Alignment was performed by eye for all loci using Se-Al v2.0a11 (Rambaut, 2007). Two regions in the β-fibrinogen sequences, where alignment was uncertain were excluded for the phylogenetic analyses. The excluded regions correspond to the nucleotides 24–34 and 431–443 in our alignment. The alignments are available from the first author upon request.

Molecular phylogenies were estimated using model-based approaches (maximum likelihood, ML, and Bayesian inferences, BI), using Phyml v2.4 (Guindon and Gascuel, 2003) and MrBayes 3.1 (Huelsenbeck and Ronquist, 2003; Ronquist and Huelsenbeck, 2003). The most appropriate likelihood models were determined with MrModeltest 2.0 (Nylander, 2004), using the Akaike Information Criterion (Akaike, 1973; Posada and Buckley, 2004). Clade supports for the ML analyses were assessed by non-parametric bootstrapping (Felsenstein, 1985) (100 replicates).

The seven loci sequenced differ considerably in their properties and substitution patterns, as inferred from the fact that the models and the 95% CI from some parameters values do not overlap among genes (Table 3). This result (heterogeneous substitution process, especially between mitochondrial and nuclear DNA among loci) was expected and has already been reported (e.g. Brown et al., 1979; Prychitko and Moore, 2000; Johnson and Clayton, 2000; Barker, 2004), albeit the amount of heterogeneity between the two genomes can vary among taxa and genes (Crawford, 2003). We therefore did not perform ML and Bayesian analyses that assume a single model of evolution for the concatenated dataset. The appropriateness of partitioning the data set by gene and codon position was determined using the Bayes Factor (Kass and Raferty, 1995; Nylander et al., 2004). We used the harmonic mean approximations of the marginal likelihood of the two compared models (M_0 and M_1) to calculate the Bayes Factors (B_F), which is the ratio between the two compared models. A value greater than 10 for $2\ln B_F$ was considered as strong evidence against the simpler model (M_0) (Kass and Raferty, 1995; Nylander et al., 2004).

Bayesian analyses for the concatenated data set were performed allowing the different parameters (base frequencies, rate matrix, shape parameter, transition/transversion ratio, proportion of invariable sites) to vary between the eleven partitions (using the prset and unlink commands, i.e. mixed-models analyses (Ronquist and Huelsenbeck, 2003; Nylander et al., 2004). Four metropolis-coupled MCMC chains (one cold and three heated) were run for five to 30 million iterations with trees sampled every 100 iterations. The number of iterations discarded before the posterior probabilities (i.e. the length of the 'burn-in' period) varied between analyses. Two 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 had reached stationarity. We checked that the potential scale reduction factor (PSRF) approached 1.0 for all parameters and that the average standard deviation of split frequencies converged towards zero. We also used Tracer v1.4

Table 1
List of taxa studied (following Dickinson, 2003)

Species	Tissue/Voucher number	Origin	Myoglobin	Fibrinogen	GAPDH	TGBF	BRM	ATP6	Cyt. *b*
Campephilus haematogaster	ZMUC 114730 (M)	Ecuador	DQ188143	AF240016	EU556898	EU556949	EU556811	EU556771	EU556846
Campethera caroli	MNHN 03-04 (B)	Cameroon	DQ188157	DQ188131	EU556912	No sequence	EU556823	EU556783	EU556857
Campethera nivosa	MNHN 01-28 (B)	Cameroon	DQ352447	AY489408	EU556917	EU556965	EU556828, EU556829	EU556788	EU556862
Celeus brachyurus	USNM 620445 (L/M)	Myanmar	DQ352417	DQ352398	EU556916	EU556964	EU556827	EU556787	EU556861
Celeus lugubris	NRM 947231 (L/M)	Paraguay	DQ352441	DQ352396	EU556905	EU556955	EU556817	EU556777	EU556852
Colaptes melanochloros	NRM 947052 (L/M)	Paraguay	DQ352436	DQ352390	EU556907	EU556957	EU556819	EU556779	EU556853
Dinopium javanense	NRM 20026532 (L/M)	Captive	DQ352421	DQ352406	EU556915	EU556963	EU556826	EU556786	EU556860
Dryocopus lineatus	NRM 967106 (L/M)	Paraguay	DQ352439	DQ352394	EU556901	EU556952	EU556814	EU556774	EU556849
Dryocopus martius	NRM 20016156 (L/M)	Sweden	DQ188140	DQ188114	EU556909	EU556959	EU556821	EU556781	EU556855
Gecinulus grantia	MNHN 05-16 (B)	Laos	DQ352444	DQ352407	EU556918	EU556966	EU556830	EU556789	EU556863
Geocolaptes olivaceus	UWBM 53192 (L/M)	South-Africa	DQ352440	DQ352408	EU556910, EU556911	EU556960	EU556822	EU556782	EU556856
Meiglyptes tristis	LSUMZ B-36352 (L/M)	Malaysia	DQ352425	DQ352386	EU556919	EU556967	EU556831	EU556790	EU556864
Mulleripicus funebris	ZMUC 114105 (B)	Philippines	DQ352433	DQ352409	EU556914	EU556962	EU556825	EU556785	EU556859
Picoides mixtus	NRM 976765 (L/M)	Paraguay	DQ188151	AF394323	EU556908	No sequence	EU556820	EU556780	EU556854
Piculus chrysochloros	NRM 966938 (L/M)	Paraguay	DQ352442	DQ352392	EU556900	EU556951	EU556813	EU556773	EU556848
Picus awokera	LSU B-16980 (L/M)	Japan	EU556930	EU556872	EU556890, EU556891	EU556942	EU556804	EU556764	EU556920*
Picus canus	MNHN 05-09 (B)	Laos	DQ188156	DQ188130	EU556899	EU556950	EU556812	EU556772	EU556847
Picus canus	NRM 976051 (L/M)	Sweden	EU556929	EU556871	EU556888, EU556889	EU556941	EU556803	EU556763	EU556839
Picus chlorolophus	USNM 620432 (L/M)	Myanmar	DQ352448	DQ352410	EU556913	EU556961	EU556824	EU556784	EU556858
Picus erythropygius	Pittsburgh Aviary (F)	Captive	EU556925	No sequence	EU556882	No sequence	EU556799	EU556759	EU556835
Picus flavinucha	NRM 20056713 (B)	Vietnam	EU556926	EU556868	EU556883, EU556884	EU556939	EU556800	EU556760	EU556836
Picus flavinucha	USNM 620313 (L/M)	Myanmar	DQ352427	DQ352411	EU556902, EU556903	EU556953	EU556815	EU556775	EU556850
Picus mentalis	LSU B-36478 (L/M)	Malaysia	DQ352446	AY279221	EU556906	EU556956	EU556818	EU556778	AY279265
Picus mentalis	ZMUC 131895 (L/M)	Captive	EU556928	EU556870	EU556887	No sequence	EU556802	EU556762	EU556838
Picus miniaceus	MNHN CG 1950-767 (T)	Malaysia							EU556921
Picus puniceus	ZMUC 134527 (L/M)	Captive	EU556924	EU556867	EU556881	No sequence	EU556798	EU556758	EU556834
Picus rabieri	NRM 20026662 (B)	Vietnam	EU556933	EU556875	EU556894	EU556945	EU556807	EU556767	EU556842
Picus rabieri	NRM 2005-6688 (B)	Vietnam	EU556934	EU556876	EU556895	EU556956	EU556808	EU556768	EU556843
Picus viridanus	MNHN 6-78 (B)	Thailand	EU556935	EU556877	EU556897	EU556948	EU556810	EU556770	EU556845
Picus viridanus	MNHN 4-2F (B)	Thailand	EU556936	EU556878	EU556896	EU556947	EU556809	EU556769	EU556844
Picus viridis sharpei	MNHN P32 (F)	Spain	EU556922	EU556865	EU556879	EU556937	EU556796	EU556756	EU556832
Picus viridis *sharpei*	MNHN P5 (F)	France	EU556923	EU556866	EU556880	EU556938	EU556797	EU556757	EU556833
Picus viridis viridis	MNHN C38 (L/M)	France	DQ188155	DQ188129	EU556904	EU556954	EU556816	EU556776	EU556851
Picus viridis viridis	NRM 976304 (L/M)	Sweden	EU556931	EU556873	EU556892	EU556943	EU556805	EU556765	EU556840
Picus vittatus	NRM 20046825 (B)	Vietnam	EU556932	EU556874	EU556893	EU556944	EU556806	EU556766	EU556841
Picus xanthopygaeus	USNM 620357 (L/M)	Myanmar	EU556927	EU556869	EU556885, EU556886		EU556801	EU556761	EU556837
Veniliornis nigriceps	ZMUC 115548 (B)	Bolivia				EU556958			

Abbreviations: LSU, Museum of Natural Science, Louisiana State University; 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.
The source of DNA is indicated between brackets in the Tissue/Voucher column (B: blood, F: feather, L/M: liver/muscle, T: toe pads).
* Pseudogene.

Table 2
List of primers used in this study

Gene	Primer	Sequence (5′–3′)	Reference
Cytochrome *b*	L15424	CGATTCTTCGCTTTACACTTCCTCC	This study
Cytochrome *b*	H15916	ATGAAGGGATGTTCTACTGGTTG	This study
ATP6	L9245	CCTGAACCTGACCATGAAC	Eberhard and Bermingham (2004)
ATP6	H9947	CATGGGCTGGGGTCRACTATGTG	Eberhard and Bermingham (2004)
Myoglobin intron 2	Myo2 Pi-F	CCTGTCAAATATCTGGAGGTATG	Fuchs et al. (2006)
Myoglobin intron 2	Myo3F	GCAAGGACCTTGATAATGACTT	Heslewood et al. (1998)
Ƀ-Fibrinogen intron 7	Fib7U	GGAGAAAACAGGACAATGACAATTCAC	Prychitko and Moore (1997)
Ƀ-Fibrinogen intron 7	Fib7L	TCCCCAGTAGTATCTGCCATTAGGGTT	Prychitko and Moore (1997)
Ƀ-Fibrinogen intron 7	Fib7U-P	GTATGTGCTTGCTTTACAC	This study
Ƀ-Fibrinogen intron 7	Fib7L-P	ATCAACACCTCCTGTACTG	This study
GAPDH intron 11	G3P14b	AAGTCCACAACACGGTTGCTGTA	Fjeldså et al. (2003)
GAPDH intron 11	G3P13	TCCACCTTTGATGCGGGTGCTGGCAT	Fjeldså et al. (2003)
GAPDH intron 11	G3PintL1	GAACGACCATTTTGTCAAGCTGGTT	Fjeldså et al. (2003)
TGFB2 intron 5	TGF5	GAAGCGTGCTCTAGATGCTG	Bures et al. (2002)
TGFB2 intron 5	TGF6	AGGCAGCAATTATCCTGCAC	Bures et al. (2002)
BRM intron 15	BRM15F	AGCACCTTTGAACAGTGGTT	Goodwin (1997)
BRM intron 15	BRM15R	TACTTTATGGAGACGACGGA	Goodwin (1997)

Table 3
Length of the alignment (minimum and maximum length of the intron sequences), numbers of variable and parsimony informative sites, mean, median and 95% credibility interval (in brackets) for the models parameters of the seven individual genes (obtained with MrBayes 3.1), ML likelihood score (obtained with Phyml) and BI likelihood score (arithmetic mean)

	ATP6	Cyt. *b*	Myoglobin	GAPDH	Fibrinogen	TGFB2	BRM
Length	684	481	664 (636–663)	443 (369–411)	852[a] (687–826)	565(521–564)	371 (337–365)
Number variable sites	307	196	128	81	155	89	66
Number of parsimony informative sites	258	166	54	40	48	38	33
Model	GTR + Γ + I	HKY + Γ + I	K80 + Γ	HKY + Γ	HKY + Γ	GTR + Γ	HKY + I
Freq. A	0.284, 0.284 (0.255–0.314)	0.276, 0.276 (0.241–0.314)	0.25	0.194, 0.194 (0.162–0.228)	0.295, 0.295 (0.268–0.323)	0.244, 0.244 (0.212–0.277)	0.323, 0.323 (0.282–0.365)
Freq. C	0.433, 0.433 (0.403–0.461)	0.443, 0.443 (0.407–0.478)	0.25	0.216, 0.216 (0.182–0.252)	0.180, 0.180 (0.158–0.204)	0.222, 0.222 (0.191–0.254)	0.153, 0.153 (0.122–0.187)
Freq. G	0.077, 0.076 (0.063–0.091)	0.086, 0.086 (0.070–0.104)	0.25	0.320, 0.320 (0.282–0.361)	0.194, 0.194 (0.171–0.278)	0.232, 0.233, (0.201–0.264)	0.218, 0.218 (0.182–0.256)
Freq. T	0.207, 0.207 (0.186–0.230)	0.194, 0.194 (0.171–0.220)	0.25	0.269, 0.269 (0.233–0.307)	0.330, 0.330 (0.303–0.359)	0.302, 0.301 (0.267–0.337)	0.306–0.306 (0.265–0.347)
A–C	0.008, 0.008 (0.005–0.013)	NA	NA	NA	NA	0.111, 0.111 (0.056–0.183)	NA
A–G	0.635, 0.637 (0.547–0.716)	NA	NA	NA	NA	0.414, 0.412 (0.301–0.505)	NA
A–T	0.019, 0.019 (0.011–0.029)	NA	NA	NA	NA	0.031, 0.029 (0.008–0.066)	NA
C–G	0.011, 0.011 (0.003–0.021)	NA	NA	NA	NA	0.096, 0.092 (0.041–0.169)	NA
C–T	0.304, 0.303 (0.230–0.388)	NA	NA	NA	NA	0.278, 0.276 (0.186–0.386)	NA
G–T	0.022, 0.020 (0.005–0.043)	NA	NA	NA	NA	0.070, 0.068 (0.031–0.124)	NA
Γ	1.489, 1.465 (1.071–2.024)	1.114, 1.092 (0.704–1.660)	0.105, 0.105 (0.094–0.118)	0.103, 0.102 (0.092–0.116)	4.265, 1.183 (0.489–37.093)	0.454, 0.390 (0.213–1.009)	NA
I	0.499, 0.499 (0.459–0.539)	0.510, 0.511 (0.452–0.562)	NA	NA	NA	NA	0.546, 0.553 (0.412, 0.639)
Ts/Tv	NA	29.528, 29.035 (21.566–40.090)	4.557, 4.476 (3.173–6.407)	3.679, 3.593 (2.408–5.431)	2.932, 2.895 (2.151–3.908)	NA	4.787, 4.635 (2.924–7.560)
ML	5679.02	3662.20	1965.09	1292.84	2382.80	1487.36	1043.73
BI	5711.02	3678.55	2187.99 (Fig. 2a)	1449.07 (Fig. 2b)	2419.52 (Fig. 2c)	1524.85 (Fig. 2d)	1084.62 (Supplementary Figure 3)
BI partitioned	5359.14 (Supplementary Figure 1)	3427.03 (Supplementary Figure 2)	NA	NA	NA	NA	NA

NA means non applicable.
[a] Alignment length is after excluding regions with ambiguous alignment.

(Rambaut and Drummond, 2007) to ascertain that our sampling of the posterior distribution had an adequate effective sample size (ESS).

We searched for important incongruence between individual gene trees by comparing the topologies and nodal support obtained under different analytical methods (ML, BI). Criteria for topological incongruence that was judged to be 'significantly supported' were set at 70% bootstrap value in ML (Hillis and Bull, 1993), and at 0.95 posterior probability in BI (Ronquist and Huelsenbeck, 2003).

3. Results

3.1. Sequences properties

The length of the alignment, numbers of variable and parsimony informative sites, model parameters values and their associated 95% credibility interval and likelihood scores for all individual loci are indicated in Table 3.

3.1.1. Mitochondrial genes

The analyzed fragments of mitochondrial origin correspond to the positions 9240–9923 (ATP6) and 15449–15929 (cytochrome *b*) of the *Gallus gallus* mitochondrial genome (X52392, Desjardins and Morais, 1990). The concatenated mitochondrial data set was 1165 bp long.

We obtained the complete sequence of the ATP6 gene (684 bp). The sequences of both *P. rabieri* individuals exhibited several double peaks. The amplification products were thus cloned and up to eight clones per PCR were sequenced. None of the sequences possess any stop-codon or indel that disrupted the reading frame and the different clones exhibited very similar base composition, e.g. G content varied between 10.7% and 11%. The cloned sequences fall into two clusters, that differ by up to 7% sequence divergence, when including them in a maximum likelihood analysis. One cluster is close to *P. xanthopygaeus*, *P. vittatus* and *P. viridanus* (bootstrap < 50%, PP = 0.80), whereas the other is sister to *P. awokera* (bootstrap < 50%, PP = 0.67) (Supplementary Figure 1). We consider the first sequence cluster to be of mitochondrial origin because the recovered relationships are similar to those recovered in the other loci analyzed, even if these relationships are not supported by bootstrap values or posterior probabilities. The five cloned sequences that we identified as of pseudogene origin were also submitted to GenBank (Accession Nos. EU556791–EU556795). After excluding the putative nuclear pseudogenes, the best-fit models selected by MrModeltest were GTR + Γ + I (non partitioned), GTR + Γ (1st codon position), HKY + I (2nd codon position) and GTR + Γ + I (3rd codon position). The non-partitioned and partitioned by codon position analyses yielded very similar 50% majority-rule consensus tree topologies. The $2\ln B_F$ was 680.20 (harmonic mean partitioned, −ln = 5407.27; harmonic mean non-partitioned −ln = 5747.37), suggesting that allowing the three codon positions of the ATP6 gene to have independent parameter values had a better fit to the data than assuming a single model of evolution for the whole ATP6.

The sequenced cytochrome *b* fragment was 481 bp long. The cytochrome *b* sequence for *Campephilus haematogaster* is rather divergent (5.4%) from the other sequences of this taxon in GenBank (Accession Nos. AY942882 and U83284; U83285 is from the same specimen as AY942882 but the two sequences are not fully identical), although they cluster together with bootstrap values greater than 70% (Phyml analysis with 156 published cytochrome *b* sequences of woodpeckers, including all *Campephilus* species – tree not shown). None of the *C. haematogaster* sequences had stop codons or indels in the reading frame, which would have suggested that they were pseudogene. The cytochrome *b* sequence of *P. awokera* (liver sample, EU556920) contained a one base-pair deletion that disrupts the reading frame, indicating that we amplified and sequenced a nuclear copy. The electropherograms were clean and we could not amplify the mitochondrial fragment, despite the use of other combinations of primer pairs. The base frequencies were in the range of what was found for other taxa, albeit the G and T contents were among the highest in the studied species and the C content was the lowest. Consequently, we analysed the cytochrome *b* data set in two ways, with and without this sequence in order to assess its impact on the phylogenetic reconstruction. The best-fit models selected by MrModeltest were HKY + Γ + I (non partitioned), GTR + Γ + I (1st codon position), HKY + I (2nd codon position) and GTR + Γ (3rd codon position). Excluding the *P. awokera* sequence had no effect on model selection. Both the analysis of the data set partitioned according to codon positions and that of the non-partitioned data set yielded the same 50% majority-rule consensus tree topology (Supplementary Figure 2). The $2\ln B_F$ was 519.16 (harmonic mean partitioned −ln = 3466.81; harmonic mean non-partitioned, −ln = 3726.39), thus strongly favouring the partitioning by codon position strategy. The BI performed with the *P. awokera* cytochrome *b* pseudogene indicates that the sequence is outside the two primary *Picus* clades, a result that is in accordance with the hypothesis of a slower rate of molecular evolution in the nuclear genome. Its position was unresolved at the base of the Malarpicini.

As expected, there were no conflicts between the ATP6 and cytochrome *b* gene trees. As we could not obtain the ATP6 sequence for *P. miniaceus*, we performed the concatenated mitochondrial analyses with and without this taxon; the resulting topologies of the two analyses were identical and we only present the results from the analyses including *P. miniaceus*. Analyses performed on the concatenated mitochondrial data set (1165 bp, BI partitioned by gene and codon position, arithmetic mean −ln = 8815.67, Fig. 1) did not confidently recover the genus *Picus* as monophyletic. Two main clusters of *Picus* species were recognized, one containing the yellow-naped species *miniaceus, flavinucha* and *mentalis* (PP = 1.0, *Picus* clade 1) and another containing all the remaining *Picus* taxa sampled (yellow-naped *puniceus* and *chlorolophus* along with all the red-crowned species, PP = 1.0, *Picus* clade 2). The relationships of these two clades relative to the African *Geocolaptes–Campethera* assemblage are unresolved in the 50% majority-rule consensus tree (Fig. 1). The posterior probabilities for the monophyly and paraphyly of *Picus* in the mitochondrial tree were 0.28 and 0.72 (*Picus* clade 1-*Geocolaptes–Campethera*: PP = 0.40; *Picus* clade 2-*Geocolaptes–Campethera*: PP = 0.32), respectively. These data suggest that all the possible relationships among *Picus* clade 1, *Picus* clade 2 and *Geocolaptes–Campethera* have equal probabilities in the mitochondrial tree.

3.1.2. Autosomal loci

We detected length variation between alleles for six individuals in the GAPDH intron-11 locus. We treated all the alleles that differ in length as operational taxonomic units (OTUs), yielding a data set of 41 OTUs in the GAPDH analyses (Fig. 2b). We could not obtain the β-fibrinogen sequence for *P. erythropygius* and the TGFB2 sequences for *Picoides mixtus, Campethera caroli, P. erythropygius, P. mentalis* (ZMUC 131895) and *P. puniceus*. Since we could not obtain the TGFB2 sequence for one of our outgroups (*Picoides mixtus*), we used a TGFB2 sequence of *Veniliornis nigriceps*, a species that is closely related to *Picoides mixtus* (Moore et al., 2006), as outgroup.

The topologies obtained from the individual loci were unevenly resolved and supported (Fig. 2a–d). The numbers of nodes supported by posterior probabilities of 0.95 or higher were 20 in myoglobin, 16 in GAPDH, 15 in TGFB2 and 9 in β-fibrinogen. We observed some discrepancies between the two methods concerning the support for some nodes. For example, the clade *P. canus* MNHN, *P. erythropygius* and *P. awokera* in the myoglobin analyses was supported by a posterior probability of 0.96 but by a bootstrap value of only 35. The lack of clear relationship between posterior probabilities and bootstrapped maximum likelihood percentages has already been highlighted (e.g. Douady et al., 2003) and some studies suggested that PP might provide a better estimate of the clade support (Wilcox et al., 2002 but see also Erixon et al., 2003). Here we mainly focus on clades that were considered significantly supported in individual gene trees by bootstrap values greater than 70% and posterior probabilities greater than 0.95.

Among the five primary lineages that were recognized by previous studies, one was recovered in all gene trees (New World *Colaptes–Piculus–Celeus–Dryocopus–Mulleripicus*), three were

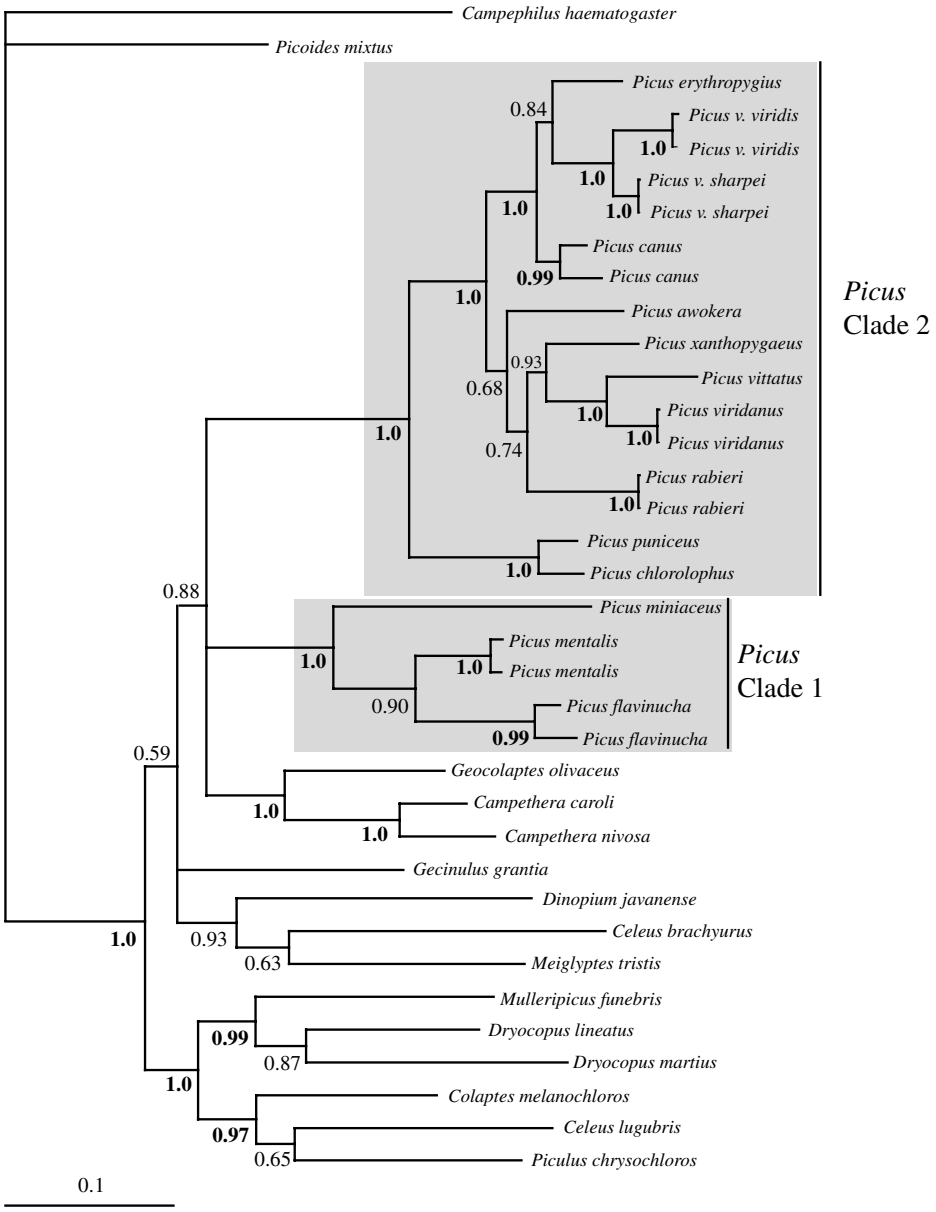

Fig. 1. Fifty percent majority-rule consensus tree (arithmetic mean −ln = 8815.67) obtained from the Bayesian Inference analyses of 1165 bp of mitochondrial data (ATP6: 684 bp, cytochrome *b*: 481 bp) using a mixed-model strategy (partitioned by gene and codon positions, six partitions). Values close to nodes represent posterior probabilities, with bold type highlighting values greater than 0.95. The taxon *P. miniaceus* was only represented by a cytochrome *b* sequence. Gray blocks represent the two main *Picus* clades that are discussed in the text.

recovered in three out of the four gene trees (*P. flavinucha–P. mentalis, P. viridis–P. chlorolophus*, and African *Geocolaptes–Campethera*). One lineage (Indo-Malayan *Micropternus–Dinopium–Meiglyptes–Gecinulus*) was only recovered in one of the individual gene trees. As in previous studies, the relationships among these five primary lineages of Malarpicini are difficult to unravel.

The Bayesian mixed-model analysis performed on the concatenated data set of the nuclear introns (2524 bp) resulted in a 50% majority-rule consensus tree topology where 15 out of the 23 resolved nodes involving species-level relationships received posterior probabilities of 0.95 or greater (arithmetic mean, −ln = 7522.45, harmonic mean, −ln = 7595.34, Fig. 3). The relationships among the primary Malarpicini lineages could not be better resolved by the concatenation of the four autosomal introns. The genus *Picus* was recovered as monophyletic, albeit posterior probabilities were low (0.57). Within *Picus*, two main clades emerged; *Picus* clade 1: *P. flavinucha–P. mentalis* and *Picus* clade 2: *P. chlorolophus–P. puniceus*-red-crowned species. This is in agreement with the findings in other studies, as well as in our analysis of mitochondrial data (Fig. 1). However, only a few relationships within *Picus* received support.

3.1.3. Z-linked locus

Length variation between alleles was detected for *Campethera nivosa*. Only a few nodes involving inter-specific relationships were resolved and well supported in the BI topology (Supplementary Figure 3). These include the relationships between *P. v. viridis* and *P. v. sharpei*, between *P. puniceus* and *P. chlorolophus*, between *Campethera caroli* and *C. nivosa*, and between *P. flavinucha* and *P. mentalis*.

3.1.4. Concatenated data set

The analysis performed on the concatenated data set (4060 bp) under a mixed-model strategy (11 partitions, arithmetic mean −ln = 17232.41) recovered an overall well-supported topology where all but six nodes received posterior probabilities of 1.0

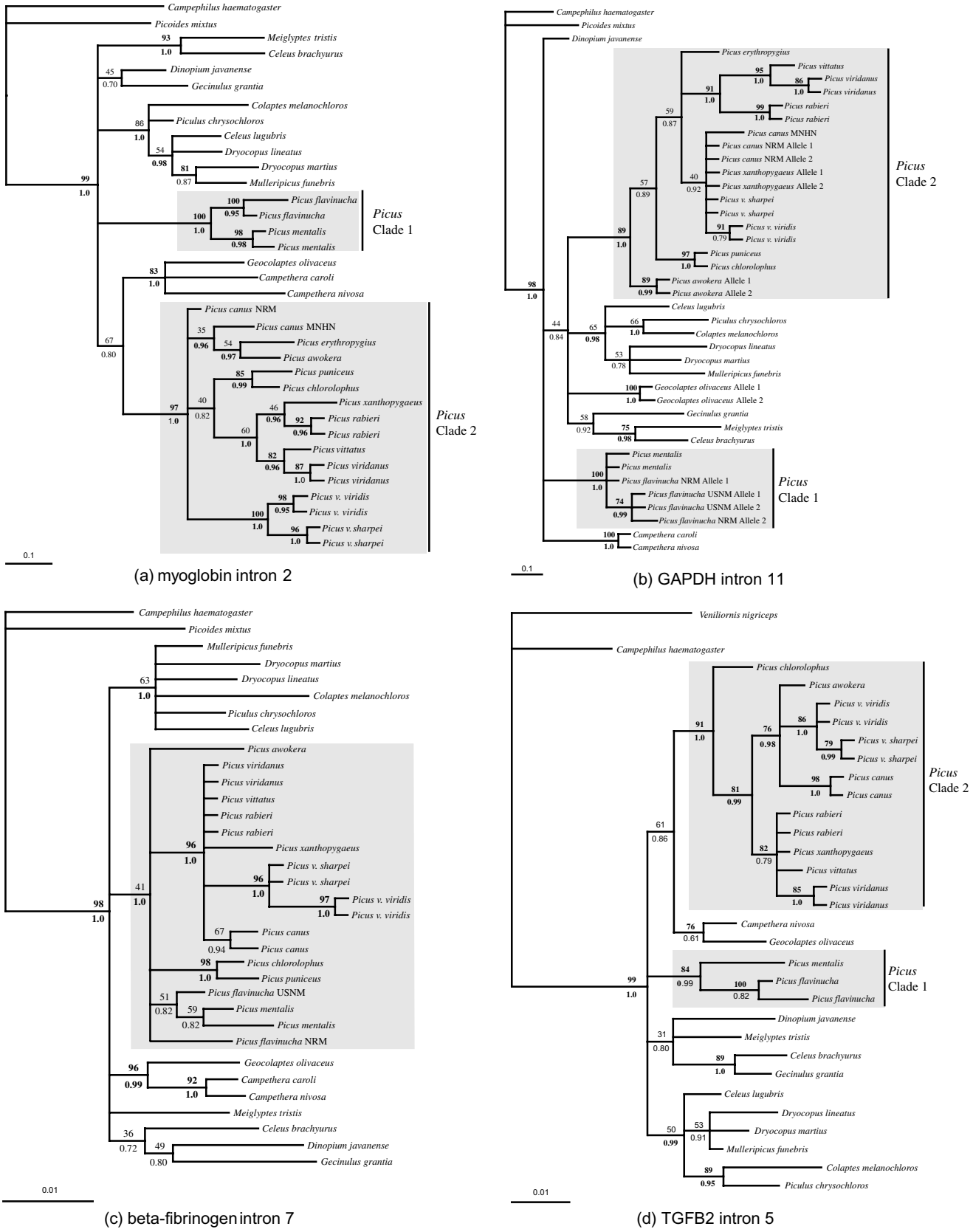

Fig. 2. Fifty percent majority-rule consensus trees obtained from the Bayesian inference analyses of the four autosomal introns. (a) Myoglobin intron 2 (664 bp, arithmetic mean −ln = 2187.99), (b) GAPDH intron 11 (443 bp, arithmetic mean −ln = 1449.07), (c) β-fibrinogen intron 7 (875 bp, arithmetic mean −ln = 2419.52), (d) TGFB-2 intron 5 (665 bp, arithmetic mean −ln = 1449.07). Length variation between alleles was detected for GAPDH (six individuals, *Geocolaptes olivaceus*, *Picus awokera*, *Picus canus* NRM, *Picus flavinucha* NRM, *Picus flavinucha* USNM and *Picus xanthopygaeus*). Bootstrap values (above) or posterior probabilities (below) greater than 70% and 0.95 are highlighted in bold. The gray blocks represent the two main *Picus* clades that are discussed in the text.

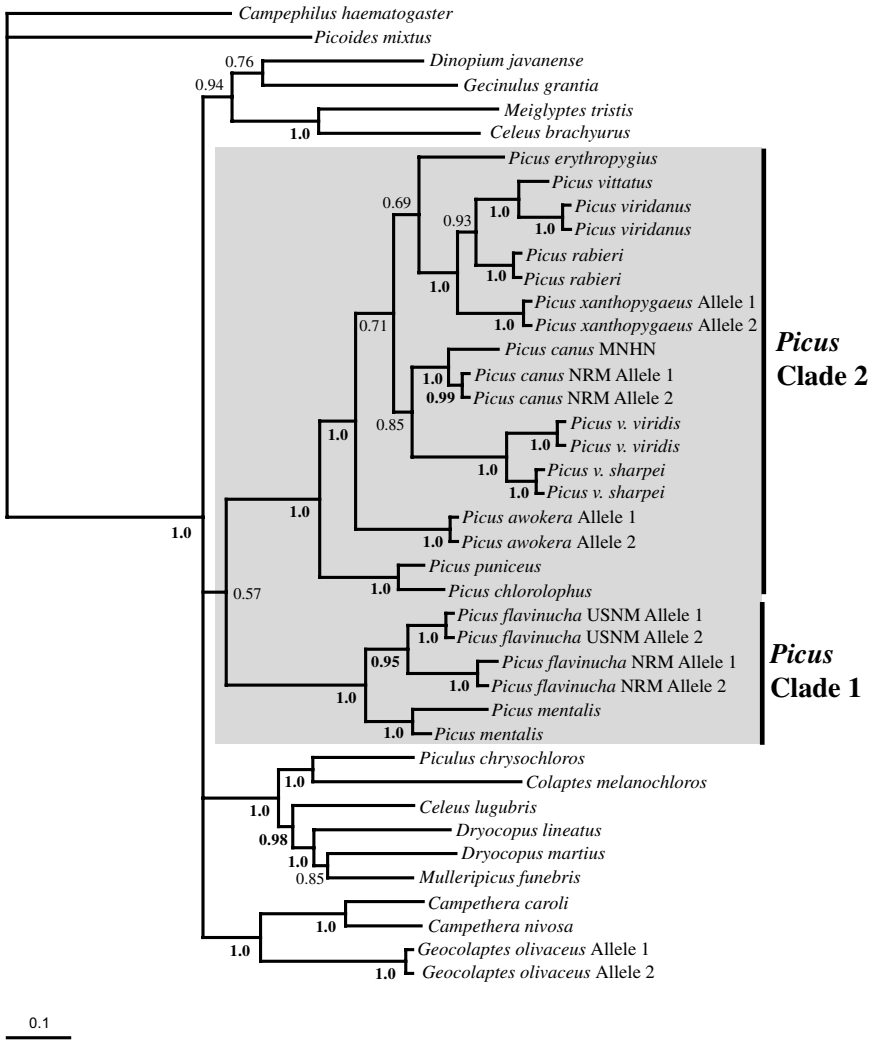

Fig. 3. Fifty percent majority-rule consensus tree (arithmetic mean −ln = 7522.45) obtained from the Bayesian inference analyses of 2524 bp of nuclear intron data (myoglobin intron 2: 664 bp, GAPDH intron 11: 443 bp, β-fibrinogen intron 7: 875 bp, TGFB2 intron 5: 565 bp) using a mixed-model strategy (partitioned by loci, four partitions). Posterior probabilities greater than 0.95 are highlighted in bold types. Gray blocks represent the two main *Picus* clades that are discussed in the text.

(Fig. 4). The five main Malarpicini clades were recovered with posterior probabilities greater than 0.95 but the relationships among these lineages remained unresolved. The genus *Picus* was recovered as paraphyletic as the African genera *Campethera* and *Geocolaptes* were nested within it. However, relationships among *Picus* clade 1, *Picus* clade 2 and *Campethera*/*Geocolaptes* did not receive strong support (PP = 0.84 and 0.89) and thus monophyly of *Picus* can not be definitively rejected. The relationships within all lineages but *Picus* clade 2 are similar to those reported in Fuchs et al. (2007). *Picus* clade 2 consists of four lineages supported by posterior probabilities greater than 0.95: yellow-naped *P. puniceus*–*P. chlorolophus*, *P. awokera*, *P. erythropygius*–*P. canus*–*P. v. viridis*–*P. v. sharpei*, and *P. xanthopygaeus*–*P. rabieri*–*P. vittatus*–*P. viridanus*, respectively. Mapping of the indels onto the tree topology indicates that several of these are synapomorphic for subclades of Malarpicini taxa, although some homoplasic indels also occur (Table 4).

4. Discussion

4.1. Phylogenetic relationships and molecular evolution within the Malarpicini

The recently erected tribe Malarpicini has been suggested to include five primary lineages with a strong biogeographic component (Webb and Moore, 2005; Benz et al., 2006; Fuchs et al., 2007): three of these lineages are endemic to the Palearctic and Indo-Malaya (the two main clades of *Picus*, and the *Dinopium*–*Gecinulus*–*Meiglyptes*–*Micropternus* clade), one is endemic to Africa (the *Geocolaptes*–*Campethera* clade) and the last one is found in both Eurasia and the New World, but with a center of diversity in the New World (the *Colaptes*–*Piculus*–*Celeus-Dryocopus*–*Mulleripicus* clade). The relationships between these lineages do not receive strong support in this study, nor in previous studies despite the use of several different combinations of genetic markers (i.e. three mitochondrial loci, Webb and Moore, 2005; one nuclear intron and two protein-coding mitochondrial genes, Benz et al., 2006; two nuclear introns and one protein-coding mitochondrial gene, Fuchs et al., 2007). The lack of resolution of higher-level relationships within Malarpicini might be attributed to several non-exclusive factors such as insufficient character sampling (soft polytomy) and/or simultaneous or nearly simultaneous origin of three or more lineages from a common ancestor (hard polytomy). All the analyses performed using our concatenated data set (4060 bp) yielded a tree where the relationships among the primary lineages remain unresolved. Rare genomic changes (RGCs), such as insertions or deletions of nucleotides in DNA sequences (indels) or retroposons, are often invoked to highlight support for clades that do not receive high support by bootstrap values or posterior prob-

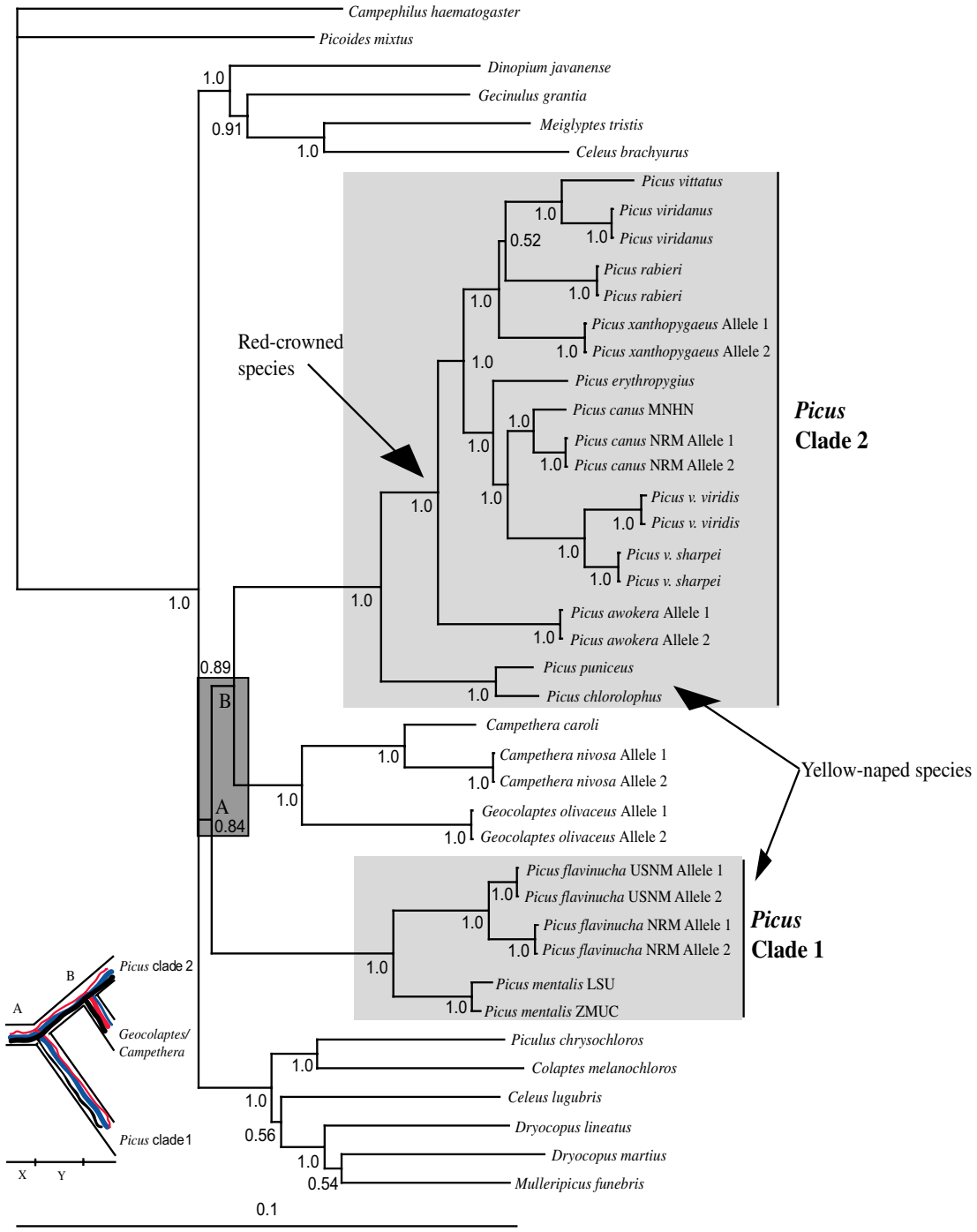

Fig. 4. Fifty percent majority-rule consensus tree (arithmetic mean −ln = 17232.41) obtained from the Bayesian inference analyses of 4060 bp of DNA (mitochondrial: 1165bp, autosomal introns: 2524 bp, Z-linked intron: 371 bp) using a mixed-model strategy (partitioned by gene and codon positions, 11 partitions). Only posterior probabilities greater than 0.95 are indicated. Light gray blocks represent the two main *Picus* clades that are discussed in the text. The schema below the phylogeny represent the lineage sorting of the individual loci for the nodes A and B (dark gray block). Lines represent the coalescence of the fibrinogen (blue bold line: allele with the deletion, blue thin line: allele without the deletion), myoglobin (red bold line: allele with the deletion, red thin line: allele without the deletion) and TGFB2 (black bold line: allele with the deletion, black simple line: allele without the deletion). When the time between several cladogenetic events (e.g. X and Y) tends toward 0, as in our case, the probability that population-level processes, such as incomplete lineage sorting, had strong influence on the topology increases (see Section 4).

abilities (Rokas and Holland, 2000). For example, an insertion in the β-fibrinogen intron 7 has been proposed to delineate primary clades within the Neoaves, the Coronaves and Metaves (Fain and Houde, 2004; see also Ericson et al., 2006 and Morgan-Richards et al., 2008 about monophyly of these two clades). Mapping of the indels onto the tree obtained from the concatenated analysis suggests several instances where indels are homoplasic or in conflict with each other (myoglobin and fibrinogen, see Table 4). One potential explanation for incongruence of indels is paralogy of at least one of the studied loci. The current knowledge about the avian genome indicates that gene duplication is rather limited (Ellegren, 2005), implying that the probability of amplifying a paralog is low. One of the indels that varies within an otherwise well-supported clade is a 16 bp insertion in the TGFB2 intron 5 shared between the African *Campethera/Geocolaptes* clade and *Picus* clade 2 with the exception of *P. rabieri*. A secondary loss of this insertion

Table 4
List of phylogenetically informative indels within Malarpicini inferred by the alignment (autapomorphic indels are excluded)

Locus	Nature of events (number of bp)	Clade supported	Synapomorphic
BRM	Deletion (6)	*Picus flavinucha*	Yes
BRM	Insertion (4)	*Picus v. viridis*/*P. v. sharpei*	Yes
FIB7	Deletion (3)	*Campethera*	Yes
FIB7	Deletion (3)	*Picus v. viridis*/*P. v. sharpei*	Yes
FIB7	Insertion (8)	*Picus v. viridis*/*P. v. sharpei*	Yes
FIB7	Deletion (113)/insertion (15)/deletion (10)	*Picus*	Ambiguous
GAPDH	Insertion (3)	*Campethera*/*Geocolaptes*	Yes
GAPDH	Deletion (10)	*Dryocopus*/*Mulleripicus*	Yes
GAPDH	Insertion (1)	*Colaptes*/*Piculus*/*Celeus*	No
GAPDH	Deletion (6)*	*Picus flavinucha*/*P. mentalis*	Yes
GAPDH	Deletion (1)*	*Picus flavinucha*/*P. mentalis*	No
GAPDH	Insertion (1)*	*P. mentalis*/*P. flavinucha* NRM Allele 1	No
GAPDH	Deletion (9)*	*P. flavinucha* except NRM Allele 2	No
Myoglobin	Deletion (8)	*Geocolaptes*/*Campethera*/*Picus* clade 2	Ambiguous
TGBF	Deletion (6)	*Geocolaptes*/*Campethera*	Yes
TGBF	Insertion (16)	*Geocolaptes*/*Campethera*/ *Picus* clade 2 (not *P. rabieri*)	No
TGBF	Insertion (6)	*Picus canus*	Yes
TGBF	Insertion (1)	*Picus xanthopygaeus*/*P. rabieri*/*P. viridanus*/*P. vittatus*	Yes

Insertions or deletions in mononucleotide repeats of more than five residues, as well as regions excluded due to uncertainties in primary homology assessment are not indicated. It should be noted that the insertion of a single G, which was previously reported to support monophyly of a clade with the New World taxa *Celeus* and *Gecinulus*/*Dinopium* (Fuchs et al., 2007), is not listed here because it occurs in a mononucleotide repeats zone that contains five G residues. The list of supported clades refers to the nodes recovered with the concatenated analyses using the 11 partitions strategy. In two cases it was not possible to determine if the indel supports a monophyletic clade or not, since they occur in an unresolved part of the tree. The asterisks indicate indels that were only observed in one of two specimens of *Picus flavinucha*.

in *P. rabieri* might, at a first glance, be rather unlikely and would suggest paralogy. Yet, our analyses recovered *P. rabieri* as being closely related to *P. xanthoypgaeus*, *P. viridanus* and *P. vittatus*, a result that was obtained with all other loci. Interestingly, an examination of the regions close to the 16 bp insertion in the TGFB2 intron 5 suggests that the insertion is a duplication of a 16 bp region. Therefore, this insertion may have been eliminated in *P. rabieri* during DNA replication in the same way as it was inserted.

Conflicting insertion/deletions events, i.e. indels that support mutually exclusive hypotheses, include the above-mentioned 16 bp insertion in the TGFB2 and an 8 bp deletion in the myoglobin sequence, which are both shared between *Geocolaptes*/*Campethera* and *Picus* clade 2 with an over one hundred base-pair deletion in β-fibrinogen intron 7 that supports the monophyly of *Picus*. Many of the clades within the Malarpicini are separated by short internodes in both the individual gene trees and in the concatenated tree. In cases of short internodes, characters can be influenced by population-level processes such as lineage sorting or hybridization (Degnan and Rosenberg, 2006) (Fig. 4). Indeed, if the effective population size (Ne) is large with respect to the length of the internode (2Ne > *T*, Avise, 2000) and if the cladogenetic events occurred far enough in the past for sorting of individual lineages to have completed, we should expect 1/*N* gene trees from independent polymorphic loci, where *N* is the number of lineages involved (McCracken and Sorenson, 2005). Therefore, the observed conflicts between the tree topology and the taxonomic distribution of the indels in TGFB2 intron 5, myoglobin intron 2 and β-fibrinogen intron 7, respectively, may be attributable to incomplete lineage sorting of the polymorphic alleles rather than paralogy. It is also worth pointing out that all combinations of conflicting indels, as well as the topology of the mitochondrial tree, suggests a close relationship between *Picus* clade 1, *Picus* clade 2 and *Geocolaptes*/*Campethera* (Fig. 4).

Due to the smaller effective population size of the mitochondrial genome, that is one fourth of the nuclear genome (Moore, 1995), we would expect the mitochondrial sequences to be more efficient in portraying the species tree (but see also Ballard and Whitlock, 2004 about possible biases, like hybridization and selection, of mtDNA in phylogenetic and phylogeographic studies). Our analyses of two mitochondrial loci could not recover a bifurcating tree as all three possible relationships among *Picus* clade 1, *Picus* clade 2 and *Geocolaptes*–*Campethera* received similar and very low posterior probabilities (between 0.28 and 0.40). Therefore, we conclude that the mitochondrial pattern is in agreement with the nuclear data and suggest multiple cladogenetic events in a short period of time at the base of the Malarpicini.

Fuchs et al. (2007) suggested that the burst of diversification within Malarpicini, as discussed above, is linked to the formation of the northern ice sheets at *c.* 8 Myr BP (Flower and Kennett, 1994). The resulting global increase in aridity and seasonality favoured the spreading of the C4 grasses throughout the world (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). We here add more data on this cladogenetic event by showing that gene flow between continental assemblages ceased abruptly, as indicated by the observation that some nuclear markers did not have enough time to sort out and become reciprocally monophyletic. The use of a newly proposed Bayesian method (Edwards et al., 2007) that accounts for the stochastic variation of gene trees obtained from unlinked loci from a single species tree may help to draw further conclusions regarding the phylogenetic relationships within the Malarpicini.

In a more general context, our study shows how misleading it can be to rely on insertion or deletions events from a single locus to infer phylogenetic relationships. If the cladogenetic events involve more than two lineages and occurred during a short period of time the gene trees can differ substantially from the species tree as a result of incomplete lineage sorting or hybridization (Degnan and Rosenberg, 2006).

4.2. Phylogenetic relationships among Picus species

Our analysis, based on DNA sequences from seven loci, recovered two main clades of *Picus* species.

4.2.1. Picus clade 1

This clade includes three yellow-naped species (*P. flavinucha*, *P. mentalis* and *P. miniaceus*) that are restricted to the Indo-Malayan realm of which the latter two are distributed south of the Isthmus of Kra. *P. miniaceus* is the only *Picus* species that does not possess any green on the upperparts and it was previously thought to be related to either *P. chlorolophus* or *P. puniceus* (Short, 1982).

P. flavinucha (continental Indo-Malaya and Sumatra) and *P. mentalis* (Peninsular Malaysia, Borneo, Java, Sumatra) are sometimes considered to form a superspecies (Winkler and Christie, 2002); their distributions are mostly parapatric and the two species only occur in sympatry in Peninsular Malaysia and Sumatra, where they are segregated by altitude (Short, 1982; Winkler and Christie, 2002). These two species were recovered as sister-taxa in all analyses. The fact that *P. flavinucha* and *P. mentalis* are very closely related is also indicated by the sharing of identical alleles in some nuclear loci (e.g. GAPDH).

4.2.2. Picus clade 2

This clade consists of a basal dichotomy involving the two remaining yellow-naped species, *P. chlorolophus* and *P. puniceus*, and all the red-crowned species.

The two yellow-naped species were recovered as sisters in all analyses. Their distributions parallel those of *P. flavinucha* and *P. mentalis*: *P. chlorolophus* is distributed in continental South-East Asia and Sumatra whereas *P. puniceus* occurs in Peninsular Malaysia, Borneo, Java and Sumatra. As for the *flavinucha/mentalis* complex, *P. chlorolophus* and *P. puniceus* are also segregated along an altitudinal gradient where they occur in sympatry (Winkler and Christie, 2002). This suggests that speciation within the two species complexes may have been synchronous, but the genetic distances and branch-lengths indicate an older age of the *P. flavinucha/P. mentalis*. This hypothesis needs further examination using comprehensive geographic sampling within each complex.

Within the red-crowned species assemblage, three main lineages emerged. The first group (the *vittatus*-group) includes four species (*P. rabieri*, *P. vittatus*, *P. viridanus* and *P. xanthopygaeus*) that are confined to Indo-Malaya. A close relationship between these four taxa was suggested by Short (1982) based on the presence of chevron bars on their underparts. Most relationships between these four taxa were poorly supported, or unresolved, in the individual and concatenated analyses. The only exception is the strongly supported relationship between *P. vittatus* and *P. viridanus*. These two taxa, which sometimes are considered to belong to the same species, differ by the presence of streaks on the breast and by habitat choice: *P. viridanus* is a lower montane forest bird whereas *P. vittatus* inhabits coastal mangroves and shrubs (Short, 1982; Winkler and Christie, 2002). Our molecular data tend to recognize both forms as genetically distinct, albeit definitive conclusions would need further sampling, especially in South-West Thailand where the two taxa occur in sympatry (Winkler and Christie, 2002). Together with the two yellow-naped species complexes, the case of *P. viridanus* and *P. vittatus* highlights the role of habitat choice in driving differentiation among these birds.

The second species group (the *canus*-group) within clade 2 includes *P. canus*, *P. viridis* (including *sharpei*) and the peculiar *P. erythropygius*. Preliminary data (J.M. Pons, unpublished) suggests that *P. vaillantii* also belongs to this clade. The group is strongly supported by mitochondrial data, where most of the signal originates from ATP6, as well as in the concatenated analyses, but its internal relationships were mostly unresolved or not supported by other loci. The affinities of *P. erythropygius* have remained uncertain for a long time, being considered to be related to either the *vittatus*-group or to *P. canus* (Short, 1982). Our study suggests that *P. erythropygius* is the sister of two species adapted to more temperate areas, *P. viridis* and *P. canus*. However, based on our current sampling it is not possible to draw any conclusions regarding how many times the Western Palearctic has been colonized by members of *Picus*. Indeed, the current distribution of species suggests two alternative hypotheses: (1) a single colonization event of the Western Palearctic by the common ancestor of *P. viridis*/*P. canus* after it had split from *P. erythropygius*, followed by a subsequent re-colonization by *P. canus* of the Eastern Palearctic and South-East Asia, or (2) an initial colonization at the time of the split between *P. canus* and *P. viridis* followed by a recent colonization of the Western Palearctic by *P. canus* originating from populations in the Eastern Palearctic and/or South East Asia. Both routes of colonization, i.e. from East to West or *vice versa*, have already been documented in other Eurasian species (e.g. *Dendrocopos major*, Zink et al., 2002; *Parus major*, Pavlova et al., 2006). Further studies on the phylogeography of *P. viridis* and *P. canus* are needed to discriminate between the two explanations.

The next primary lineage within the red-crowned species in *Picus* clade 2 concerns the singular *P. awokera*, endemic to Japan. Short (1982) hypothesized that this species is closely related to *P. canus, P. squamatus* or *P. viridis*. Our phylogenetic analyses suggest a more complex pattern for *P. awokera*. Indeed, only some gene trees (TGFB2, myoglobin and, to a lesser degree, BRM) suggest that *P. awokera* is closely related to the *canus*-group, in accordance with the opinion of Short (1982). While the relationships of *P. awokera* is unresolved in the ATP6, fibrinogen and GAPDH gene trees suggest that *P. awokera* should have a more basal position within the red-crowned species group. Indeed, the analysis of the concatenated data set yielded a tree where *P. awokera* is the sister-group of all other red-crowned species, in accordance with the fibrinogen and GAPDH gene trees. Such a result would require a new perspective on the origin of the biodiversity in the Japanese archipelago, and relationships between endemic taxa and their closest relatives in South-East Asia. Here, we regard the systematic position of *P. awokera* as unresolved. Although we could not include *P. squamatus*, we suggest that the fact that it possesses chevrons on the underparts favors a closer relationship to the *vittatus*-group. Short (1982) suggested that it is "forming a link between the groups of *viridis*, *awokera* and *canus* and the *vittatus–xanthopygaeus* group".

4.3. Proposed taxonomic changes

We here propose to divide the species currently referred to the genus *Picus* into two genera. Our recommendation is based on the fact that: (1) our analyses of seven loci recovered two primary clades within *Picus* that may not form a monophyletic assemblage and (2) the molecular divergence observed between these two clades is as high or higher than the divergence observed between other well recognized genera (e.g. between *Celeus* and *Colaptes* or between *Geocolaptes–Campethera* and any of the two *Picus* clades). We here suggest that this classification scheme provides a better description of the biodiversity encountered within the Picinae.

- **Genus *Chrysophlegma* Gould, 1850**
- *Chrysophlegma miniaceus* (Pennant, 1769)
- *Chrysophlegma flavinucha* (Gould, 1834)
- *Chrysophlegma mentalis* (Temminck, 1825)
- **Genus *Picus* Linnaeus, 1758**
- *Picus chlorolophus* Vieillot, 1818
- *Picus puniceus* Horsfield, 1821
- *Picus awokera* Temminck, 1826
- *Picus viridis* Linnaeus, 1758; including the subspecies *sharpei* (Saunders 1872). We temporarily treat *sharpei* as a subspecies of *P. viridis* but unpublished results (Pons et al. *in progress*) suggests that this taxon may be more closely related to *P. vaillantii* than to *P. viridis*).
- *Picus vaillantii* (Malherbe, 1847)
- *Picus canus* Gmelin, 1788
- *Picus erythropygius* (Elliott, 1865)
- *Picus squamatus* Vigors, 1831
- *Picus viridanus* Blyth, 1843
- *Picus vittatus* Vieillot, 1818
- *Picus xanthopygaeus* (Gray and Gray, 1846)
- *Picus rabieri* (Oustalet, 1898)

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.03.036.

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