Phylogeny of *Laniarius*: Molecular data reveal *L. liberatus* synonymous with *L. erlangeri* and "plumage coloration" as unreliable morphological characters for defining species and species groups

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ABSTRACT

Laniarius is one of the larger genera within the avian bush-shrike radiation, the family Malaconotidae. Fairly homogenous by size and shape but highly variable by colours, these have been classified mainly on basis of plumage colours. In the present study, which is the first taxon-dense analysis of the genus *Laniarius* based on molecular sequence data (nuclear BRM15 intron-15, and mitochondrial ND2 and ATPase6 genes), we investigate interrelationships between 16 species and 34 subspecies of *Laniarius*. Altogether 2094 bp were aligned and subjected to maximum likelihood and Bayesian inference analyses. Results strongly support the monophyly of *Laniarius*, and place it close to *Chlorophoneus*, but without outlining a precise sister-group. In a generally well-resolved phylogeny of *Laniarius*, *L. leucorhynchus* and *L. atrococcineus* constitute deep branches and the remaining species form five clades which are not concordant with previously defined superspecies. The black and white boubous belong to two different clades. *L. aethiopicus* appears polyphyletic and our results support the resurrection of *Laniarius major*, *Laniarius erlangeri* and *Laniarius sublacteus*. We also find that *L. liberatus*, described in 1991 based on the only known live individual, is identified as an unusual colour morph from *L. erlangeri*. The black boubous are not monophyletic; *L. funebris* and *L. leucorhynchus* appear as isolated species whereas *L. poensis* and *L. fuelleborni* are sister-taxa. We recovered the polyphyly of crimson boubous and new hypotheses on their relationships have been generated. Overall, the variation in pigments and patterns does not follow phylogenetic lineages. The plumage coloration could be thoroughly subject to modification and it could not reflect exactly colour plumages of the parents. From then on, the plumage coloration appears as an unreliable morphological character for defining species and species groups.

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1. Introduction

The genus *Laniarius* (boubous and gonoleks) constitutes a fascinating group of African bush-shrike rather uniform by size and shape but with strikingly different combinations of plumage colours and patterns. Sixteen species and thirty-four subspecies are currently recognized, all endemic to Africa (Dickinson et al., 2003). With few exceptions, sexes are alike and they often interact with well developed vocal duets, comprising ringing whistles combined with harsh croaking or snoring (for males) and harsh tearing or clicking calls (for females), with some variation between species

and populations (Field, 1979; Harris and Franklin, 2000). Solitary or in pairs, they skulk at different strata in the vegetation. Like the other members of the Malaconotidae family, boubous and gonoleks feed like giant warblers, moving around by powerful hops and gleaning inside dense vegetation, mostly in the understorey of forest and thickets or on the ground under dense vegetation (Benson et al., 1971; Harris and Franklin, 2000).

Hall and Moreau (1970) tried to group species of *Laniarius* on the basis of a set of external characters and ecology. They recognized three lineages that differed primarily in their plumage coloration and secondarily in habitat selection. The first of these comprises all black boubous, which mainly inhabit forest, the second lineage the pied boubous, mostly of scrub thickets and dense riparian bush, and the third lineage the crimson boubous, which occupy a range of habitats from thorn bush to papyrus swamps. This grouping was accepted by several later authorities until very recently, but other arrangements have also been proposed (Table 1).

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Table 1
Different taxonomies of *Laniarius* according to several authors

Species	Number of subspecies (Dickinson et al. 2003)	Hall and Moreau (1970)	Field (1979)	Wolters (1980)	Sibley and Monroe (1990)	Harris and Franklin (2000)	Fry et al. (2000)
L. leucorhynchus	0	leuc	leuc	IS	fuel	leuc	leuc
L. poensis	3*	leuc	leuc	IS	fuel	leuc	leuc
L. fuelleborni	2*	leuc	leuc	IS	fuel	leuc	leuc
L. funebris	0*	leuc	leuc	IS	IS	leuc	IS
L. aethiopicus	7	ferr	ferr	ferr	ferr	ferr	ferr
L. ferrugineus	6	ferr	ferr	ferr	ferr	ferr	ferr
L. bicolor	3	ferr	ferr	ferr	ferr	ferr	ferr
L. turatii	0	ferr	ferr	ferr	ferr	ferr	ferr
L. erythrogaster	0	barb	barb	IS	barb	ferr	barb
L. barbarus	2	barb	barb	IS	barb	ferr	barb
L. mufumbiri	0	barb	barb	IS	IS	ferr	barb
L. ruficeps	3	barb	barb	IS	IS	ferr	IS
L. atroflavus	0*	barb	barb	IS	IS	ferr	IS
L. atrococcineus	0	barb	barb	IS	barb	ferr	barb
L. luehderi	3*	barb	barb	IS	lueh	ferr	lueh
L. liberatus	0	NR	NR	NR	NR	ferr	IS
L. amboimensis	0	NR	NR	NR	lueh	NR	lueh
L. brauni	0	NR	NR	NR	lueh	NR	lueh
Number of species		15	15	15	17	16	18
Number of Superspecies		3	3	1	4	2	4
Number of independent species		None	None	11	4	None	4

(∗) Variable according to other authors; IS, Independent Species; NR, not recognized; leuc, belong to Laniarius leucorhynchus superspecies; ferr, belong to Laniarius ferrugineus superspecies; barb, belong to Laniarius barbarus superspecies; fuel, belong to Laniarius fuelleborni superspecies; lueh, belong to Laniarius luehderi superspcies; In bold, regarded as subspecies or species according to authors.

Although the classification of species and subspecies has been relatively stable, it was never supported by a clear character analysis. Thus, Hall (1954) characterized representatives of the proposed *Laniarius ferrugineus* superspecies (*L. ferrugineus*, *L. aethiopicus*, *L. turatii* and *L. bicolor*) by the contrasting plumage, black with white to pinkish or buffy underparts. Hall and Moreau (1970) characterized the crimson boubous by the presence of olive-yellow, bright yellow or red on crown and underparts. Mackworth-Praed and Grant (1960) and Hall and Moreau (1970) differentiated four blackish species (*L. leucorhynchus*, *L. poensis*, *L. fuelleborni* and *L. funebris*) of their *Laniarius leucorhynchus* superspecies by the length and depth of their bills, and by the saturation of their melanin pigment. Thus, the classification was mainly based on feather pigments, which may comprise several components of melanins and carotenoids (Tyczkowski and Hamilton, 1986; Stradi, 1998; McGraw, 2006) known to be modified by various physiological factors (Burtt and Ichida, 1999; McGraw and Wakamatsu, 2004). In this genus, numerous taxa are similar in appearance and have complex distributions; nevertheless several attempts, based on the vocalization, habitat and plumage colour differences, were made to establish their relationships (Chapin, 1954; Hall, 1954; Harris and Franklin, 2000). The monophyly of *Laniarius* has never been doubted and a molecular study has recently confirmed this monophyly and has suggested a close relationship with a clade comprising *Chlorophoneus*, *Telophorus* and *Rhodophoneus* (Fuchs et al., 2004). However, the taxon sampling was insufficient to assess relationships within *Laniarius* and the validity of the suggested superspecies.

The recent-known Bulo Burti Bush Shrike *Laniarius liberatus*, described on the basis of one live individual from Bulo Burti in central Somalia (Smith et al., 1991) has caused particular controversy (LeCroy and Vuilleumier, 1992; Ash and Miskell, 1998). This bird was distinctive in its plumage characters and its mitochondrial DNA but the taxon sampling did not take into account the possibility that the species with which it was compared could be para- or polyphyletic. We will here reassess the possibility status of this bird, using a comprehensive taxon sampling. In order to assess the possibility of a hybrid origin of this bird we used a combination of mitochondrial and nuclear genes.

In this paper, we analyze 2094 aligned positions of two mitochondrial genes (ND2 and ATPase6) and one nuclear intron (intron-15 of Brahma Protein) to clarify phylogenetic relationships of species and subspecies of *Laniarius*. Furthermore, we investigate whether a phylogenetic hypothesis can throw new light upon their reliability of grouping of species based on plumage coloration and habitat.

2. Materials and methods

2.1. Taxon sampling

Samples were obtained from 41 taxa representing all the recognized species and subspecies of *Laniarius* in Dickinson et al. (2003) (Table 2). Additionally, we added several taxa which are closely related to *Laniarius* (Harris and Franklin, 2000; Fuchs et al., 2004) (Table 2). As an outgroup taxon, we used sequences from *Nilaus afer* which is outside the *Laniarius* taxa sampled here (Fuchs et al., 2004). A total of 51 Operational Taxonomic Units were included in our analyses and they are listed in Table 2.

2.2. Laboratory procedures and sequence alignment

We extracted DNA from skin specimens (toe pads) or fresh tissue (blood or muscle) using a CTAB-based protocol (Winnepenninckx et al., 1993). We amplified and sequenced three genes. The ND2 gene (1041 bp) was amplified as a single fragment using primers L5219Met and H6313Trp (Sorenson et al., 1999) or as several smaller fragments using internal primers. The ATPase6 gene (684 bp) was amplified as a single fragment using primers A8PWL and C03HMH (Eberhard and Bermingham, 2004) or as several smaller fragments using internal primers. The BRM15 intron-15 (370 bp) was amplified as a single fragment using primers BRM15F and BRM15R (Borge et al., 2005) for all samples because of their short fragment size. This intron, localized on the Z

chromosome, has often been used to clarify the reproductive isolation between species, particularly in the flycatchers of the genus *Ficedula* (see Saetre et al., 2003 and Borge et al., 2005). All these primers are detailed in Table 3.

For amplifications, cycling conditions were standard for these three markers (Nguembock et al., 2007). After controlling the amplification products by electrophoresis (on a 1.5% agarose gel with visualization under UV light with ethidium bromide), we purified the PCR products using the 'QiaQuick PCR Purification Kit' (Qiagen, Holden, Germany) and cycle-sequenced using the 'CEQ Dye Terminator Cycle Sequencing' (Beckman Counter, Inc, Fullerton, CA, USA) or the 'Big Dye' (Applied Biosystems Inc, Forster City, CA, USA) terminator chemistries kits with the same primers used for PCR amplifications in both directions. Sequences were obtained on a DNA Analysis System sequencer Beckman (version 4.3.9) or an ABI3100. The two DNA strands of same gene for same taxon were assembled together for authentification using the sequence alignment program Sequencher 3.1 (Gene Codes Cooporations Version 3.1, 1998). All unique sequences of ND2 and ATPase6 genes were aligned in a general contig with Sequencher 3.1. No insertions, deletions and stop-codons were detected in the reading frame of these two protein-coding genes suggesting that our sequences are of mitochondrial origin and not nuclear pseudogenes (Sorenson and Quinn, 1998). For BRM15, unique sequences were put in a general contig and exported in BioEdit (version v7.0.8; Hall, 2007) where they were aligned more precisely by eye. The alignment indicated the presence of several insertions

Table 2
Names of taxa (following Dickinson et al., 2003), country for individual specimens, museum voucher or tissue numbers and GenBank accession numbers of DNA sequences used in this study

Taxon	Country	No. Accession	Origin of the tissue	ND2	ATPase6	BRM15
Laniarius						
L. aethiopicus aethiopicus	Ethiopia	MNHN CG 1976-1385	Toe pads	—	EU554478	EU554519
L. aethiopicus ambiguus	Tanzania	ZMUC 116804	Blood	EU328354	EU554456	EU554495
L. aethiopicus ambiguus	Kenya	ZMUC 116798	Blood	EU328355	EU554493	EU554496
L. aethiopicus ambiguus	Kenya	MNHN CG 1955-133	Toe pads	EU328369	—	—
L. aethiopicus ambiguus	Kenya	ZMUC 124202	Blood	EU328370	EU554472	EU554513
*L. aethiopicus erlangeri**	Somalia	MNHN CG 1964-1416	Toe pads	EU328368	EU554471	EU554512
L. aethiopicus major	Chad	MNHN CG 1979-385	Toe pads	EU328380	EU554483	EU554525
L. aethiopicus major	RDC	ZMUC 128512	Flesh	EU328371	EU554473	EU554514
L. aethiopicus major	RDC	ZMUC 128601	Flesh	EU328372	EU554474	EU554515
L. aethiopicus mossambicus	Zimbabwe	MNHN CG 1990-872	Toe pads	—	—	EU554524
L. aethiopicus sublacteus	Tanzania	ZMUC 119358	—	EU328353	EU554455	EU554494
L. aethiopicus sublacteus	Tanzania	FMNH 356738	Blood	EU328362	EU554464	EU554505
L. atrococcineus	South Africa	MNHN CG 1979-1389	Toe pads	—	EU554486	—
L. atrococcineus	South Africa	MNHN CG 1979-1390	Toe pads	—	—	EU554527
L. atrococcineus	South Africa	Van ZylsrusBH09819	Flesh	EU328388	EU554492	EU554533
L. atroflavus atroflavus	Cameroon	MNHN 40-2	Flesh	EU328365	EU554467	EU554508
L. atroflavus craterum	Cameroon	MNHN 40-1	Flesh	EU328364	EU554466	EU554507
L. atroflavus craterum	Cameroon	MNHN 40-30	Flesh	EU328383	EU554487	EU554528
L. barbarus barbarus	Senegal	MNHN CG 1968-911	Toe pads	EU328367	EU554469	EU554510
L. bicolor sticturus	Namibia	MNHN CG 1979-1388	Toe pads	EU328375	EU554477	EU554518
L. erythrogaster	Chad	MNHN CG 1976-1479	Toe pads	EU328381	EU554484	EU554526
L. erythrogaster	Uganda	ZMUC 132575	Blood	EU328373	EU554475	EU554516
L. ferrugineus natalensis	South Africa	Rauri Bowie RB1563	—	EU328361	EU554463	EU554504
L. ferrugineus savensis	South Africa	MNHN CG 1981-1380	Toe pads	EU328376	EU554479	EU554520
L. ferrugineus savensis	South Africa	MNHN CG1981-83	Toe pads	EU328377	EU554480	EU554521
L. fuelleborni fuelleborni	Tanzania	ZMUC 118836	Blood	EU328356	EU554457	EU554497
L. fuelleborni fuelleborni	Tanzania	ZMUC 120597	Blood	EU328357	EU554458	EU554498
L. funebris degener	Kenya	ZMUC 124145	Blood	EU328358	EU554459	EU554499
L. funebris funebris	Tanzania	ZMUC 123466	Blood	AY529957	—	—
L. leucorhynchus	Gabon	MNHN CG 1983-804	Toe pads	—	EU554470	EU554511
L. leucorhynchus	RCA	MNHN CG 1983-61	Toe pads	—	EU554481	—
L. liberatus	Somalia	ZMUC 118877	Blood	EU328359	EU554460	EU554500
L. luehderi castaneiceps	Uganda	ZMUC 119044	Blood	AY529958	EU554461	EU554501
L. luehderi luehderi	Uganda	FMNH 355504	Blood	EU328363	EU554465	EU554506
L. luehderi luehderi	Gabon	MNHN CG 1983-816	Toe pads	EU328366	EU554468	EU554509
L. luehderi luehderi	Tanzania	ZMUC 135292	Blood	EU328374	EU554476	EU554517
L. mufumbiri	Tanzania	Rauri Bowie RB1560	—	EU328360	EU554462	EU554503
L. poensis camerunensis	Cameroon	MNHN CG 1982-TEZA	Toe pads	EU328382	EU554485	—
L. poensis holomelas	Burundi	MNHN CG 2000-135	Toe pads	EU328379	EU554482	EU554523
L. ruficeps rufinuchalis	Kenya	MNHN	Toe pads	EU328378	—	EU554522
L. turatii	Guinea	MNHN 40-32	Flesh	EU328384	EU554488	EU554529
L. turatii	Guinea	MNHN 40-33	Flesh	EU328385	EU554489	EU554530
Other Malaconotidae						
Chlorophoneus bocagei	Cameroon	MNHN 40-28	Flesh	EU328386	EU554490	EU554531
Chlorophoneus dohertyi	Burundi	FMNH 358005	Blood	AY529945	EU554454	EU554449
Chlorophoneus nigrifrons	Tanzania	ZMUC 120151	Blood	AY529946	EU554453	EU554448
Chlorophoneus sulfureopectus	Malawi	MNHN CG 1998-823	Toe pads	AY529947	EU554452	EU554451
Dryoscopus gambensis	Kenya	ZMUC 124320	Blood	AY529953	EU554534	EU554502
Rhodophoneus cruentus	Kenya	Rauri Bowie US-002	—	AY529970	—	—
Tchagra australis	Cameroon	MNHN 40-29	Flesh	EU328387	EU554491	EU554532
Telophorus zeylonus	South Africa	FMNH 390107	Blood	AY529973	EU554445	EU554447
Outgroup						
Nilaus afer	Botswana	FMNH uncatal	Blood	AY529963	EU554446	EU554450

Acronyms are: FMNH, Field Museum of Natural History (Chicago); ZMUC, Zoological Museum of the University (Copenhagen); MNHN, Museum National d'Histoire Naturelle. (∗): for black morph.

and deletions events. The occurrence of Single Nucleotide Polymorphisms (SNPs) in the BRM15 was suggested by the presence of double peaks; theses double peaks were coded using the appropriate IUPAC codes.

2.3. Phylogenetic analyses

Two methods were used to infer the phylogenetic relationships: Maximum Likelihood (ML) and Bayesian Inference (BI). They were conducted for each data set separately (ND2, 45 taxa; ATPase6, 46 taxa; BRM15, 45 taxa) and for the combined data set (40 taxa). Gaps were treated as missing data. The topologies and parameters estimated were obtained for the ML and BI with PhyML v2.4 (Guindon and Gascuel, 2003) and MrBayes v.3.1 (Huelsenbeck and Ronquist, 2003), respectively. Likelihood models were estimated with MrMODELTEST 2.0 (Nylander, 2004) and the best-fit models were selected using the Akaike Information Criterion (Akaike, 1973). The selected models and parameters estimated (base frequencies, rate matrix, shape parameter, proportion of invariable sites) are indicated in Table 4. Nodal supports in ML were estimated with 1000 bootstrap replicates (Felsenstein, 1985). For the Bayesian inference, four incrementally Metropolis coupling MCMC chains (3 heated and 1 cold) were run for two million generations with trees sampled every 100 generations (20,001 trees sampled). With our sample, the first 200,000 generations (2000 trees) were discarded ('burn-in' period) and the posterior probabilities were estimated for the remaining sampled 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 warrant that chains had reached stationarity. For combined Bayesian analysis, we used the selected models in a partitioned analysis (genes and codon positions) with the "unlink" command.

The topologies and nodal supports obtained from the different models based on methods (ML and BI) were compared to detect incongruences. Criteria for valid support were set at 70% for the bootstrap values (Hillis and Bull, 1993), and at 0.95 for posterior probabilities (Huelsenbeck and Ronquist, 2001).

2.4. Evolution of plumage coloration

To analyze the evolution of plumage coloration in *Laniarius*, we superimposed the types of colour and pattern, as judged from published illustrations and examination of voucher specimens directly on to the modified phylogenetic tree obtained with the combined dataset (inserting the species for which the molecular dataset was incomplete in Fig. 2). This method has often been used but with colour symbols (Price and Pavelka 1996; Omland and Lanyon, 2000). We combined this method with another one used by Johnson and Lanyon (2000) with the blackbirds. We scored each species of this genus for the presence of plumage patches of carotenoid (yellow, orange, or red) or not (black, or black and white) pigment on the surface of the plumage. We created several categories of colour pigmentation and we reconstructed this presence/absence character using MacClade (Maddison and Maddison, 1992) with unordered parsimony over the rooted ingroup phylogeny for the species of *Laniarius*. In order to analyze the potential correlation with the habitat, we use the same method with the different types of habitat, as suggested by Sonnenschein and Reyer (1984) in *Laniarius*.

3. Results

3.1. Individual and concatenated data sets

We obtained between 778 (*L. poensis camerunensis*) and 1041 bp (majority of taxa), for a final alignment of 1041 bp of ND2. Out of this full alignment, 420 and 547 were parsimony informative and variable sites, respectively. MrMODELTEST selected the GTR + Γ + *I* as the best-fit model. For ML analyses, the parameters estimated are detailed in Table 4. Concerning BI, the mean of the four runs resulted in −ln = 9808.28 with a SD = 1.29. Both methods supported the same topology which received high bootstrap and posterior probabilities (Fig. 1a).

For the ATPase6 gene, sequence lengths ranged from 388 (skin sample of *L. atrococcineus* MNHN CG 1979-1389) to 684 bp (majority of taxa), for a final alignment of 684 bp. The aligned ATPase6 sequences comprised 684 characters, of which 269 and 331 were parsimony informative and variable sites, respectively. MrMODELTEST selected the GTR + Γ + *I* as the best-fit model. Concerning ML analyses, the parameters estimated by PhyML for this model are indicated in Table 4. For BI, the mean of the four runs resulted in −ln = 6248.05 with a SD = 1.25. Both methods supported the same topology which received high bootstrap values and posterior probabilities (Fig. 1b).

We obtained 317 (*L. luehderi castaneiceps*) and 370 bp (majority of taxa) for the BRM15 intron-15. Indels were observed: an autapomorphic indel includes an insertion of one base in *L. luehderi* of East Africa, and notably an insertion of one base shared by several taxa of ingroup and outgroup where the primary homology was doubtful corresponding to the site number 302 from our aligned BRM15 sequences deposited in GenBank. This ambiguous insertion was deleted in our analyses giving a final alignment of 369 bp. The aligned BRM15 sequences comprised then 369 characters, of which 35 and 93 were parsimony informative and variable sites, respectively. MrMODELTEST selected the TVG + Γ as the best-fit model which approached to GTR + Γ + *I* model. For ML analyses, the parameters are estimated by PhyML and detailed in Table 4. Concerning BI, the mean of the four runs resulted in −ln = 1357.57 with a SD = 0.32. Both methods supported the same topology which received moderate bootstrap and posterior probabilities (Fig. 1c).

For a few specimens, we were unable to obtain ND2, ATPase6, and BRM15 sequences (Table 2) and these were excluded from the combined analyses. Finally, our concatenated data set included only 40 UTOS. For a better resolution, the concatenated data set was partitioned by gene and codon positions (seven partitions) for the combined Bayesian analysis (Fig. 1d). The tree obtained was the best resolved where 21 out of 30 nodes within the genus *Laniarius* were supported by posterior probabilities greater than 0.95.

3.2. Phylogenetic results

The different phylograms (Fig. 1a–d) are mostly congruent and with many well supported groupings, but with a low resolution in the BRM15 tree. The concatenated tree (Fig. 1d) has the highest support values, over all, and we therefore focus on this tree, but comment on taxa which were only represented in one or two gene trees. All our analyses support the monophyly of *Laniarius*. According to the combined tree, *C. nigrifrons*, *C. sulfureopectus* and *C. bocagei* constitute the sister group of *Laniarius*, but this node is not well supported. Within *Laniarius*, *L. atrococcineus* and *L. leucorhynchus* are together in a basal position in the ATPase6 tree, but in the poorly resolved BRM15 tree only *L. leucorynchus* is at the base, and in the ND2 tree *L. atrococcineus* is basal but *L. leucorhynchus* is lacking. Thus, the combined tree provides strong support only for a basal position of *L. atrococcineus*.

The remaining ingroup taxa can be divided into six clades (A, B, C, D, E and F) of which the first two may represent the deepest branches.

Clade A, with the two blackish species *L. poensis* and *L. fuelleborni*, has 1.0 posterior probability in the combined tree and with ND2 and BRM15, and high support also with the ATPase6 and bootstrap values. The combined analysis and ND2 place this clade as the sister group of all the following clades.

Table 3
Primers sequences used for amplification and sequencing in this study

Primers	Gene	Sequence	References
L5219Met	ND2	5′ CCC ATA CCC CGA AAA TGA TG 3′	Sorenson et al. (1999)
H6313Trp	ND2	5′ CTC TTA TTT AAG GCT TTG AAG GC 3′	Sorenson et al. (1999)
297F	ND2	5′ AGCCATTTCAATSAAACTAGG 3′	This study
356R	ND2	5′ AAGGCTCATCYRTAATA 3′	This study
480F	ND2	5′ CRCAGCYAYTGGAGGATG 3′	This study
595R	ND2	5′ CTAGCCCTACTAAARTTCTAC 3′	This study
776R	ND2	5′ TCCTCCCYAAYTGACCATT 3′	This study
A8PWL	ATPase6	5′ CCTGAACCTGACCATGAAC 3′	Eberhard and Bermingham (2004)
CO3HMH	ATPase6	5′ CACATAGTRGACCCCAGCCCATG 3′	Eberhard and Bermingham (2004)
322F	ATPase6	5′ TCAATAAACCTYGCCCTAGC 3′	This study
374F	ATPase6	5′ CTRCGAAAYCARCCATCAATCTC 3′	This study
379R	ATPase6	5′ GAGATTGATGGYTGGTTTCGYAG 3′	This study
455F	ATPase6	5′ ATCGARACAATCAGCYTRCTYAT 3′	This study
466F	ATPase6	5′ ACAATCAGCRTACTRATRCGACC 3′	This study
502F	ATPase6	5′ ACGRCTAACSGCYAAYYTAACAGC 3′	This study
541F	ATPase6	5′ GTGGCYGTRGAGATTAGYTGRAT 3′	This study
BRM15F	BRAHMA PROTEIN intron-15	5′ AGCACCTTTGAACAGTGGTT 3′	Borge et al. (2005)
BRM15R	BRAHMA PROTEIN intron-15	5′ TACTTTATGGAGACGACGGA 3′	Borge et al. (2005)

Table 4
Estimated parameters for the BRM15, the ND2, the ATPase6 and the combined dataset from Bayesian Inferences (BI), and Maximum Likelihood (ML)

Gene	BRM15	ND2	ATPase6	Combined data set
Number of bases	369	1041	684	2094
Number variable/informative	93/35	547/420	331/269	943/688
Model selected	TVG + Γ	GTR + Γ + I	GTR + Γ + I	GTR + Γ + I
RA-C	1.44	0.34	0.36	0.53
RA-G	5.19	11	11.15	11.19
RA-T	0.6	0.62	0.58	0.68
RC-G	2.99	0.36	0.12	0.44
RC-T	3.57	5.88	5.87	6.51
RG-T	1	1	1	1
α	3.25	0.59	1.2	0.52
I	0.11	0.29	0.47	0.33
−ln likelihood (ML)	1221.27	9769.12	6203.81	16196.8
BI model	TVG + Γ	GTR + Γ + I	GTR + Γ + I	NA
BI score	1357.57	9808.28	6248.05	NA
(SD)	(0.32)	(1.29)	(1.25)	NA
Partitioned BI score	NA	NA	NA	16223.61
(SD)	NA	NA	NA	1.36

Likelihood scores were estimated with PhyML. α corresponds to shape parameter and I to the proportion of invariable sites. Score of the Bayesian analyses represents the mean of the four independent runs with the associated Standard Deviations. NA, not applicable.

Clade B is strongly supported in all analyses but its content varies as *L. ruficeps* is only represented by ND2 and BRM15. The other taxa in this clade are *L. aethiopicus erlangeri* and *L. liberatus*, which are almost identical with the Z-linked and mitochondrial markers.

Clade C includes the two subspecies of the sooty-grey *L. funebris*, *L. f. funebris* and *L. f. degener*. The large clade D consists of members of the pied species *L. aethiopicus*, *L. turatii*, *L. ferrugineus*, and *L. bicolor*. Within this clade, *L. aethiopicus* is polyphyletic by the separate positions of its currently recognized subspecies (Fig. 1a, b and d). *L. turatii* appears closest to *L. aethiopicus major* and *ambiguus* (Fig. 1a and d). The placement of *L. aethiopicus aethiopicus* (Fig. 1b), *L. a. mossambicus* (Fig. 1c) and *Laniarius* sp from Arabuko-Sokoke forest in Kenya (Fig. 1a–d) are unresolved. The studied subspecies of *L. ferrugineus* are monophyletic and seem to be close to *L. aethiopicus sublacteus* and away from other forms currently referred to *L. aethiopicus*, but this relationship is moderately supported (Fig. 1a, b and d). The position of *L. bicolor* remains unresolved in all our topologies.

Clade E consists of two subspecies of *L. luehderi*. In all our analyses (except with the BRM15), these taxa formed two groups; one including the three samples of East Africa which are sister-group to the *L. l. luehderi* sample of West Africa (Fig. 1a, b and d). The sixth clade F includes the "crimson" species *L. atroflavus*, *L. mufumbiri*, *L. barbarus*, and *L. erythrogaster* except *L. atrococcineus*. This clade

consists of two subclades: the first comprises the two sub-species of *L. atroflavus* which appear sister-group to *L. mufumbiri* but this relationship remains moderately supported and is retrieved only with the concatenated data (Fig. 1d); the second subclade includes *L. barbarus* and *L. erythrogaster*, and is strongly supported in all our topologies except with the BRM15 tree (Fig. 1a, b and d).

3.3. Evolution of plumage coloration

For the presence of carotenoid (or not) onto the plumage of species of *Laniarius*, we obtained only one topology with MacClade (Fig. 2). The carotenoid and eumelanin plumages appear several times independently in this topology and seem to point out a relative convergence in the evolution of this character (Fig. 2). We thus notice that the plumage coloration does not strictly follow the phylogenetic branching with a mix of the two pigments (carotenoid and eumelanin) in some clades (Fig. 2). But at the base of our phylogeny, we retrieve these two pigments, represented by the black *L. leucorhynchus* and the crimson *L. atrococcineus* (Fig. 2). In keeping with the habitat, our analyses show a relative correlation between a few black and white boubous (except *L. turatii*) and their habitat (Fig. 2). In return in the black and crimson boubous, the plumage coloration does not show a correlation with a particular type of habitat (Fig. 2).
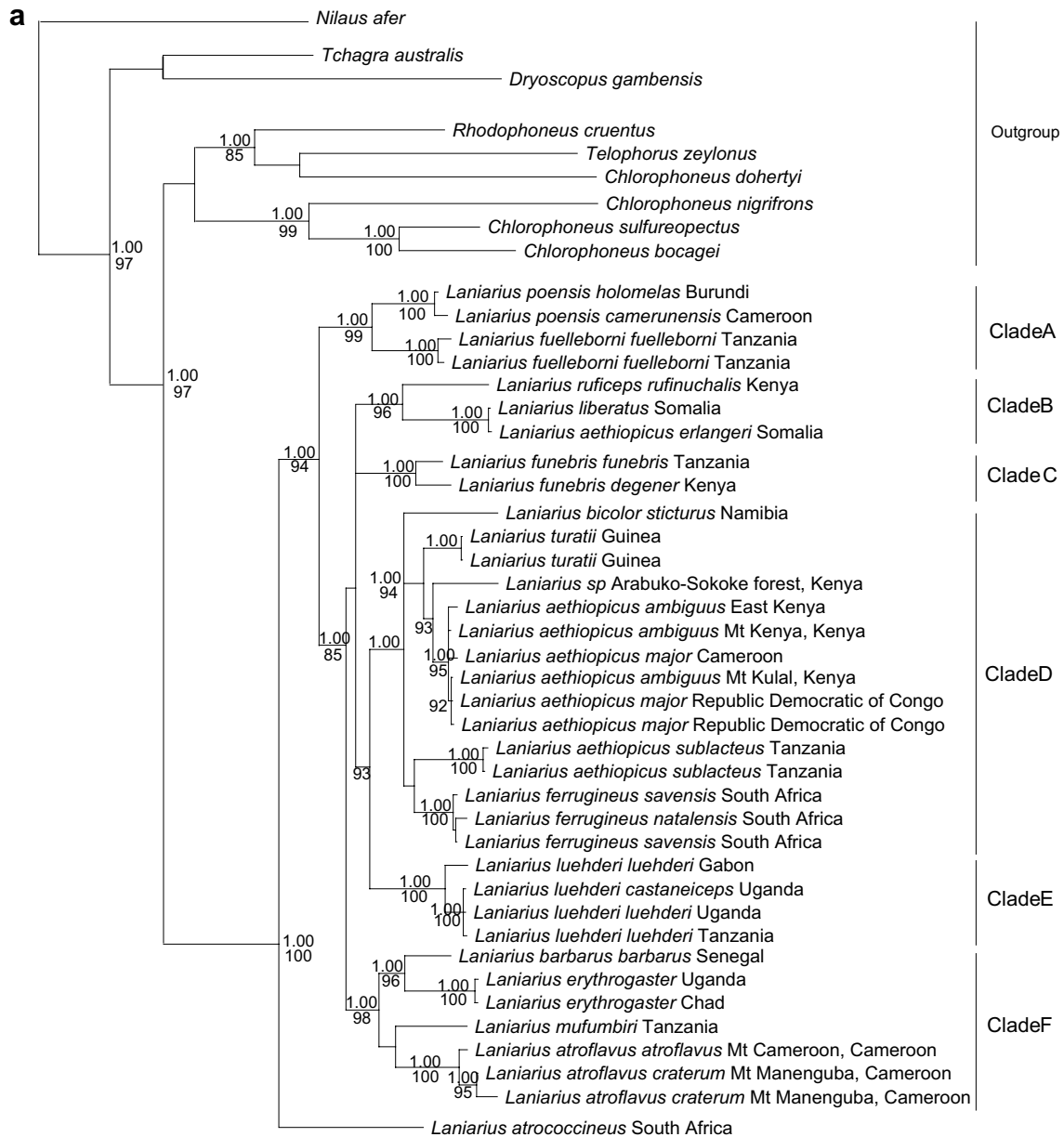
Fig. 1. (a) Bayesian inference tree (−ln = 9808.28, SD = ±1.29) showing phylogenetic relationships among species and subspecies of *Laniarius* and obtained from the mitochondrial ND2 sequences under GTR + Γ + *I* model of sequence evolution. Posterior Probabilities (⩾0.95) are indicated above the branches, and PhyML bootstrap values (⩾0.70) below the branches. Base frequencies: *A* = 0.33, *C* = 0.36, *G* = 0.09, *T* = 0.21, revmatrix = 0.34, 11, 0.62, 0.36, 5.88, *α* = 0.59, *I* = 0.29 (parameters estimated by PhyML) and Maximum likelihood tree (−ln = 9769.12) obtained from the mitochondrial ND2 gene is not shown. (b) Bayesian inference tree (−ln = 6248.05, SD = ±1.25) showing phylogenetic relationships among species and subspecies of *Laniarius* and obtained from the mitochondrial ATPase6 sequences under GTR + Γ + *I* model of sequence evolution. Posterior Probabilities (⩾0.95) are given above the nodes, and PhyML bootstrap values (⩾0.70) below the nodes. Base frequencies: *A* = 0.36, *C* = 0.37, *G* = 0.07, *T* = 0.19, revmatrix = 0.36, 11.15, 0.58, 0.12, 5.87, *α* = 1.2, *I* = 0.47 (parameters estimated by PhyML) and Maximum likelihood tree (−ln = 6203.81) obtained from the mitochondrial ATPase6 is not shown. Some names of taxa in this figure take into account the suggested taxonomic revision of *Laniarius*. (c) Bayesian inference tree (−ln = 1357.57, SD = ±0.32) showing phylogenetic relationships among species and subspecies of *Laniarius* and obtained from the nuclear intron BRM15 sequences under TVG + Γ model of sequence evolution. Posterior Probabilities (⩾0.95) are indicated above the branches, and PhyML bootstrap values (⩾0.70) below the branches. Base frequencies: *A* = 0.34, *C* = 0.19, *G* = 0.13, *T* = 0.34, revmatrix = 1.44, 5.19, 0.6, 2.99, 3.57 (parameters estimated by PhyML) and Maximum likelihood tree (−ln = 1221.27) obtained from the nuclear intron BRM15 is not shown. (d) Bayesian inference tree (−ln = 16223.61, SD = ±1.36) obtained from 2094 bp of nucleotide sequence data derived from the two mitochondrial protein-coding genes and one nuclear intron showing phylogenetic relationships among species and subspecies of *Laniarius*. Posterior Probabilities (⩾0.95) are indicated above the nodes, and PhyML bootstrap values (⩾0.70) below the nodes. Base frequencies: *A* = 0.34, *C* = 0.34, *G* = 0.09, *T* = 0.22, revmatrix = 0.53, 11.19, 0.68, 0.44, 6.51 (parameters estimated by PhyML) and maximum likelihood tree (-ln16196.8) obtained from these three markers is not shown.

4. Discussion

4.1. Monophyly of Laniarius and their sister-group

Our study strongly supports the monophyly of the genus (Fig. 1a–d). *Laniarius* was traditionally considered closer to *Telo-* *phorus* and *Chlorophoneus* than to other representatives of the 'core malaconotids' (Harris and Franklin, 2000). Fuchs et al. (2004) confirmed this, and added *Rhodophoneus* to this group of close relatives. Our results, which are congruent with these of Fuchs et al. (2004), seem to confirm the close relationship with the genus *Chlorophoneus* but still do not confidently define its sister group.
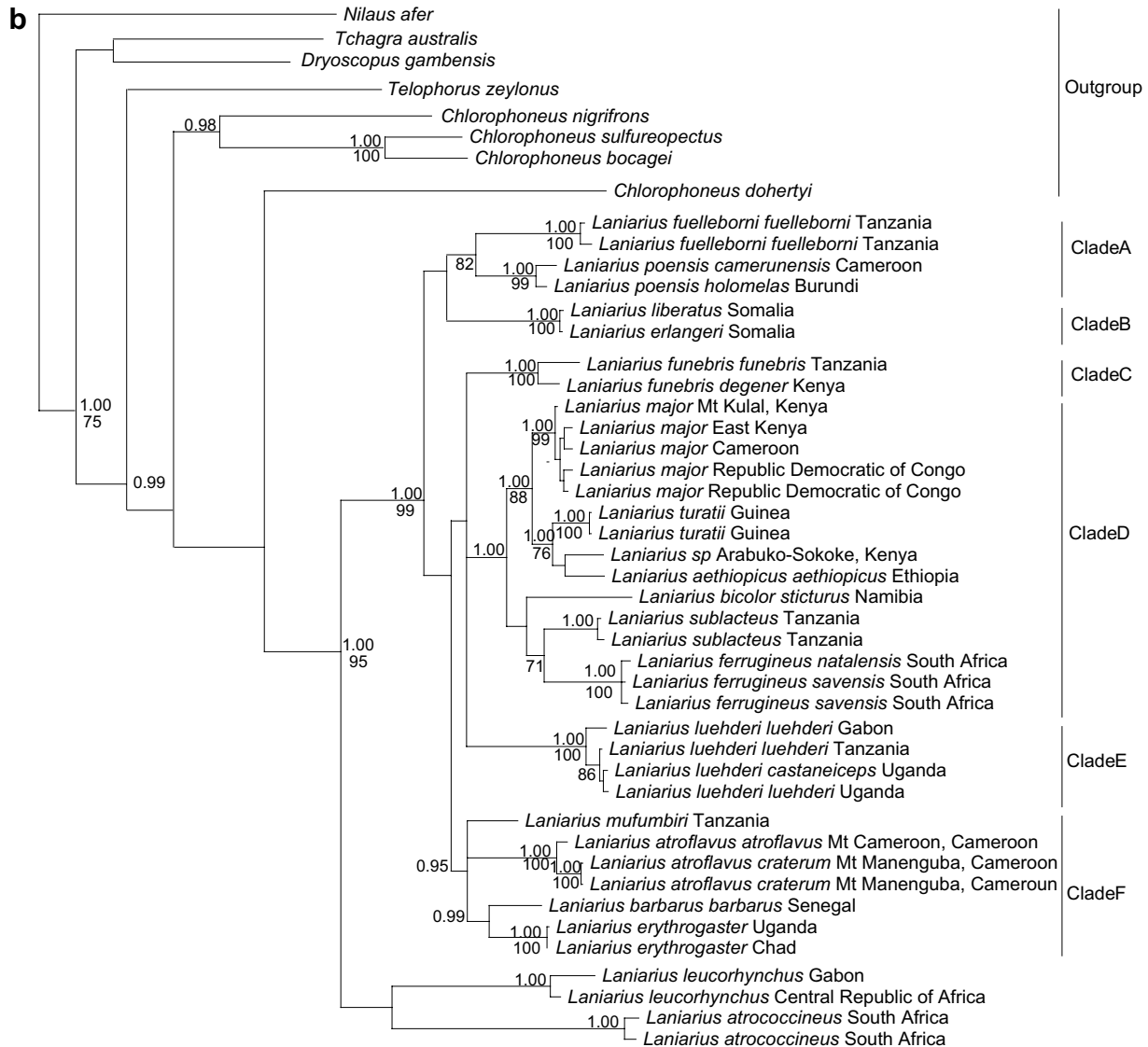
Fig. 1. (b)

A further study, involving all taxa of *Chlorophoneus* and related species, is then desirable to confidently define the sister group of *Laniarius*.

4.2. Relationships and taxonomic revisions

4.2.1. Relationships within Laniarius

Due to similar general colour and size, some authors united *L. fuelleborni* and *L. funebris* as a single species (Rand, 1957). Chapin (1954) treated them as conspecific, and other authors put them in the same superspecies with the other black boubous (Table 1). In our study, all analyses place *L. fuelleborni* with *L. poensis* and apart from *L. funebris*. This last species is morphologically the most divergent member of the proposed *leucorhynchus* superspecies (Table 1). Sibley and Monroe (1990) noted that its status was uncertain while Fry et al. (2000) ranked it among the four independent species of *Laniarius* (Table 1). Our results contradict the view of Hall and Moreau (1970) that place it in their proposed *leucorhynchus* superspecies. *L. funebris* appears as an isolated species, unrelated to other black boubous, and its position remains unresolved in this study.

The suggested *Laniarius ferrugineus* superspecies of Hall and Moreau (clade D) consists of four species in the current classification (Table 1). We confirm that *L. ferrugineus*, *L. bicolor* and *L. turatii* represent distinct lineages but then identify additional groupings that may also qualify as independent species. Within this clade, all analyses support the close relationship and an incomplete lineage sorting between *L. aethiopicus major* and *L. aethiopicus ambiguus*. These forms are rather similar morphologically except the short wing-stripe in *ambiguus* and slight differences in colour hue of the underparts and proportions, and some intergradation between them is reported (Hall, 1954; Harris and Franklin, 2000). Furthermore, one individual (ZMUC 116798) from Arabuko-Sokoke forest, in the center of the *sublacteus* range, groups with the Ethiopian *L. aethiopicus aethiopicus* or the West African *L. turatii* in different phylogenies and could represent yet another species. But a further documentation must be compiled before this species can be formally described and named. We notice that the costal region of East Africa is a geologically complex area with a complex mosaic vegetation, and thus we cannot exclude that the large Arabuko–Sokoke forest, which is exceptionally rich in endemic species (Fishpool and Evans, 2001), could hold a relict population that is distinct from those of the surrounding habitat matrix. Nevertheless, much denser taxon sampling and collection of other kinds of data will be needed to work out the historical population structure and
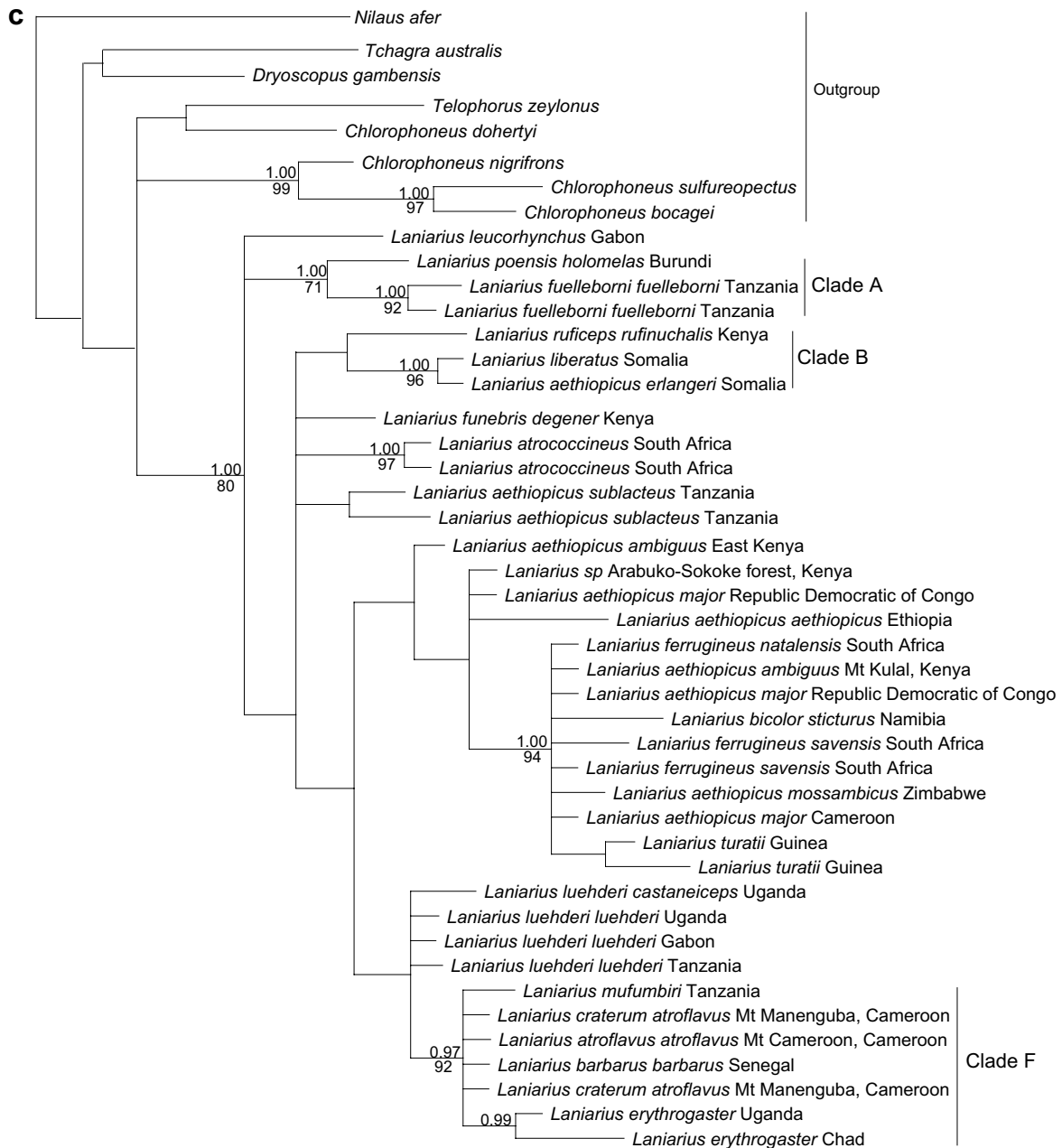
C

Fig. 1. (c)

species limits in this part of Africa. The position of *L. ferrugineus* like that of *L. bicolor* are less certain in this study and a further study including more members of these species is desirable.

In keeping with the clade E, *L. luehderi*, according to several authors (Sibley and Monroe, 1990; Fry et al., 2000; Dickinson et al., 2003), comprises three or four races of which two *L. l. brauni* and *L. l. amboimensis*, are highly distinctive and very locally distributed in Angola. Unfortunately, these two forms were unavailable to us. The differentiation noted between the Eastern and Western populations could give rise a taxonomic revision, but a denser sampling will be needed to define historical lineages. All taxa in the *barbarus* superspecies of Hall and Moreau (1970) are in clade F, except *L. ruficeps* and *L. atrococcineus* which are outside of this clade. *L. barbarus* and *L. erythrogaster* were often treated as conspecific (Sibley and Monroe, 1990; Harris and Franklin, 2000). Our results confirm a sister relationship between these two species. Concerning *L. mufumbiri*, its systematic affinities have always been consid-

ered controversial. It was seen as a small counterpart of *L. barbarus* (Chapin, 1954), regarded as conspecific with nominate *barbarus* (Mackworth-Praed and Grant, 1955), and considered as member of the *barbarus* group (Hall and Moreau, 1970; Fry et al., 2000). Sibley and Monroe (1990) treated it as separate species outside of the *barbarus* superspecies. According to our analysis, *L. mufumbiri* does not appear closely related to *L. barbarus* and this result seems to agree with the morphology, such as size (being smaller than *L. barbarus*), (small) white wing-stripe and white undertail-coverts for *mufumbiri* with regard to *barbarus* (see Fry et al., 2000; Harris and Franklin, 2000). A close relationship with *L. atro-flavus*, an endemic resident of the mountains of West Cameroon and East Nigeria, is moderately supported in this study (Fig. 1d). This relationship seems to be supported by few morphological characters such as size and plumage pattern (see Harris and Franklin, 2000; Fry et al., 2000) but it ought to be confirmed by other studies.
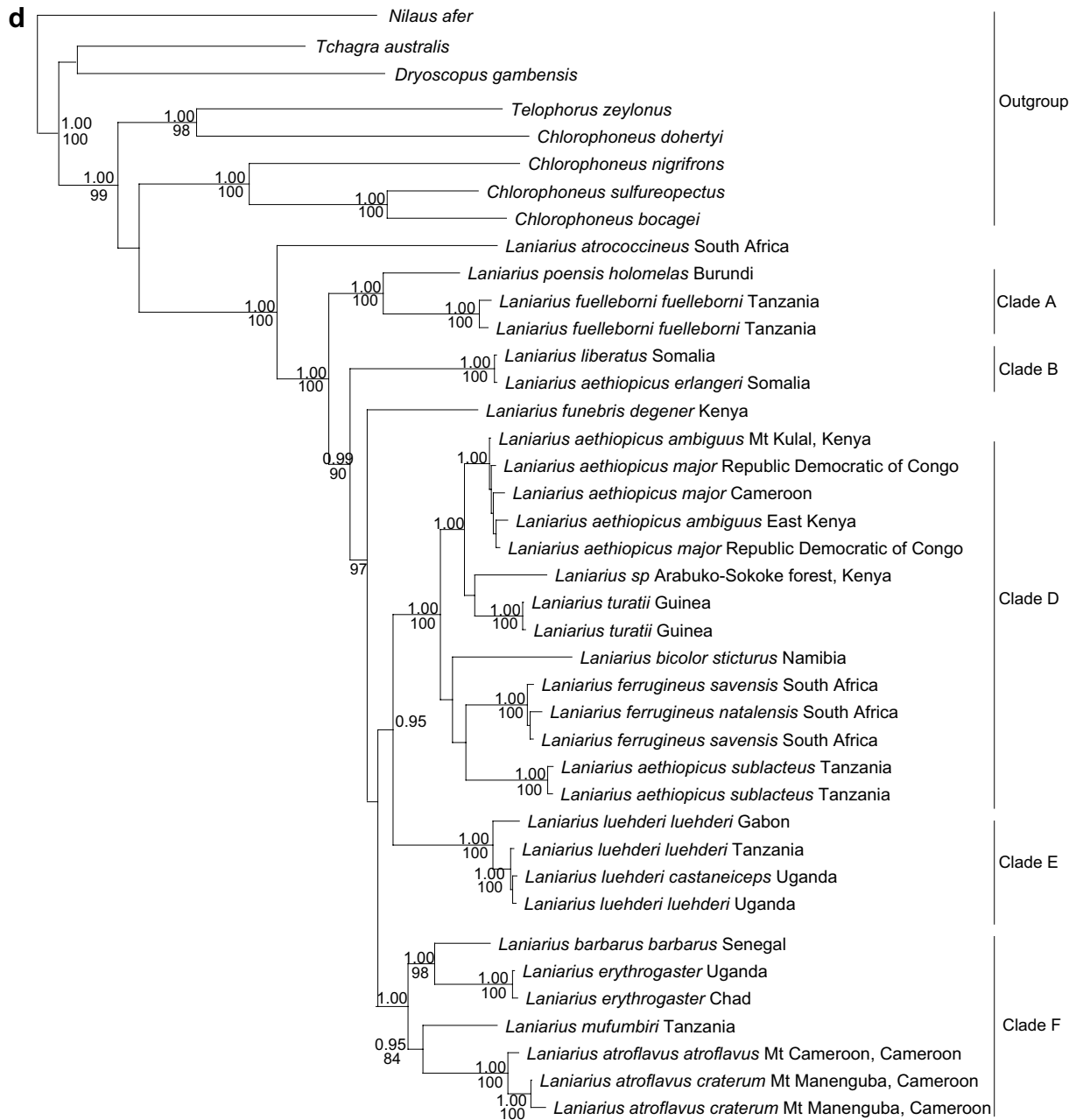
Fig. 1. (d)

L. atrococcineus most closely resembles *L. barbarus* and *L. erythrogaster,* and the three were usually put in the same superspecies (Table 1). Its isolated position in our analyses (Fig. 1a, b and d) is surprising at first glance but is in fact consistent with several morphological and ecological traits. Contrary to other crimson gonoleks, *L. atrococcineus* has a long white wing-stripe, very deep crimson-red underparts including undertail-coverts, and thighs black (Mackworth-Praed and Grant, 1960; Fry et al., 2000). Its nest is woven of shreds of bark (Harris and Franklin, 2000), and its habitat appears different (Sonnenschein and Reyer, 1984). This grouping, with the other crimson taxa, was thus simply a convergence. For *L. leucorhynchus,* often merged in the same superspecies as the other black boubous (Table 1), appears to be distantly related to these and our analyses thus fail to support this suggested superspecies. Like *L. atrococcineus,* *L. leucorhynchus* is different to other black boubous; ecologically separated, it lives mainly in lowland

forest (Sonnenschein and Reyer, 1984). Morphologically, it differs in details of the plumage coloration, proportions, calls and its juvenile has a whitish bill, unlike other young black boubous (Hall and Moreau, 1970; Fry et al., 2000). These differences support a grouping by convergence of these black boubous in the traditional authors (see Table 1).

4.2.2. Taxonomic implications and revisions

Laniarius aethiopicus erlangeri has long been known as a polymorphic form (with black and pied colour morphs) of *L. aethiopicus.* Our study shows clearly that *L. a. erlangeri* is not closely related to any other member of the suggested *Laniarius ferrugineus* superspecies. It is distinctly smaller than adjacent forms of *L. aethiopicus* more greenish glossed, and differs from them in ecology (Mackworth-Praed and Grant, 1960; Fry et al., 2000). With *L. a. erlangeri* placed far away from the type species of *Laniarius,* the Ethiopian
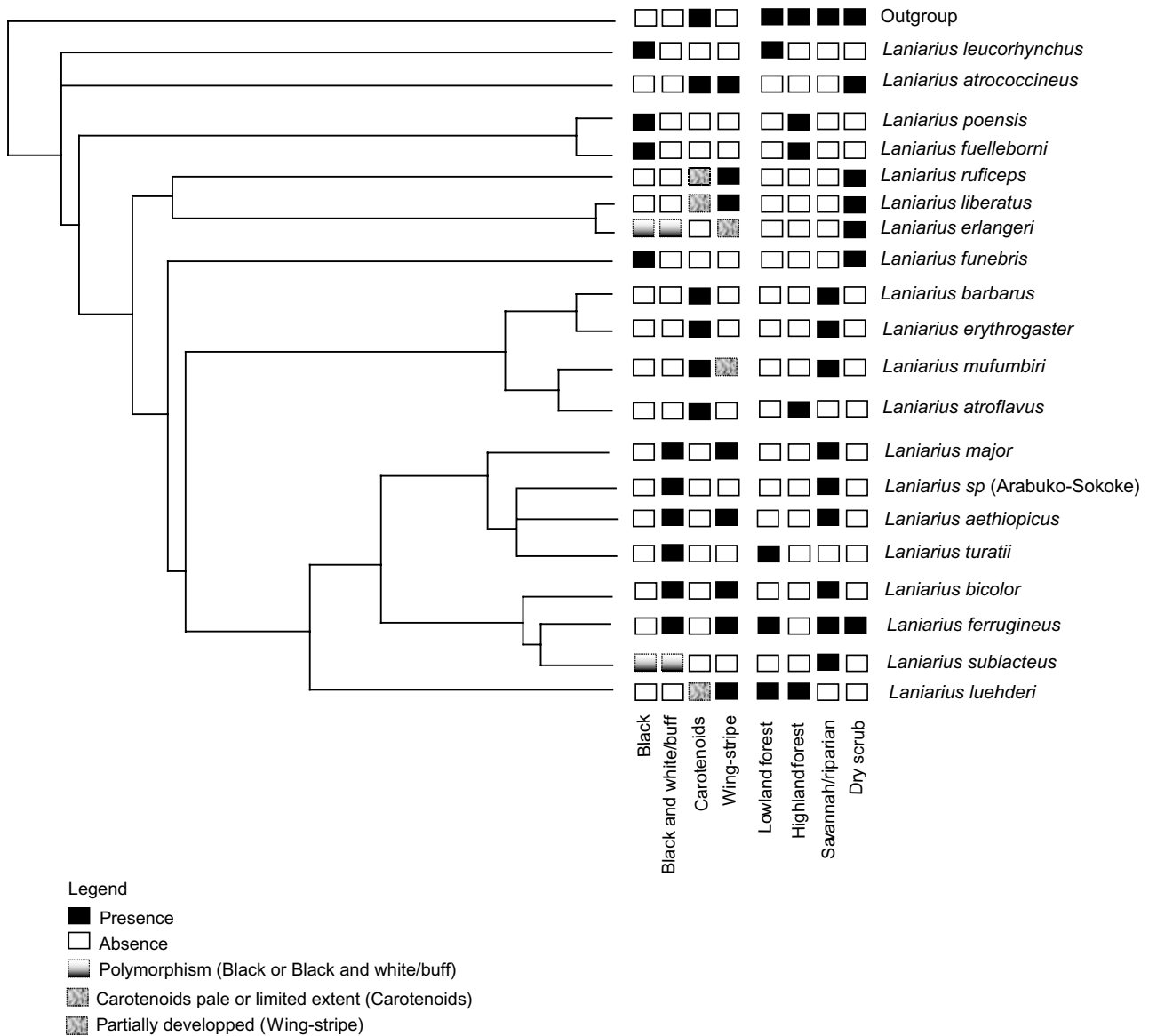
Fig. 2. Obtained tree after reconstruction of the presence of carotenoid (yellow, orange, or red) or not (black, or black/white) plumage patches in species of *Laniarius* using unordered parsimony with MacClade (Maddison and Maddison, 1992). This tree also includes the result obtained of the correlation between the plumage coloration and the different types of habitat of the taxa of *Laniarius*.

L. a. aethiopicus (Fig. 1b), we propose to resurrect the name *Laniarius erlangeri* Reichenow, 1905, refers to *L. a. erlangeri* of the present study.

Laniarius liberatus is known from one bird trapped in 1988 in central of Somalia (see Section 1). No traditional type specimen was collected, but 130 moulted feathers are kept, mounted according to feather tracts. In the initial molecular analysis of Smith et al. (1991), it was found that this bird could not be a hybrid with *L. ruficeps* or *L. a. aethiopicus*, but *L. a. erlangeri* was not included in this study. Later, Prinzinger et al. (1997) suggested that *L. liberatus* could be closely related either to *L. barbarus*, or to the *L. turatii* and *L. aethiopicus* clade, but even in this case the taxon sampling was incomplete. According to morphological characters, certain authors raised the possibility that it could be a hybrid involving *L. ruficeps* (Dowsett and Dowsett-Lemaire, 1993; Harris and Franklin, 2000), and Ash and Miskell (1998) suggested that it could be related to the polymorphic *L. a. erlangeri*. The position of *L. liberatus* found by Prinzinger et al. (1997) is contradicted by our data (Fig. 1a–d). Since similar results were obtained with the Z-linked

intron of nuclear DNA and with mitochondrial markers, we can exclude the possibility that *L. liberatus* is a hybrid and we conclude that *L. liberatus* is a synonymous with *L. erlangeri*.

Within the clade D, *L. a. sublacteus* does not cluster with the Ethiopian *L. a. aethiopicus*, or with the *ambiguus/major* group in this study. Morphologically, it differs from these by having completely black wings without any trace of a white wing-stripe, and underparts of its juvenile have no barring (Hall and Moreau, 1970; Fry et al., 2000). *L. a. sublacteus* also has a black morph in the area around the mouth of the Tana River in Kenya (Stresemann, 1947). We propose to resurrect the name *Laniarius sublacteus* Cassin, 1851, for the populations hitherto referred to as *L. a. sublacteus* of the present study. Otherwise, as the type species of *Laniarius* does not cluster together with *L. a. major* and *L. a. ambiguus*, which are clearly distantly related to it (Fig. 1b), we suggest to resurrect the name *L. major* Hartlaub, 1848 for the populations hitherto referred to as *L. a. major* and *L. a. ambiguus*. However, considering the amount of the individual variation in transition zones (between *major*, *ambiguus*, *mossambicus* and the boubou from

Arabuko-Sokoke forest), we strongly recommend that a more detailed phylogeographic study is made in order to confirm our suggestion and define species limits more precisely.

4.3. Evolution of morphological character 'plumage coloration'

Avian plumage colours have long been of interest to ecologists in a variety of studies—mate choice, parent-offspring signalling, sexual dichromatism, etc.—and especially the process of sexual selection (Darwin, 1871; Andersson, 1994). It has also been an important part of the evidence for classifying species and subspecies.

To understand coloration plumage evolution in *Laniarius*, we have considered several hypotheses. Plumage colour has long been considered as signs of quality used in the sexual selection by the female for the choice of males (Darwin, 1871; Andersson, 1994). In *Laniarius*, sexes are generally alike and when the sexual dimorphism exists, it is often just a matter of intensity (of pigmentation) or size. In the absence of sexual dichromatism, which is genetically controlled by sex chromosomes (Mundy 2006), we exclude the hypothesis that the evolution of plumage color in *Laniarius* is mainly due to sexual selection. According to several authors (Hamilton and Zuk, 1982; McGraw, 2006; Hill, 2006), the carotenoid and melanin pigments should have immune-system-signalling properties. However, no relevant information exists to judge this possibility. Gloger (1833) noticed that feathers should tend to be darkest in habitats where the relative humidity is high and pale where it is low. However, dark plumage (as in *L. funebris*) or strikingly coloured (black and dark red of *L. atrococcineus*) can also be found in dry habitats, and bright colours (such as yellow underparts of *L. atroflavus*) can be found in humid forest. Thus this hypothesis is not confirmed in *Laniarius*.

Biochemically, it is documented, for the color polymorphism found in birds, that genes, coding for some enzymes which catalyze stages in the synthesis of both carotenoids and bare-parts (and in some cases melanin), may be not working properly (Völker, 1964; Ochii et al., 1992; Stradi, 1998; Hill, 2000; Stradi et al., 2001). According to these authors, after stop- or loss-of-function of these genes, mutations can cause other color patterns or intermediate forms. This could also explain why we have different kinds of plumage colour in *Laniarius* and why some closely related species or even subspecies present intermediate forms. An example directly in keeping with *Laniarius* illustrates this hypothesis (see Völker, 1964), but only a further biochemical and physiological study, using several polymorphic species of *Laniarius*, can really confirm this hypothesis.

Phylogenetically, in order to come closer to an interpretation of the confusing variation in colours and patterns in *Laniarius* species, we studied how the plumage characters varied in relation to the phylogeny. Few other studies (Johnson and Lanyon, 2000; Moore et al., 2006) have so far tried to approach plumage evolution using phylogenetic contrasts and they proposed a correlation with marsh nesting or strictly habitat type whereas others in this case that of Dumbacher and Fleischer (2001) revealed a convergent evolution for colour patterns. Our analyses do not confirm the first hypothesis but the second is not clearly rejected (Fig. 2). However, other hypotheses can also be developed. In view of the generally intense carotenoid colours in *Chlorophoneus* and very intense red (or yellow) in *Laniarius atrococcineus*, it is likely to assume that these colours are plesiomorphic in *Laniarius*. The white wing-stripe (although found in many other corvoid bird-group) is an apomorphy in *Laniarius*, as illustrated by its presence in *L. atrococcineus*, and furthermore in clades B, D and E (and vestigial in one member of clade F). The possibility that also *L. leucorhynchus* represents a deep branch (Fig. 2) may suggest, along with the position of *L. poensis*, *L. fuelleborni*, *L. erlangeri* (with a black morph) and

L. funebris (Fig. 1d), also that a black plumage appeared very early in the evolution of the genus. Thus, ancestral forms of *Laniarius* may have been black (in agreement with the needs for wear-resistant feathers in their typical microhabitat), but also had a disposition for evolving striking red or yellow colours for clear visual communication within the dense undergrowth. This means that the true novelties in the diversification of plumage colours are the cases of loss of pigments, such as appearance of paler colours or white underparts. Thus, the reduced melanisation is seen in two species inhabiting the thornbush of arid regions, the sooty grey *L. funebris*, and *L. ruficeps*, which has a grey mantle and white underparts. And the white underparts appear in clades B, D and E (with some pale yellow in *liberatus*, and more or less buffy or pinkish hues in *L. ruficeps* and other members of clade D).

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