

Climatic change as an engine for speciation in flightless Orthoptera species inhabiting African mountains

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Abstract

Many East African mountains are characterized by an exceptionally high biodiversity. Here we assess the hypothesis that climatic fluctuations during the Plio-Pleistocene led to ecological fragmentation with subsequent genetic isolation and speciation in forest habitats in East Africa. Hypotheses on speciation in savannah lineages are also investigated. To do this, mitochondrial DNA sequences from a group of bush crickets consisting of both forest and savannah inhabiting taxa were analysed in relation to Plio-Pleistocene range fragmentations indicated by palaeoclimatic studies. Coalescent modelling and mismatch distributions were used to distinguish between alternative biogeographical scenarios. The results indicate two radiations: the earliest one overlaps in time with the global spread of C4 grasslands and only grassland inhabiting lineages originated in this radiation. Climatically induced retraction of forest to higher altitudes about 0.8 million years ago, promoting vicariant speciation in species inhabiting the montane zone, can explain the second radiation. Although much of the biodiversity in East Africa is presently threatened by climate change, past climatic fluctuations appear to have contributed to the species richness observed in the East African hot spots. Perceiving forests as centres of speciation reinforces the importance of conserving the remaining forest patches in the region.

Keywords: climate change, East Africa, hot spots, Orthoptera, radiation, speciation

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Introduction

High mountain systems are continental versions of oceanic island archipelagoes and are attractive model systems for phylogeographical studies due to restricted gene flow between populations on individual mountains (Knowles 2001; Bowie *et al.* 2004, 2006; Kebede *et al.* 2007; Knowles *et al.* 2007). Such continental island archipelagoes are found all over the world but the high mountains of East Africa (Kenya, Tanzania and Uganda) are especially well known for being particularly rich in species (Fjeldså & Lovett 1997; Burgess *et al.* 2007; Hemp 2007). These mountains are of different ages and many of them are of a volcanic origin, created by volcanic activity from the Miocene to the Late Pliocene (Griffiths 1993). The vegetation along the slopes of

these mountains is typically divided into different zones: the afro-alpine zone, the ericaceous zone and the montane forest zone. A typical savannah landscape is found at lower altitudes below the montane forest at the plains lying between the mountains. The plants and animals inhabiting the different vegetation zones above the savannah therefore have a very patchy distribution throughout East Africa (Bowie *et al.* 2004, 2006; Hemp 2007; Kebede *et al.* 2007; Schultz *et al.* 2007).

The impact of climate change on species divergence and distributional shifts of populations and species during the Pleistocene glacial cycles in the northern hemisphere has been amply studied (see, e.g. Hewitt 2000). Climatic fluctuations have undoubtedly contributed in shaping the distribution of species also in East Africa in the past (Hamilton 1982; Gottelli *et al.* 2004). However, compared to the northern hemisphere, fewer studies have actually addressed the impact of climatic fluctuations on phylogeographical

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patterns in East Africa (but see Bowie *et al.* 2004, 2006; Hemp 2005b; Stanley & Olson 2005; Kebede *et al.* 2007; Schultz *et al.* 2007). A lot of palaeoclimatic research has been conducted in Africa, especially in the eastern part of the continent. Apart from the intrinsic scientific interest of palaeoclimate, another reason for this massive research on historical climate in this particular region is due to the fact that climatic change is believed to have had an important influence on evolution and community change in mammals in general and in hominids in particular (see, e.g. Dennell 2003; Finlayson 2005). As *Homo* and its ancestors have undergone significant evolutionary change the last five million years, the geological epochs of Plio- and Pleistocene have been particularly well studied with respect to climatic history (Fernandez & Vrba 2006). These studies indicate that East Africa has experienced a drying trend the last millions of years (deMenocal 1995; Fernandez & Vrba 2006), with humid periods superimposed on this long-term aridification (Trauth *et al.* 2005). The drying climate reduced the forest cover in the region and the expanding areas of savannah during the Plio-Pleistocene are thought to have had a significant effect on the evolutionary history of mammals (e.g. Flagstad *et al.* 2001) including our own genus (Trauth *et al.* 2005; Behrensmeyer 2006). However, during the wetter and warmer periods it is assumed that the forest cover expanded from the mountains to lower altitudes. More humid periods occurred from about 2.7–2.5, 1.9–1.7 and 1.1–0.9 million years ago (Ma; Trauth *et al.* 2005) and originally isolated forest patches may have become connected during these periods, allowing allopatric forest inhabiting populations to meet and potentially mix and spread. During dry and cold periods, the forest probably retracted to higher altitudes and was replaced by savannah landscape between the mountains. The Plio-Pleistocene climate in East Africa may therefore be characterized as a continuum of alternating wet and dry conditions (DeMenocal 1995), climatic fluctuations which surely influenced the forest cover in the region. This is especially so for the mid- and late Pleistocene where large-amplitude glacial cycles affected the tropical African vegetation (Dupont *et al.* 2001). However, the impact of climatic fluctuations on distribution and divergence of species living on the mountains in East Africa may vary extensively as the mountains provided relatively stable habitats despite changes in climate (Fjelds  & Lovett 1997; Burgess *et al.* 2007; Fjelds  & Bowie 2008).

This paper investigates to what degree major climatic changes in East Africa during the last millions of years might have influenced speciation, radiation and dispersal (i.e. the evolutionary history) of a group of flightless Orthoptera species. Flightless insects have reduced ability to disperse and are thus conservative indicators of past biogeographical change. Most of the species used in the study either inhabit forests, forest edge or forest clearings of the montane zone or savannah grasslands. Forests are

more or less absent in the lowland and each of the species in the study inhabiting the montane zone is restricted to a particular mountain or mountainous area (Fig. 1). In contrast, the savannah and the species living there mostly have a wider distribution. All species used in the study belong to the subfamily Phaneropterinae (Orthoptera: Tettigoniidae).

Several hypotheses are addressed in this study. First, as forest seems to predate savannah in East Africa in the Miocene (deMenocal 1995), we hypothesize that grassland adopted lineages evolved from forest-living species, that is, that forest habitat is the original habitat for this group of species. A global spread of C4 grasslands at the expense of C3 vegetation occurred about 4–7 Ma (Cerling *et al.* 1993; Morgan *et al.* 1994; Latorre *et al.* 1997; Barry *et al.* 2002). This invasion probably formed opportunities for adaptive speciation of insects to the new radiating plant variants or to the niches left vacant after extinction of insects dependent on the eradicated C3 plant species communities. The grassland species included in this study all depend on different plant communities and therefore differ from each other ecologically as well as morphologically. We therefore hypothesize that many grassland inhabiting lineages originated at the same time as the spread of C4 grasslands, that is, around the Miocene–Pliocene boundary. A further goal with this study is to investigate if the patchily distributed Orthoptera species inhabiting the montane zone diverged because of (i) climatically induced ecological fragmentation of the montane forest, with subsequent genetic isolation of forest species on different mountains; or (ii) through adaptation to novel habitats on newly formed volcanoes. The vicariant speciation hypothesis predicts more or less contemporary speciation events in montane forest species in the dry periods following the more humid periods, that is, when the forest bridges connecting montane niches were replaced by savannah because of the drier climatic conditions. In contrast, the adaptive speciation hypothesis predicts speciation events approximately at the same time as the volcano inhabiting the species was formed, as it is well known that new and unoccupied niches on emerging volcanoes often lead to speciation when invaded by species (Jordan *et al.* 2003; Coyne & Orr 2004). Another contrasting prediction between the two hypotheses is the distribution of the species events in time: vicariant speciation of montane forest lineages due to the same climatically induced forest fragmentation is expected to happen within a restricted time window of a few hundred thousand years. Many speciation events within such a short time frame are not necessarily expected within the adaptive speciation hypothesis as many of the volcanoes are of different age.

To test our hypotheses we examined phylogeographical patterns by inferring phylogenetic relationships from mitochondrial sequence data and compared these relationships with the species' present distribution. To distinguish

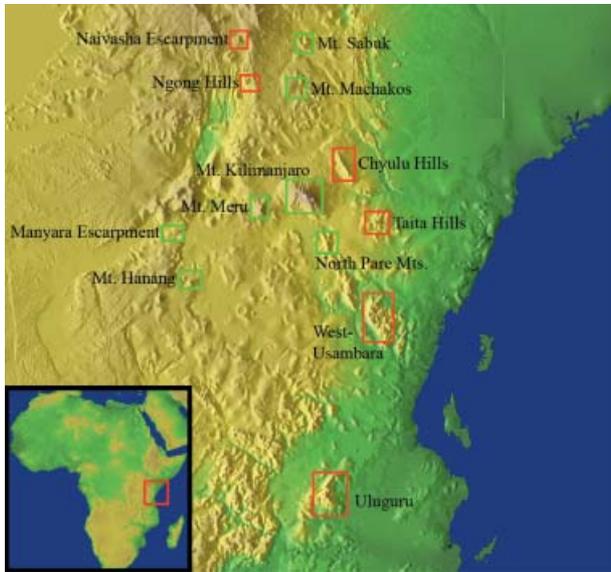


Fig. 1 Map showing the location of mountains, volcanoes and other geological formations inhabited by species included in the study (see Table 1 for more detailed species information). Forest species are all restricted to one or a few of these elevated formations and the green squares indicate where we sampled the bush crickets inhabiting the montane zone (Mount Kilimanjaro, *Monticolaria kilimandjarica*, *Horatosphaga montivaga*; Mount Meru, *Monticolaria meruensis*, *H. montivaga*; Manyara Escarpment, *Monticolaria manyara*, *Horatosphaga nou*; Mt. Hanang, new species X; North Pare Mountains, *Horatosphaga parensis*; Mount Sabuk, *Horatosphaga sabuk*; Mount Machakos, *H. sabuk*). Most of the grassland species have a more widespread distribution compared to the montane species.

between alternative biogeographical hypotheses and to date speciation events, we used coalescent modelling and mismatch distributions together with a molecular-clock approach.

Materials and methods

Study system

The bush crickets included in our study represent four different genera belonging to the subfamily Phaneropterinae. All species used are restricted to East Africa. The genus *Monticolaria* consists of species occurring in submontane and montane forest, forest edge and clearings while the genus *Horatosphaga* comprises a mix of species inhabiting montane forest and savannah habitats. Species belonging to the genera *Lamecosoma* and *Peronura* are all grassland inhabitants. While the montane species in this study are endemic to a single mountain or volcano, the open-land species have a much more widespread distribution (Fig. 1, Table 1). Two species previously not described (named new species X and Y) are included in the study. All specimens

are part of the collection of Claudia Hemp, including all specimens of the two new species. Information on holotypes and their location will be given in publications describing the new species.

Sampling and laboratory analyses

The analysed specimens were collected between 1997 and 2006, either by hand or by the use of catching nets. Sample sizes are for the most part small, reflecting the rarity of many of the species. We removed one hind leg from each specimen for DNA extraction and cut it into small pieces. DNA was extracted using a standard phenol–chloroform protocol (Sambrook *et al.* 1989). Using polymerase chain reactions, we amplified segments of the mitochondrial gene cytochrome oxidase I (COI) using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994) and segments of the third domain of 12S gene using the primers SR-J-14233 (5'-AAGAGCGACGGCG ATGTGT-3') and SR-N-14588 (5'-AAACTAGGATTAGATA CCCTATTAT-3') (Simon *et al.* 1994) (see Appendix S1, Supporting information for further details on laboratory analyses). Sequences were aligned by eye using MEGA version 3.1 (Kumar *et al.* 2004). In total, we sequenced 558 bp from 80 individuals of the COI gene and 398 bp from 34 individuals for the 12S gene. Because of huge morphological differences within the ingroup, we chose outgroup species from outside the Phaneropterinae subfamily to ensure that the ingroup species were more closely related to each other than to the outgroup. Sequences of *Usambilla olivacea*, *Altiusambilla modicicrus* and *Rhainopomma usambaricum* were used as outgroup for the COI data set while a sequence of *Locusta migratoria* was used as an outgroup for a combined COI and 12S data set. All sequences used are available in GenBank (for accession numbers, see Table 1).

Phylogenetic analysis

Bayesian inference (BI) analysis (Huelsenbeck & Ronquist 2001) was performed in MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001) on both the COI sequence data set and the combined COI/12S data set. A general time reversible model with invariable sites and a gamma distribution (GTR + I + Γ) fitted both the COI and 12S sequences best according to the Akaike information criterion in ModelTest version 3.7 (Posada & Crandall 1998). For each of the two analyses in MrBayes we therefore set the priors to match this model, but we did not fix any of the parameters. Two independent analyses were run simultaneously, each starting from different random trees. Each search was run with four Markov chains for 3 000 000 generations. Trees were sampled every 1000 generation. We discarded the first

Table 1 Species table

Species	Current range	Region	Collection location (number of specimens)	Habitat type	Source; references	GenBank Accession nos
<i>Horatosphaga heteromorpha</i> Karsch, 1889	Widespread	East Africa	North Pare (1), Kilimanjaro (1), Taita Hills (2), Uluguru (1), Meru (1), Pangani/Mwera (1), W-Usambara (1)	Colline to montane bush- and grassland	Hemp (2005b)	EU286954, EU309732–08
<i>Horatosphaga</i> new species X	Narrow/endemic	Mount Hanang, Tanzania	(5)*	Herbaceous vegetation along montane forest edges and clearings		EU309748–52
<i>Horatosphaga nou</i> Hemp, 2006	Narrow/endemic	Manyara Escarpment, Tanzania	(3)*	Herbaceous vegetation along montane forest edges and clearings	Hemp (2006)	EU309739–41
<i>Horatosphaga parensis</i> Hemp, 2002	Narrow/endemic	North and South Pare Mountains	North Pare only (2)	Herbaceous vegetation along montane forest edges and clearings	Hemp (2002)	EU309756–57
<i>Horatosphaga tenera</i> Hemp, 2007	Narrow	Ngong Hills, Kenya	(3)*	Savannah grasslands	Hemp (2007)	EU309764–66
<i>Horatosphaga regularis</i> Bolivar, 1922	Widespread?	Kenya	Ngong Hills (4) and Kikuyu Escarpment (2)	Submontane savannah to montane grasslands	Ragge (1960)	EU286957, EU309759–63
<i>Horatosphaga montivaga</i> Sjöstedt, 1909	Narrow/endemic	Mount Meru and Mount Kilimanjaro, Tanzania	(7)*	Herbaceous vegetation along montane forest edges and clearings	Hemp & Hemp 2003	EU286955, EU309742–47
<i>Horatosphaga meruensis</i> Sjöstedt, 1909	Narrow/endemic	Mount Meru and Mount Kilimanjaro, Tanzania	(1)*	Colline and submontane grasslands	Hemp (2007)	EU309758
<i>Horatosphaga sabuk</i> Hemp, 2006	Narrow	Kenyan Highlands (Mount Machakos, Mount Sabuk)	(4)*	Herbaceous vegetation along montane forest edges and clearings	Hemp 2006	EU286958, EU309753–55
<i>Lamecosoma inermis</i> Ragge (1961)	Widespread?	Tanzania, Kenya	Kilimanjaro (3)	Colline to montane grasslands	Ragge (1961)	EU286959, EU309793–94
<i>Lamecosoma</i> new species Y	Narrow?	Mount Hanang, Tanzania	(5)*	Montane grasslands		EU309788–92
<i>Peronura clavigera</i> Karsch, 1889	Widespread	Kenya, Tanzania	North Pare (4), Kilimanjaro (6), Taita Hills (3)	Herbaceous vegetation from the colline to the montane zone		EU286956, EU309776–87
<i>Peronura uguenoensis</i> Hemp, 2002	Narrow/endemic	North Pare Mountains, Tanzania	(10)*	Grassland intermingled with herbs, forest edges	Hemp 2002	EU302812, EU309767–75
<i>Monticolaria kilimandjarica</i> Sjöstedt, 1909	Narrow/endemic	Mount Kilimanjaro, Tanzania	(4)*	Disturbed submontane forests and disturbed and undisturbed montane forests and clearings	Hemp & Hemp 2003	EU286960, EU302802–04
<i>Monticolaria meruensis</i> Sjöstedt, 1909	Narrow/endemic	Mount Meru, Tanzania	(3)*	Disturbed submontane forests and disturbed and undisturbed montane forests and clearings		EU286961, EU302805–06
<i>Monticolaria manyara</i> Hemp, 2008	Narrow/endemic	Manyara Escarpment (Nou forest reserve) and Mount Hanang, Tanzania	(3)*	Montane forests and clearings	Hemp <i>et al.</i> in press 2008)	EU286962, EU302807–08

Summary of current range, region, collection locations and habitat type of the species included in the study. Star (*) indicates that species were collected in the region given in the table.

750 000 generations as burn-in. Plots showing generation vs. the log probability of the data given the parameter values, were analysed for both data sets to ensure the two independent analyses had reached stationarity before the end of the burn-in.

Estimation genetic distances and of divergence rate

The timing of divergences between species and populations provides an important test of phylogeographical hypotheses. In this respect, the time since they split must be consistent with the historical events that putatively have driven their divergence. In order to estimate when lineages diverged, however, it is necessary to have an accurate estimate of the evolutionary rate in which mutations accumulate in the DNA sequences used.

Using COI consensus sequences made for each species in BioEdit version 5.0.9 (Hall 1999), we applied Tajima's 1D and 2D relative rate tests (Tajima 1993) to examine if the substitution rate varied between species, as implemented in MEGA (Kumar *et al.* 2004). Less than 5% of the tests indicated deviations from the molecular-clock hypothesis. Large differences in divergence rates between lineages induced by, for example, environmental stress, seem therefore not to be a major problem. We then computed genetic distances (both corrected and uncorrected using PAUP* (Swofford 2001) (Table S1, Supporting information). The reliability of such genetic distances often decreases with increased sequence divergence due to multiple substitutions. We therefore plotted transitions and transversions vs. corrected genetic distance to search for mutation saturation (Fig. 2). Nucleotide substitutions seem to accumulate linearly up to a corrected genetic distance of approximately 0.2.

Calibrated divergence rates applicable to the mitochondrial genome vary to some extent (e.g. Brower 1994; Fleischer *et al.* 1998). If possible, evolutionary rates should therefore be estimated in each study separately, if only to verify whether the divergence rate found seems to be about the same as found in previous studies. Previous calibrations of the COI divergence against geological data have found an evolutionary rate of 2% divergence between lineages per million years for Coleoptera species (Juan *et al.* 1995, 1996). A 2% divergence rate has also often been used in phylogeographical studies on insects using the COI gene (Juan *et al.* 1995, 1996; Trewick & Morgan-Richards 2005; Spooner & Ritchie 2006). To investigate if this divergence rate was suitable in our study, we compared COI divergence for endemic species with the approximate geological age of the volcanoes these species inhabit. Mount Kilimanjaro and Mount Meru are both assumed to be the result of the same geological processes and the oldest lavas of Mount Kilimanjaro are about 1.0 million years old (Griffiths 1993). These two volcanoes are inhabited by *Monticolaria kili- mandjarica* and *Monticolaria meruensis*, respectively, while

Horatosphaga montivaga exists on both volcanoes. Exactly half of the corrected divergence rates for these species lie very close to 2.0% (1.6, 2.0 and 2.9%, see Table S1). The other corrected distances were larger (e.g. 12%). However, our phylogenetic analyses strongly indicate that these values belong to species that diverged much earlier than the time of the orogenetic process creating the mountains they now inhabit. A 2.0% divergence rate therefore seems reasonable to use also in our case.

Distinguishing between alternative biogeographical hypotheses and dating of speciation events

Distinguishing between alternative biogeographical hypotheses is often difficult. We used two methods to differentiate between contrasting historical biogeographical scenarios and to estimate the timing of the divergence between different species. Every approach for estimating divergence times has certain shortcomings. Accordingly, one should try to use more than one method in order to investigate the reliability of the estimate found. In this study, we therefore use two coalescent methods and a mismatch distribution approach to estimate the divergence times between some of the recent lineages in our data. The coalescent methods and the mismatch distribution approach differ a lot from each other in how they calculate parameters. Congruence in time estimates found by the two methods may therefore indicate that the estimates can be considered reliable.

Ancestral and old lineage splitting events do often not conform to the assumptions made by the models applied in the mismatch and coalescent approaches used here. The timing of old and basal speciation events were therefore estimated by the use of a traditional molecular-clock approach. To ensure linearity, we only considered events where the corrected genetic distance between sister clades or species was less than 0.2 (Fig. 2).

Coalescent modelling. In order to examine to what extent gene flow occurs between newly formed species and to date divergence times between these species, we applied the coalescent approach implemented in the MDRV software package (Nielsen & Wakeley 2001). In Nielsen & Wakeley's (2001) method, a coalescent model which employs Bayesian inference in addition to a likelihood framework is established using a Markov chain Monte Carlo (MCMC) method to search parameter space while jointly estimating three parameters: theta (θ) ($2N_e\mu$, i.e. two times the effective population size times the mutation rate), T (the divergence time between the two populations scaled by the population size) and M (the migration rate between the two populations, also scaled by population size). Inference regarding the parameters is based on the posterior probability distribution of the parameters, $p(\theta, T, M | x)$, where x is the DNA

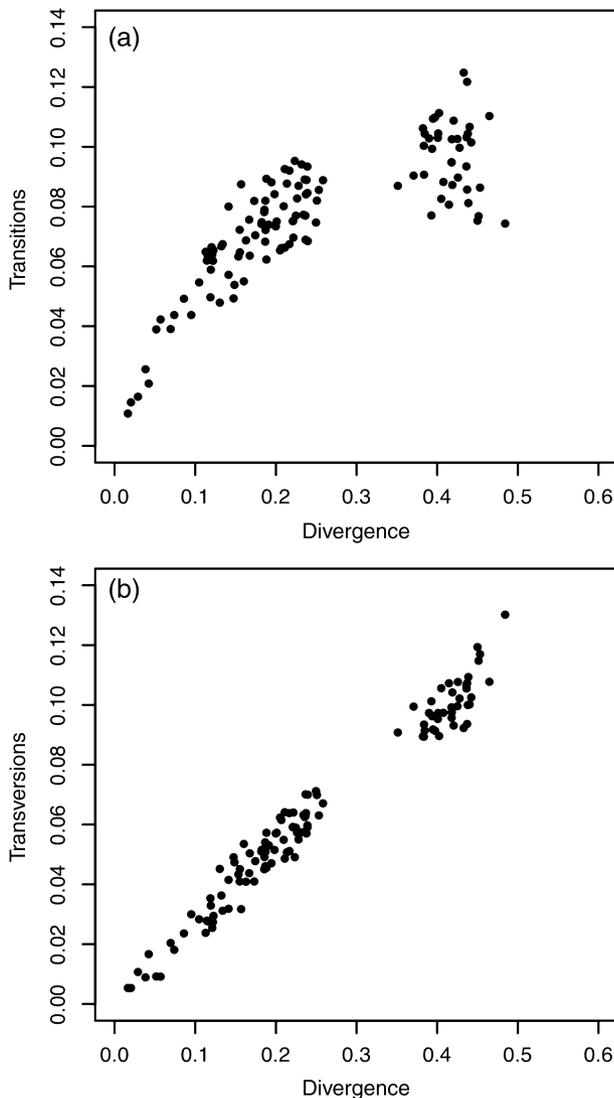


Fig. 2 Transitions (a) and transversions (b) vs. corrected genetic distance (using a GTR + I + Γ model) for the COI data set. Each species is represented with one consensus sequence.

sequence data using uniform priors. The parameter space needs to be constrained to ensure that the posterior distributions are appropriate (i.e. biological plausible) and the maximum values for the parameters to be estimated must therefore be defined before the analysis. Analyses were run over again with a higher prior max value if the estimated parameter lied close to the prior maximum value chosen. We also conducted reruns with different preset maximum values to check if alternative preset values affected the posterior distribution of parameter estimates. No such effect was detected.

We conducted four pairwise comparisons using COI sequences from five different species. Species comparisons were only conducted within monophyletic groups, and

only on species pairs that were separated by relatively low genetic distances. θ , M and T were estimated using 5 000 000 generations of MCMC with 500 000 burn-in cycles. The parameters were all estimated from the mode of their respective posterior distribution. Confidence intervals were calculated using Akaike's information criteria (AIC; Burnham & Anderson 1998). Coalescent time units were converted to years using:

$$T_{\text{div}} = (T \times \theta) \div (2 \times \mu),$$

where μ is equal to the expected number of mutations that will occur in a sample of n base pairs per generation. MDIV also gives a point estimate of the expected time to the most recent common ancestor (TMRCA) of all the sequences given the data. TMRCA was converted to years as follows:

$$\text{tRMCA} = (\text{TRMCA} \times \theta) \div (2 \times \mu)$$

We also used the program MSBayes (Hickerson *et al.* 2007) to explicitly test for simultaneous divergence in multiple population pairs. This program employs an approximate Bayesian computation (ABC) under a hierarchical coalescent model to estimate three hyper-parameters (φ). These hyper-parameters characterize the degree of variability in divergence times across population pairs while allowing for variation in various within population-pair demographic subparameters (Φ) that can affect the coalescent. For full details of the model, see Hickerson *et al.* 2006 and Appendix S1. We used the hierarchal ABC model to estimate the number of possible divergence times per Y taxon-pair (Ψ), the mean divergence time across the Y taxon-pairs [$E(\tau)$] and the ratio of the variance of τ to the mean of τ [$\Omega = \text{var}(\tau)/E(\tau)$]. The hyper-posteriors were obtained from 1000 accepted draws from 500 000 simulated draws from the hyper-priori using the acceptance/rejection with local regression algorithm (Beaumont *et al.* 2002). Reruns of the prior simulations with different values of the prior distribution of parameters were conducted to check if alternative preset values affected the posterior distribution of the parameter estimates, but no such effect was detected. We estimated the time of divergence in generations by using:

$$T_{\text{div}} = \tau \times (\theta_{\text{AVE}} \div \mu),$$

where θ_{AVE} is a constant determined by the mean of the subprior for θ and μ is defined as the per gene per generation mutation rate. We assume one generation per year.

The same five species analysed using MDIV were also investigated using MSBayes. A total of four species pairs hypothesized to have diverged simultaneously were included in the analysis.

Mismatch distribution. To test for historical radiations of new species/lineages within haplogroups (a group of haplotypes that share a common ancestor) which showed phylogenetic signals compatible to such a scenario (e.g. groups with polytomies and/or where short branch lengths separated clades), we used a mismatch distribution approach (Slatkin & Hudson 1991; Rogers & Harpending 1992). A mismatch distribution approach is normally used on samples from different populations to test for, and estimate the timing of, intraspecific population expansion. However, the approach can also work on groups that have experienced recent speciation events since species radiation within a short period of time to a large extent is genetically equivalent to a regular intraspecific population expansion (Sturmbauer *et al.* 2005).

We calculated the mismatch distributions as implemented in Arlequin version 2.000 (Schneider *et al.* 2000). This program estimates the parameters of the demographic expansion by a generalized nonlinear least-square approach, as proposed by Schneider & Excoffier (1999). The model assumes that a stationary population at equilibrium experiences a sudden expansion in population size from N_0 to N_1 t generations ago after which the population rapidly reaches a new stationary phase. Based on the mean and variance of all the pairwise haplotype differences, the general nonlinear least-square approach is used to estimate the three demographic parameters $\tau = 2\mu t$, $\theta_0 = 2\mu N_0$ and $\theta_1 = 2\mu N_1$, where μ is the mutation rate for the whole haplotype. Approximate confidence intervals for the three parameters are obtained by a parametric bootstrap approach (Schneider & Excoffier 1999). The validity of the estimated stepwise expansion model is tested using the same parametric bootstrap approach, where the sum of square deviation (SSD) between the observed and the expected mismatch distribution is used as a test statistic. With a trusted model, the timing of the putative expansion event can be estimated from τ , which represents the expected number of differences between two randomly drawn haplotypes at time t since the population expansion. τ is defined as $2\mu t$ and simple rearrangements give $t = (\tau \text{ differences per haplotype}) / [(\text{divergence rate per million years} \times \text{number of nucleotides}) / 1 \text{ million years}]$.

Ninety-five per cent confidence intervals were estimated from the same parametric bootstrap approach that was used to test the validity of the expansion model, using 10 000 replicates.

Arlequin also computes the raggedness index of the observed distribution, as defined by Harpending (1994). This index indicates the shape of the mismatch distribution as it takes larger values for multimodal distributions than for unimodal distributions. A small raggedness index value indicates low raggedness, typical for recently non-stationary expanding populations. The significance of the raggedness value is tested as described above for SSD using 10 000 bootstrap replicates.

Results

Phylogenetic analyses

The Bayesian inference based on the COI and the COI/12S data sets is very similar (Figs 3 and 4). Four distinct haplogroups with high clade support are evident in both trees and each haplogroup seem to share a particular ecological zone. *Monticolaria* (haplogroup A) is the sister group to the rest of the ingroup and consists of species living within a montane habitat. All species in the monophyletic clade consisting of *Horatosphaga nou*, *H. montivaga* and new species X (haplogroup B) also inhabit the montane zone. The two remaining haplogroups (C & D) consist of grassland inhabiting species only. The main difference between the two trees is the location of the species *Horatosphaga parensis* and *Horatosphaga sabuk*. These two species are therefore not part of any of the four defined haplogroups.

Paraphyly is common at the genus level and *Monticolaria* is the only monophyletic genus in both phylogenetic trees. Also, intraspecific lineages of *Lamecosoma inermis* and *Horatosphaga heteromorpha* do not form monophyletic clades in the COI tree.

The low support for some of the old lineages observed in the phylogenetic trees is not desirable from a taxonomic point of view. More data are probably needed to better understand the lineage splitting events in these parts of the phylogeny. However, low clade support can also suggest a radiation of new species originating more or less simultaneously. To address this hypothesis, we computed genetic distances and their equivalents measured in million years between the lineages *Peronura clavigera*, *P. uguenoensis* and *Horatosphaga regularis* and between the different main lineages within haplogroup B (for details, see Fig. S1, Supporting information). All these lineages gave rise to savannah inhabiting species and seem to have originated between 4.0 and 6.1 Ma.

Coalescent analyses

For all pairwise comparisons, estimates of migration rates suggest zero or extremely low levels of recent gene flow between the species compared (Fig. S2, Supporting information). The posterior distributions of both divergence time and the population mutation rate parameter show a very large degree of overlap in all pairwise comparisons (Fig. 5) and the point estimates suggest that the three *Horatosphaga* species originated during a time period of less than 250 000 years (Table 2).

A conventional molecular clock estimates the time to the most recent common ancestor in a sample of haplotypes. In contrast, the divergence time dates back to the time when gene flow no longer occurred between the two popula-

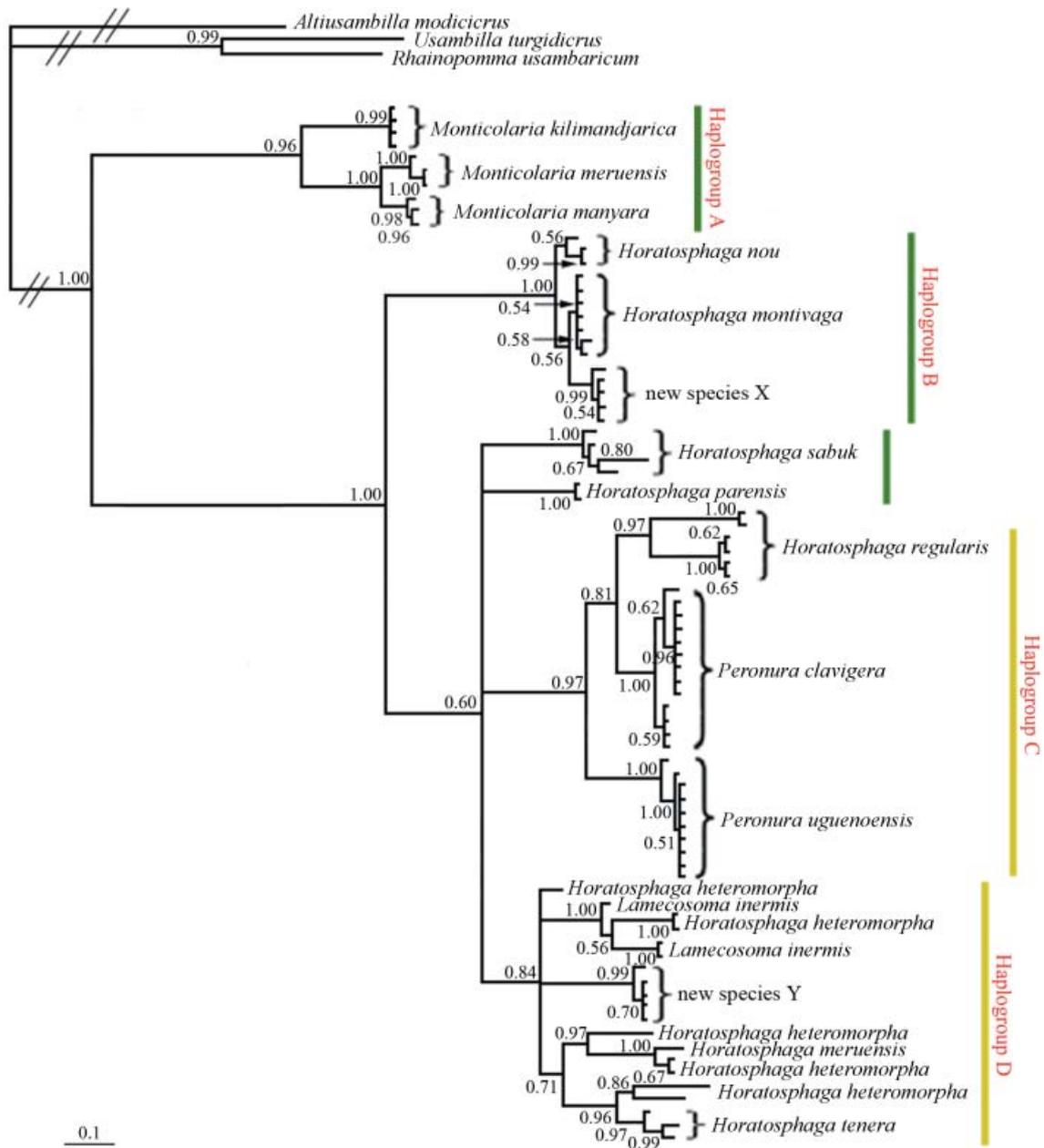


Fig. 3 Bayesian inference of the phylogeny of the species included in the study, based on a fragment of the COI-gene. Priors were set to match a GTR + I + Γ model. *Usambilla olivacea*, *Altiusambilla modicicrus* and *Rhainopomma usambaricum* were used as outgroup. Branch lengths are unconstrained with a uniformly distributed prior. Support values are indicated for nodes that were supported in 50% or more of the sampled trees. Colour bars to the right of the tree indicate habitat type: green, species within the montane zone; yellow, grassland inhabiting species. The different haplogroups are also indicated.

tions. By definition within coalescent theory, the time to common ancestry (tMRCA) must always be longer than the time since divergence (T_{div}) between two populations. This is confirmed by the values for tMRCA and T_{div} computed (Table 2). However, we found *H. montivaga* and new species X to have a lower tMRCA value than *H. nou* and *H. montivaga*, although the estimated T_{div} values suggest

that *H. montivaga* diverged later from *H. nou* than from new species X. This discrepancy might indicate that these three species originated approximately at the same time. Indeed, the ABC analysis performed in MSBayes indicates that the data are most consistent with one simultaneous divergence event for all the four species pairs analysed. The estimate of the number of divergence times for the four species pairs

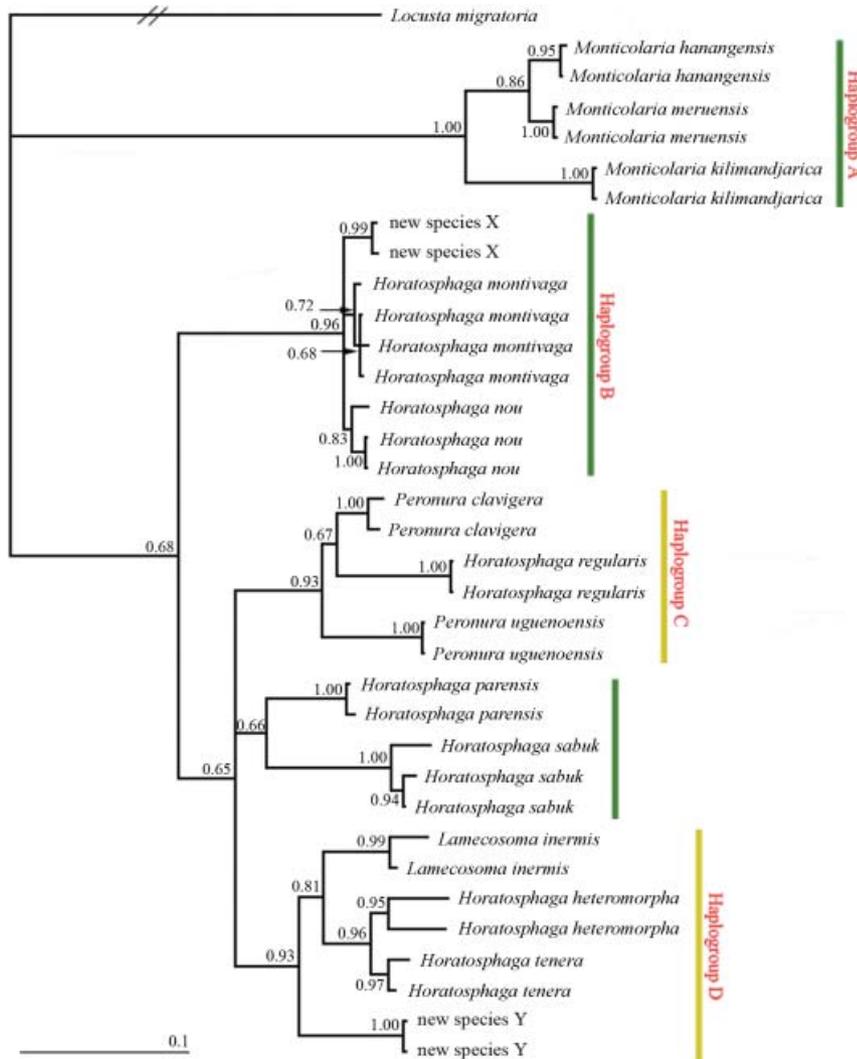


Fig. 4 Bayesian inference of the combined COI/12S data set. Priors were set to match a GTR + I + Γ model. *Locusta migratoria* was used as an outgroup. Branch lengths are unconstrained with a uniformly distributed prior. Support values are indicated for nodes that were supported in 50% or more of the sampled trees. Colour bars to the right of the tree indicate habitat type: green, species within the montane zone; yellow, grassland inhabiting species. The different haplogroups are also indicated.

Table 2 Divergence times and times to most recent common ancestor

	T_{div} (ybp)	Lower (ybp)	Upper (ybp)	tMRCA (ybp)
<i>Horatosphaga nou</i> and new species X	698 683	235 200	2 026 874	938 630
<i>Horatosphaga nou</i> and <i>H. montivaga</i>	464 132	162 940	1 268 957	713 085
<i>Horatosphaga montivaga</i> and new species X	504 288	181 317	1 388 209	645 197
<i>Monticolaria manyara</i> and <i>M. meruensis</i>	925 408	347 028	3 416 892	1 379 911

Estimates of time since divergence (T_{div}) and time to most recent common ancestor (tMRCA) are given in years before present (ybp). Lower and upper time since divergence represent the extreme values defined by 2 AIC units (ΔAIC) on either side of the point estimate.

(Ψ) is 1.057 (95% confidence interval: 1.000–1.968, see Fig. S3, Supporting information) and also the value of uniformity in τ across the four taxon pairs ($\Omega = 0.005$, 95% confidence interval: 0.000–0.028) provide strong support for one simultaneous divergence time for all four species pairs

(Fig. 6). The estimate of mean τ for all four population pairs [$E(\tau)$] is 0.252 (95% confidence interval 0.130–0.370). According to this estimate of $E(\tau)$, the divergence of the four population pairs happened approximately 900 000 years ago (95% confidence interval: 471 774–1 342 742).

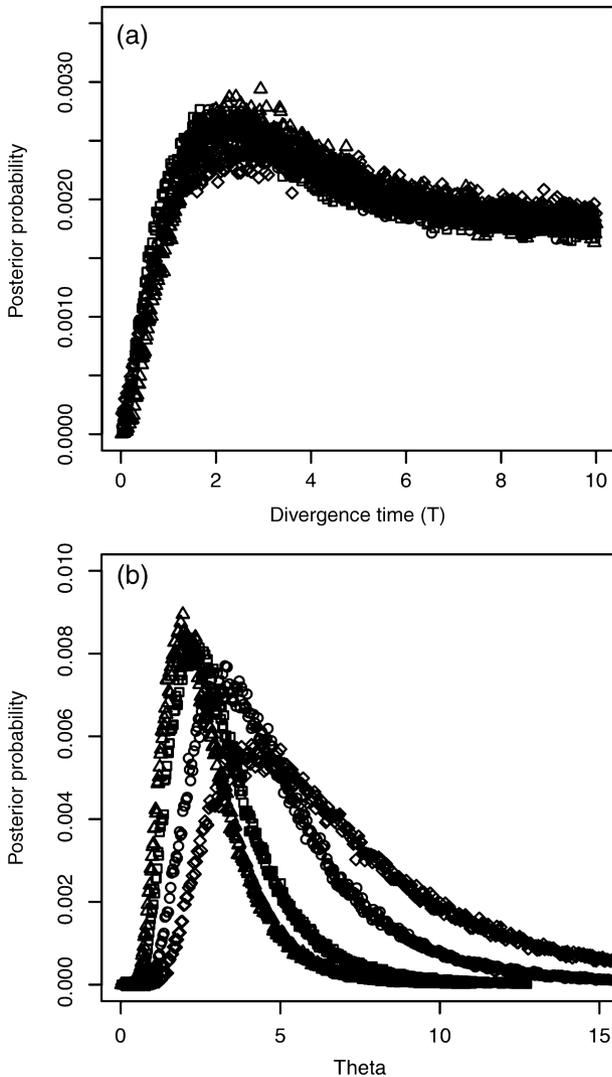


Fig. 5 One of the three independent replicates of the posterior probabilities (pp) for divergence time (T) (upper) and for theta (θ) (lower) for each of the four pairwise comparisons between species; \circ , *Horatosphaga nou* & new species X; \square , *H. montivaga* & *H. nou*; Δ , new species x and *H. montivaga*; \diamond , *Monticolaria hanangensis* and *M. meruensis*.

Mismatch distributions

Mismatch distributions were calculated for haplogroups B and D as these clades showed phylogenetic signals compatible with historical radiations of new species/lineages. Two peaks can be seen in the observed mismatch distribution of haplogroup B (*H. nou*, *H. montivaga* and new species X) (Fig. 7). The three bars on the left side of the histogram represent expected intraspecific variation of the three species. Excluding these three bars makes the distribution unimodal, a pattern which is consistent with

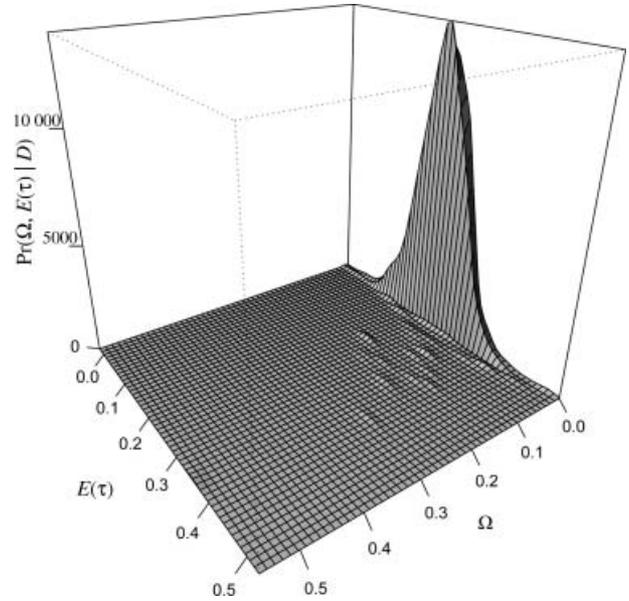


Fig. 6 Three-dimensional joint posterior probability density for $E(\tau)$ and $\Omega = \text{Var}(\tau)/E(\tau)$.

a historic demographical expansion. The sum of squared deviations (0.031, $P = 0.228$) and the raggedness index (0.042, $P = 0.475$) support this interpretation. Given the estimated tau of about 7.8, we calculated the population expansion to have happened about 680 000 years ago (95% confidence interval: 307 672–1 090 829). This estimate falls within the upper and lower divergence estimates found in the coalescent analysis.

The overall shape of the mismatch distribution of haplogroup D (*Horatosphaga heteromorpha*, *H. meruensis*, *H. tenera*, *Lamecosoma inermis* and new species Y) also shows a unimodal pattern. Neither the raggedness index (0.023, $P = 0.076$) nor the sum of squared deviations (0.016, $P = 0.057$) contradicts this interpretation but the support for an expansion of new species and lineages is not as strong as for haplogroup B. We estimated tau to about 43.9 and the population expansion to have happened almost 4 Ma (95% confidence interval: 3 151 523–4 261 648).

Discussion

Species within each of the four haplogroups in the evolutionary tree share the same habitat type. Species within *Monticolaria* (haplogroup A) and the clade comprising *Horatosphaga nou*, *H. montivaga* and new species X (haplogroup B) are all inhabitants of the montane zone, while the two last haplogroups (C & D) consists solely of grassland inhabitants of lower zones. Paraphyly is evident for all examined genera, except *Monticolaria*. Also the intraspecific lineages of *Lamecosoma inermis* and *Horatosphaga heteromorpha* do not form monophyletic clades in the

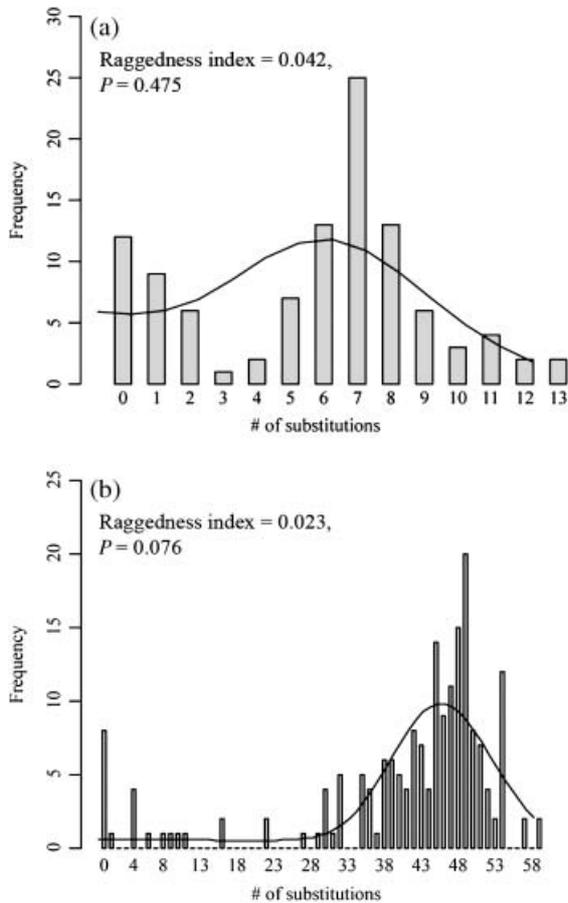


Fig. 7 The expected (line) and observed (bars) mismatch distribution given a null distribution compatible with sudden population expansion for (a) haplogroup B and (b) haplogroup D. Harpending's raggedness index (Rogers & Harpending 1992) with significance levels are indicated for each of the two haplogroups.

phylogenetic tree based on the COI data set. Inconsistencies of the traditional taxonomy have long been anticipated (Ragge 1960; Hemp 2007) and a reconsideration of the present taxonomy at the genus level therefore seems advisable if analyses of one or more nuclear markers confirm the taxonomic deviations detected in this study (see Appendix S1 for an extended taxonomic discussion). It should be noticed, though, that the deviations from the current taxonomy detected in the phylogenetic analysis do not affect the conclusions of this paper.

The evolutionary scenario of grassland inhabiting species evolving from forest living species is the most parsimonious explanation compared to the alternative hypothesis considering both phylogenetic trees constructed in this study. Furthermore, the forest dependent genus *Monticolaria* is very distinct both morphologically and genetically and appears as the ancestral group in the phylogeny. The genetic distances between *Monticolaria* and the other lineages

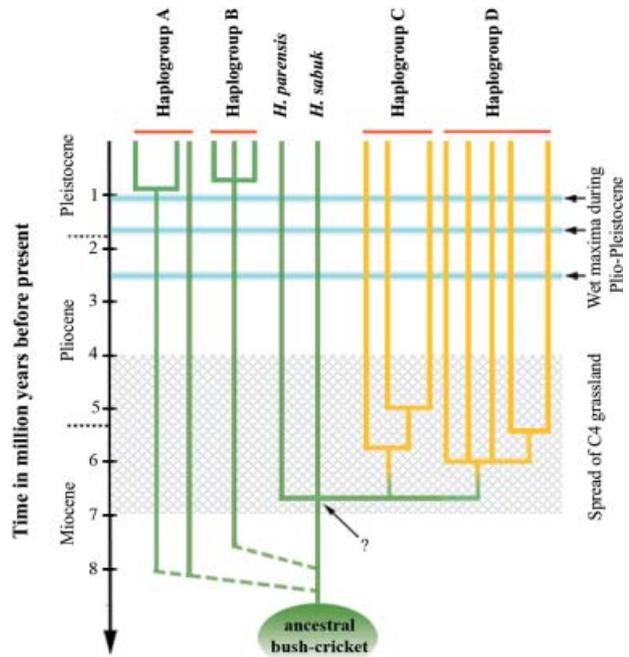


Fig. 8 Major evolutionary events within the bush crickets as related to the onset of the spread of C4 grassland in the late Miocene and Pliocene and the shorter wet periods in the Plio-Pleistocene. Question mark indicates uncertainty regarding time of speciation.

confirm an old common ancestor of the ingroup as a whole. Even the common ancestor of the remaining ingroup species seems to be quite ancient as is indicated by the large genetic distances between the remaining main clades. It is therefore interesting, in light of the old age of the ingroup, that nearly all speciation events within the four haplogroups mainly distribute themselves in two distinct and relatively short periods of time.

Spread of grasslands and adaptive radiation of savannah inhabiting lineages

Some parts of the phylogenetic tree show weak clade support (i.e. posterior probabilities below 0.50). Such low support in molecular phylogenetic analyses is often interpreted as a signal of insufficient data, inappropriate choice of genes or both. However, low clade support might also indicate a radiation of new species over a relative short period of time. The molecular-clock approach suggests that several different lineages originated between 4.0 and 6.1 Ma. Interestingly, all of these new lineages appear to have been grassland inhabiting species. A population expansion of grassland lineages about 4 Ma is also indicated by the mismatch distribution analysis. The profound increase in grassland lineages during the late Miocene and early Pliocene corresponds well to a substantial increase in C4

grasslands in widely separate parts of the globe from 7 to 4 Ma (Cerling *et al.* 1993; Morgan *et al.* 1994; Latorre *et al.* 1997; Barry *et al.* 2002). C4 grasses are characteristic of seasonal, arid and warm environments and are more tolerant of lower atmospheric CO₂ than C3 plants, indicating that the spread of C4 plants are climatically controlled (Jacobs *et al.* 1999). Extinction of the out-competed C3 plants may have led to further extinction of a variety of species, making niches earlier occupied by these species available for new species to invade. Adaptive speciation to the new niches promoted by the invading C4 species or adaptive speciation by invasion of the niches left vacant after extinction of C3 dependent species therefore seems as an obvious and plausible explanation for the contemporary massive burst of new grassland adopted lineages (Fig. 8).

Recent radiation of species within the montane zone

All species within haplogroups A and B are trapped in montane refuges at higher altitudes on mountains and volcanoes. Our analyses revealed that five out of six of these montane species have a quite recent origin. The coalescent analyses also strongly indicate that most of these species originated more or less simultaneously within a very short period of time, as might explain why we are not able to resolve the internal relationships between *H. nou*, *H. montivaga* and new species X. All these results point towards the existence of one or a few recent historical events which played an important role in speciation of the montane species.

East Africa has experienced an increased aridification over the last millions of years. Especially during the late Pliocene and Pleistocene African climate became cooler, drier and more seasonal, which triggered the spread of savannah in the eastern parts of the continent (deMenocal 1995). Vegetation shifted from closed canopy to open savannah, and evidence suggests a continuous presence of savannah from about 2.5 Ma and onwards (Fernandez & Vrba 2006). However, despite the overall trend of a more arid and cooler climate in this region, climatic fluctuations with more humid periods occurred from about 2.7–2.5, 1.9–1.7 and 1.1–0.9 Ma (Trauth *et al.* 2005). The Plio-Pleistocene climate in the region may therefore be characterized as a continuum of alternating wet and dry conditions, climatic fluctuations which surely influenced the forest cover in the region. While montane forests retracted to higher altitudes during dry and cold periods this was reversed during warm and wet periods when forests expanded down from the mountains towards lower altitudes. Accordingly, the periodically cooler and drier East African conditions after about 2.5 million years and their subsequent intensification about 1.7 and 0.9 Ma might have established opportunities for ecological fragmentation with subsequent genetic isolation of forest dependent species (deMenocal 1995).

The large-amplitude glacial cycles restricted in time to the last 900 000 years also caused large variations in the vegetation in East Africa (Dupont *et al.* 2001). Between 0.9 and 0.6 million years, interglacials were characterized by warm dry conditions and glacials were characterized by cool humid conditions while cold and arid glacial cycles do not seem to have developed before polar icecaps of certain size had developed in the late Pleistocene (Dupont *et al.* 2001).

The coalescent modelling conducted in MDIV and the mismatch distribution suggest the three species *H. nou*, *H. montivaga* and new species X, all of them endemic to respective mountains or mountainous areas, diverged more or less simultaneously between 500 000 and 700 000 years ago. The same coalescent approach indicates that the endemic *Monticolaria meruensis* and *Monticolaria manyara* diverged about 900 000 years ago, suggesting that these two *Monticolaria* species diverged some hundred thousand years earlier than *H. nou*, *H. montivaga* and new species X, but a single divergence event for all of these species is supported by the analysis conducted in MSBayes. However, all these speciation events happened shortly after the humid maximum in the region about 1 Ma (Fig. 8). Furthermore, if the climate induced retraction of forest was the main force of speciation we would not necessarily expect strict age-equality between the different species. This is because the time when a barrier interrupts gene flow will differ between ecologically different species (Knowlton *et al.* 1993). Although *H. nou*, *H. montivaga*, new species X and the two *Monticolaria* species are all inhabitants of the montane zone, they do not share the same ecological niche. *H. nou*, *H. montivaga* and new species X are herbaceous vegetation dwellers and need more open conditions, while *Monticolaria* species, although they also prefer forest edge and clearings, tend to inhabit the tree and bush layer in forests. The climate induced spread of grassland and the consequential establishment of isolated forest refuges after this last wet period therefore seem as the primary driving force of speciation in this group of forest dependent species.

Studies of birds in East Africa have concluded that the montane zone is a region of recent speciation and population diversification and that the majority of montane speciation and diversification has happened within the past 2.8 million years (Roy 1997; Bowie *et al.* 2006). Finding the same pattern in a much less mobile group of organisms may therefore be an argument in the debate for conservation priorities.

Adaptive speciation to new heterogeneous habitats on geologically new volcanoes represents an alternative explanation to the climate-induced vicariant speciation hypothesis. Other studies focusing on the evolutionary history in Orthoptera species have found adaptive speciation to play an important role in speciation processes (Trewick *et al.* 2000). However, our data reveal few convincing links

between the age of the volcanoes and the age of the species which inhabit them. For example, Mount Kilimanjaro is assumed to have appeared about 1 Ma, but *Monticolaria kilimandjarica*, endemic to this volcano, seems to be several million years old. Both *M. meruensis* and *M. manyara* have more than a 12% genetic distance to *M. kilimandjarica*, suggesting that *Monticolaria* is an old genus. The lineage leading to *M. kilimandjarica* probably originated in the Miocene when the climate in East Africa still favoured a more continuous forest cover. However, the lineage was probably also affected by the drying climate in Plio-Pleistocene since the species has only been found on Mount Kilimanjaro. Around the time of uplift of Kilimanjaro and Meru Mountains, there may have been episodes with much more wider distribution of humid habitats than now around the Tanzania-Kenya border area. It therefore seems reasonable to assume that the lineage had a more widespread distribution before the climate caused the forest to retract and finally secluded *M. kilimandjarica* on Mount Kilimanjaro.

Also on Mount Hanang and the Manyara Escarpment of the Rift Valley system, there is no convincing link between speciation and mountain formation. The timing of the rifting and associated volcanism creating these formations probably started in the Pliocene but the topology seen today was also largely affected by geological activity in the Pleistocene (Griffiths 1993). *Horatosphaga nou*, new species X and *M. manyara*, which all inhabit one or both of these formations, diverged within a short time frame. A scenario where these species originate more or less simultaneously as an adaptive response to a mountain building process which probably lasted some million years seems less probable compared to the vicariant speciation hypothesis. This is also in agreement with studies of birds from the same area where isolation of species seems not to be closely linked with orogenetic processes (Fjeldså & Bowie 2008). However, although our findings seem to favour the vicariant speciation hypothesis, adaptive speciation may still have played an important part in the speciation processes reported here, as both drift and adaptation can cause two independently evolving populations to develop reproductive isolation (Coyne & Orr 2004).

Conclusions

Shrinking of habitats with subsequent fragmentation of species into small and isolated populations poses a major threat to the survival of species. However, this scenario may also lead to genetic diversification and possibly speciation, given enough time and given that the habitats persist. Some of the species in this study are represented by a relative low sample size and some caution should therefore be called upon when interpreting our findings. However, the multiple and simultaneous vicariant speciation

events within species inhabiting the montane zone about 800 000 years ago documented in this study seem to have been mediated by climatically induced barriers. Although several studies invoke climate change during Plio-Pleistocene as an important determinant of the observed biodiversity of insects in East Africa (e.g. Brühl 1997; Fattorini 2007), our study is one of the first to demonstrate how a specific climate change event influenced speciation in a group of montane insects. Climate change has therefore probably been an important promoter in creating much of the species which today are threatened by the changing climate (Hemp 2005a; Thuiller 2007). This study thus demonstrates how climate can act as a double-edged sword upon biodiversity. Such a perspective only reinforces the importance of conserving the remaining forest patches in East Africa. If these forests are eradicated, we will not only lose major hot spots of biodiversity but also historically important centres of speciation.

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K.L. Voje is a biologist with special interests in speciation and evolutionary theory. Wrent projects are exploring how evolutionary models with a strong foundation in what we currently know about evolutionary processes fit fossil time series (reconstructions of tempo and mode of phenotypic evolution). Other projects focus on the relative importance of current adaptation and phylogenetic inertia in evolution of traits. C. Hemp has an interest in the ecology (habitat demands, saltatoria (grasshoppers, locusts and bush crickets)). Our working group (A. Hemp, University of Potsdam, K.-G. Heller, University of Erlangen, W. Zech, University of Bayreuth, W. Wägele, Museum Koenig Bonn) uses flightless saltatoria as a model group to reconstruct the climatic and vegetational history of East Africa and to understand mechanisms of speciation. N.C. Stenseth is an ecologists and evolutionary biologists working on a broad spectrum of issues, including studies on how climate variation affect ecology and evolution.

Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1 Climatic change as an engine for speciation in flightless Orthoptera species inhabiting African mountains

Table S1 Genetic distances

Fig. S1 Genetic distances (above darkened diagonal) and their equivalents in million years (below darkened diagonal) between some of the lineages in two different monophyletic groups (A and B) using a molecular-clock approach (2% divergence between lineages per million years).

Fig. S2 Three independent replicates of the posterior probabilities of migration (M) for each of the four pairwise species comparisons.

Fig. S3 Result from the approximate Bayesian computation under a hierarchical coalescent model using MSBayes: posterior probability density (solid line) of the distribution of the number of different possible divergence times (Ψ) across the four taxon pairs analyzed (the prior distribution is indicated by the dashed line).

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