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## New frog family from India reveals an ancient biogeographical link with the Seychelles

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About 96% of the more than 4,800 living anuran species<sup>1</sup> belong to the Neobatrachia or advanced frogs<sup>2–4</sup>. Because of the extremely poor representation of these animals in the Mesozoic fossil record, hypotheses on their early evolution have to rely largely on extant taxa<sup>5–7</sup>. Here we report the discovery of a burrowing frog from India that is noticeably distinct from known taxa in all anuran families. Phylogenetic analyses of 2.8 kilobases of mitochondrial and nuclear DNA unambiguously designate this frog as the sister taxon of Sooglossidae, a family exclusively occurring on two granitic islands of the Seychelles archipelago<sup>8</sup>. Furthermore, molecular clock analyses<sup>9</sup> uncover the branch leading to both taxa as an ancient split in the crown-group Neobatrachia. Our discovery discloses a lineage that may have been more diverse on Indo-Madagascar in the Cretaceous period, but now only comprises four species on the Seychelles and a sole survivor in India. Because of its very distinct morphology and an inferred origin that is earlier than several neobatrachian families<sup>10</sup>, we recognize this frog as a new family.

Amphibia L., 1758  
 Lissamphibia Haeckel, 1866  
 Anura Rafinesque, 1815  
 Neobatrachia Reig, 1958  
 Nasikabatrachidae fam. nov.  
*Nasikabatrachus* gen. nov.

*Nasikabatrachus sahyadrensis* gen. et sp. nov.

**Etymology.** Nasika (Sanskrit) meaning nose, batrachus meaning frog, and Sahyadri, being synonymous for the Western Ghats (the hills along the west coast of the Indian subcontinent).

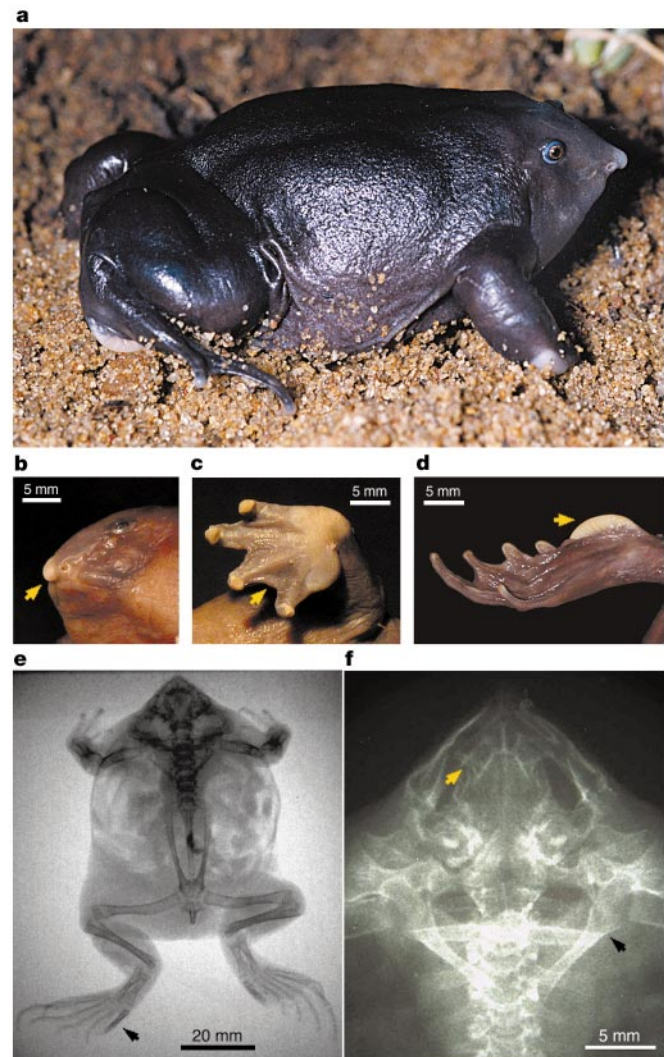
**Holotype.** Bombay Natural History Society (BNHS; Mumbai), BNHS 4202, an adult female, snout–vent length 70.1 mm, collected July 2000 by S.D.B. (Fig. 1a).

**Type locality.** Disturbed secondary forest near a cardamom planta-

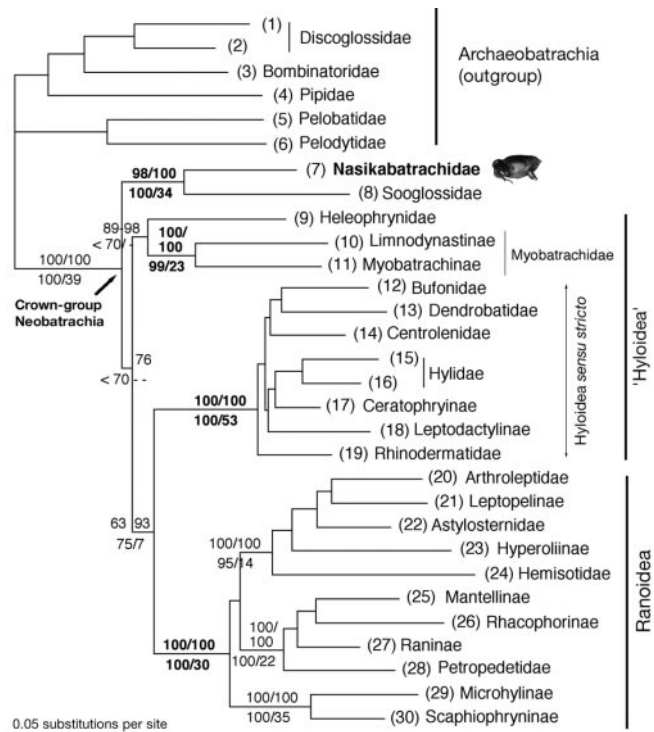
tion at Kattappana (09° 45' N, 77° 05' E, altitude approximately 900 m), Idukki district, Kerala, Western Ghats, India.

**Diagnosis.** The diagnosis is valid for the family, genus and species. A relatively large frog with a bloated general appearance, smooth skin and an overall black coloration dorsally and dark grey ventrally; the head (Fig. 1b) is pointed and short relative to the body; the snout has a distinct white protrusion. The eyes are small with a rounded, horizontal pupil; no apparent tympanum; the forelimbs are short, the hands (Fig. 1c) are rudimentarily webbed, the tips of fingers are rounded, without disks. The hindlimbs are short, feet (Fig. 1d) are about 3/4 webbed, and the tips of toes are rounded, without disks. A large, white inner metatarsal tubercle is present on both feet (detailed measurements of external morphology are provided as Supplementary Information).

The skeleton (Fig. 1e) is characteristic of a burrowing frog and displays bones with a well-calcified cortical area, a skull with strongly ossified neurocranial and dermal elements (Fig. 1f), a short tibiae and fibulae, strong and short tibiofibular bones, and



**Figure 1** Holotype of *Nasikabatrachus sahyadrensis*. **a**, *Nasikabatrachus sahyadrensis* in life. **b**, Detail of head, showing slender mouth and distinct protrusion on snout. **c**, Detail of hand, showing rudimentary webbing. **d**, Detail of foot showing the large, white inner metatarsal tubercle. **e**, X-ray photograph showing strongly calcified bones. The arrow indicates the prehallux. **f**, X-ray photograph showing strongly ossified skull and pectoral girdle. The yellow arrow indicates the presumed neopalatine bone; the black arrow indicates the coracoid, the lateral end of which is wider than the medial.



**Figure 2** Bayesian consensus phylogram for the analysis of 2,325 bp of DNA. Numbers before (sub)familial names correspond to species as given in the Methods section. Numbers on branches indicate bayesian posterior probabilities and metaGA posterior branch support values (above), respectively, and maximum parsimony bootstrap (10,000 replicates) values and decay indices (below), respectively. The sister group relationship of Nasikabatrachidae with Sooglossidae from the Seychelles is very strongly supported. Although the exact order of early divergences remains tentative, the strong support for several groups (clade support indicated in bold) implies that the Nasikabatrachidae/Sooglossidae lineage is one of the basal-most splits in Neobatrachia.

a well developed and highly calcified prehallux. The following osteological characters were tentatively interpreted from the X-rays: neobatrachian synapomorphies include fusion of the third carpal to other carpals, presence of a neopalatine bone (Fig. 1f) and absence of a parahyoid bone. The pectoral girdle with slender

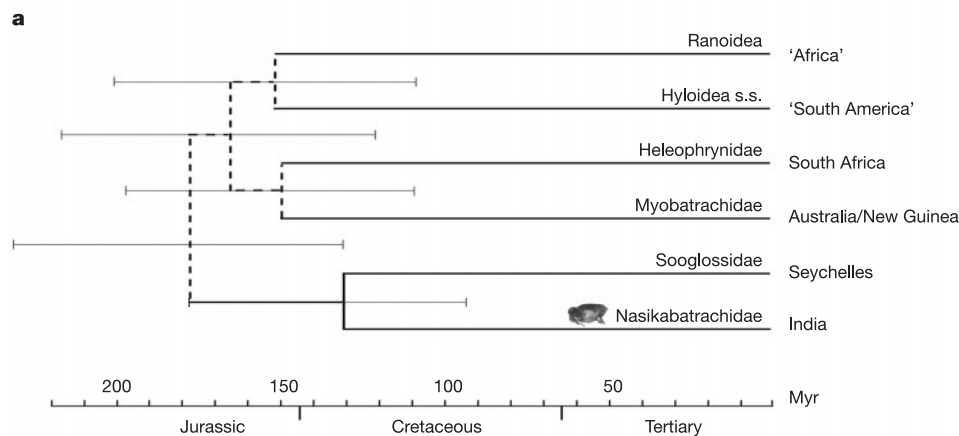
Table 1 Bayesian divergence time estimates

Divergence	Time estimate (Myr ago)
Origin of Ranoidea and Hyloidea s.s.	152 (108, 202)
Origin of Heleophryinae and Myobatrachidae	150 (109, 198)
Origin of Sooglossidae/Nasikabatrachidae lineage	178 (131, 233)
Sooglossidae–Nasikabatrachidae split	131 (93, 177)

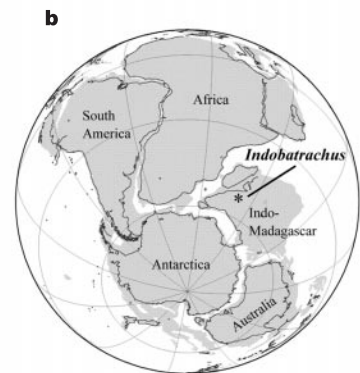
See Methods and Supplementary Information for a description of the calibrations used for estimating node times. Numbers in parentheses represent 95% credibility intervals.

coracoids—the lateral end of which is wider than the medial (Fig. 1f)—excludes Nasikabatrachidae from Ranoidea<sup>3</sup>; a bony element situated at the medio-distal aspect of the tibiale at the level of the third metatarsal, if homologous with the os sesamoides tarsale, links the new family to Sooglossidae<sup>3</sup>. The combination of the above external and skeletal characters makes Nasikabatrachidae distinct from all anuran families. In particular, the new lineage differs from its sister group Sooglossidae in many aspects, some of the most obvious being the absence of toe disks, a much larger size and the numerous adaptations to a burrowing lifestyle described above.

The phylogenetic position of Sooglossidae has been a point of debate for several decades, and they have been placed variously in Ranoidea<sup>11</sup>, Hyloidea<sup>6</sup> or in a trichotomy with the former<sup>5</sup>. Regardless of the optimality criteria used, our mitochondrial DNA, nuclear DNA and combined DNA analyses (see Supplementary Information for analyses of individual genes) strongly support Hyloidea *sensu stricto* and Ranoidea clades, and three additional main lineages: Myobatrachidae, Heleophryinae and the Sooglossidae/Nasikabatrachidae clade (Fig. 2). In the absence of rate constancy, we used a relaxed molecular clock<sup>9</sup> to estimate their divergence times under different possible topologies (derived from the bayesian posterior probabilities), using multiple calibration points. All analyses resulted in similar dating estimates indicating that the major neobatrachian lineages originated relatively rapidly in the Middle/Late Jurassic and Early Cretaceous periods (Table 1). Around that time, the Gondwanan supercontinent broke up into two landmasses—western Gondwana (Africa and South America) and eastern Gondwana (Australia, Antarctica and Indo-Madagascar)—which rapidly disintegrated further into their respective components<sup>12</sup>. These geological events probably isolated the stem group leading to the Nasikabatrachidae/Sooglossidae clade on the Indo-Madagascan fragment of eastern Gondwana (Fig. 3).



**Figure 3** Early evolution of advanced frogs. **a**, Evolutionary tree showing estimates of divergence time for the major lineages, estimated from 1,443 bp of nuclear gene fragments. Analyses were performed on all taxa, but only basal stocks of modern Neobatrachia are shown. Dashed lines indicate uncertain phylogenetic relationships. Thin horizontal lines denote the 95% credibility interval for the corresponding node time. For



each lineage, the current distribution is indicated. Ranoidea and Hyloidea are now wide ranging, but their origin is associated with Africa and South America, respectively<sup>7</sup>. s.s., *sensu stricto*. **b**, Tectonic reconstruction of Gondwana 130.0 Myr ago. The locality of the fossil *Indobatrachus* from the Eocene epoch is indicated with an asterisk.

The endemism of Sooglossidae on the Seychelles has been a constant biogeographical mystery<sup>5,6,8</sup>. One hypothesis postulates that this family is derived from an extant or extinct ancestral stock on Africa<sup>11,13</sup>. Alternatively, it has been proposed that Sooglossidae were present on Indo-Madagascar<sup>6</sup> during its trans-Tethys drift, and subsequently became extinct on India and Madagascar (if they had been present there). The phylogenetic position of Nasikabatrachidae, in combination with our dating estimates, clearly favour the second hypothesis. Furthermore, our analyses indicate that Nasikabatrachidae and Sooglossidae diverged well before the break-up of India and the Seychelles at the Cretaceous/Tertiary boundary (Table 1). Our findings thus uncover the existence of an early stock of advanced frogs, which consisted of at least two major lineages on drifting Indo-Madagascar. It is possible that this assemblage was composed of a much greater diversity during the Cretaceous period on parts of these Gondwanan fragments. Offshoots of this clade may even have existed on Australia and Antarctica, as some tectonic models propose a connection of the latter with Indo-Madagascar until the late Cretaceous period<sup>14</sup>.

It has already been suggested that India played a significant role in the passage of amphibians to southeast Asia<sup>4,15,16</sup>. The phylogenetic pattern revealed here provides independent evidence for this landmass being an important setting for the evolution of advanced frogs. There are no Mesozoic fossil records that testify to the existence of this early assemblage on eastern Gondwana. The only possible palaeontological evidence of a member of this group is *Indobatrachus pusillus* (Owen, 1847) from the Eocene epoch of India. This species has been regarded as a myobatrachid<sup>4,17,18</sup> or sooglossid<sup>18,19</sup>, but its osteology is still not completely known<sup>17</sup>. Comparison of additional fossil material with extant species of the Nasikabatrachidae/Sooglossidae clade may therefore provide new insights on the phylogenetic position of *Indobatrachus*.

Mesozoic fossils sometimes have important implications for understanding the early history of vertebrate groups<sup>20,21</sup>. Yet, there are still very few palaeontological findings that have contributed to our knowledge on the origin of advanced frogs. Our discovery of an ancient extant frog lineage in India discloses a clade that significantly adds to the understanding of early neobatrachian biogeography. However, knowledge on how extensively the Indo-Madagascan lineage has radiated (both in the biogeographical and phylogenetic sense) will have to await the recovery of fossils from the Cretaceous period of Gondwanan landmasses. □

**Methods**

**Osteological analyses**

In order to perform a preliminary, non-destructive analysis of skeletal characters of the holotype, we made several X-ray photographs. We used a Philips Optimus M200 X-ray system and image intensifier (41 kV, 800 mA). Images were recorded digitally using a Redlake motion Pro high-resolution camera. Furthermore, an MPG 65 generator and a RSN 620 X-ray tube (General Electric) (56 kV, 200 mA) were used to study cranial elements in more detail.

**Taxon selection and DNA methods**

Our data set includes the following 30 species, sampled from all continental regions (including Madagascar and the granitic Seychelles) and forming a fair representation of the major anuran lineages recognized. Numbers correspond to (sub)familial names in Fig. 2: (1) *Alytes obstetricans boscai*, (2) *Discoglossus pictus*, (3) *Bombina orientalis*, (4) *Pipa pipa*, (5) *Pelobates cultripes*, (6) *Pelodytes punctatus*, (7) *Nasikabatrachus sahyadrensis*, (8) *Nesomantis thomasseti*, (9) *Heleophryne purcelli*, (10) *Limnodynastes salmini*, (11) *Myobatrachus gouldii*, (12) *Bufo melanostictus*, (13) *Dendrobates auratus*, (14) *Centrolene prosoblepon*, (15) *Hyla arenicolor*, (16) *Phrynolias venulosa*, (17) *Ceratophrys ornata*, (18) *Leptodactylus melanonotus*, (19) *Rhinoderma darwini*, (20) *Arthroleptis variabilis*, (21) *Leptopelis kivuensis*, (22) *Trichobatrachus robustus*, (23) *Hyperolius* sp., (24) *Hemisus marmoratus*, (25) *Boophis xerophilus*, (26) *Philautus wynaadensis*, (27) *Meristogenys kinabaluensis*, (28) *Petropedetes cf. parkeri*, (29) *Microhyla ornata*, (30) *Scaphiophryne marmorata*. After preliminary analyses using salamanders as outgroups, Archaeobatrachia (species 1 to 6) were selected as outgroup taxa because of the evidence (osteological and molecular) for monophyly of Neobatrachia (including Nasikabatrachidae).

Two mitochondrial DNA gene fragments—covering part of 12S ribosomal RNA, complete transfer RNA<sup>Val</sup> and part of 16S rRNA—were sequenced, which together yielded a data matrix of 1,329 base pairs (bp). Additionally, a fragment of the following

nuclear DNA genes was sequenced: exon 1 of rhodopsin, single exon of *Rag1*, and exon 2 of *Cxcr4*, yielding alignments of 315, 555 and 675 bp, respectively. Methods for DNA extraction, amplification (including primers) and sequencing are given elsewhere<sup>22</sup>. The following primers were developed for this study: Rag1-B, 5'-ATGGGAGATGTGAGTGA RAARCA-3'; Rag1-C, 5'-GGAGATGTTAGTGAAGAARCAAYGG-3'; Rag1-D, 5'-GCTGC ATTTCCRATRTCACATGTG-3'; Rag1-E, 5'-TCCGCTGCATTTCCRATGTGTCRCA-3'; and Rag1-F, 5'-CCAAATGTCGCAGTGCAGCAARGCRT-3'. CXCR4-A, 5'-CTGCTGGGCGAT CATTGGRAAYGG-3'; CXCR4-B, 5'-ATCATTGGCAATGGAYTRGT-3'; CXCR4-C, 5'-GTCATGGGCTAYCARAAGAA-3'; CXCR4-D, 5'-AGGACAATGACWGYAATA-3'; CXCR4-E, 5'-AGGACAATGACWGYAAGTA-3'; CXCR4-F, 5'-TTGAATTTGGCCCR AGGAARGC-3'; and CXCR4-G, 5'-AGGCAACAGTGGAARAANGC-3'.

**Alignment and phylogenetic analyses**

Sequences were aligned using ClustalX 1.64 and ambiguous sections were excluded for subsequent analyses. Phylogenetic analyses were performed using PAUP\* 4.0b10 (ref. 23). Plots of transitions and transversions against uncorrected and GTR-corrected pairwise distances indicated that none of the fragments showed saturation. Partition homogeneity tests revealed no significant incongruence among different fragments. Heuristic maximum parsimony searches were executed in 10,000 replicates with all characters unordered and equally weighted. Clade support under maximum parsimony was calculated using decay indices and nonparametric bootstrapping. Appropriate likelihood models were determined using the software Modeltest 3.06 (ref. 24). We also conducted 250 replicated metaGA searches<sup>25</sup> using MetaPIGA 1.0.2b, each with strict consensus pruning among four populations, using a HKY + Γ + I model (the most parameter-rich model implemented in MetaPIGA) with the  $T_i/T_c$  ratio optimized every 200 generations. The 1,000 resulting trees were used to compute a majority-rule consensus tree and calculate posterior branch support values. Bayesian analyses were performed using the software MrBayes 2.01 (ref. 26) under the GTR + Γ + I model. Four chains were run simultaneously for 1,000,000 generations and trees were sampled every 100 cycles. Likelihood scores reached stationarity well before 100,000 generations, but to be on the safe side we discarded the first 2,000 trees as the 'burn in'. Hence bayesian posterior probabilities were estimated as the 50% majority-rule consensus tree of the 8,000 last sampled trees.

**Dating estimates**

Mean and 95% credibility interval values for node times were estimated from 1,443 bp of nuclear DNA on the consensus phylogram (Fig. 2) using a bayesian method that allows correlated rate changes between the nodes of a tree<sup>9</sup>. After incorporating in our data set the homologous fragments of the nuclear genes of *Danio rerio*, *Homo sapiens* and *Gallus gallus* (retrieved from GenBank), as well as the caudates *Hynobius formosanus* and *Salamandra salamandra*, we were able to use three calibrations simultaneously: (1) the divergence of amniotes from amphibians at a minimum of 338 Myr ago<sup>27</sup>; (2) the divergence of diapsids from synapsids at about 310 Myr ago<sup>28</sup>; and (3) the minimum age of Cryptobranchioidea (164 Myr ago; here represented by the *Hynobius-Salamandra* split)<sup>29</sup> (see Supplementary Information).

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## Females increase offspring heterozygosity and fitness through extra-pair matings

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Females in a variety of species commonly mate with multiple males, and there is evidence that they benefit by producing offspring of higher genetic quality<sup>1–3</sup>; however, the nature of these genetic benefits is debated<sup>1–4</sup>. Enhanced offspring survival or quality can result from intrinsic effects of paternal genes—‘good genes’—or from interactions between the maternal and paternal genomes—‘compatible genes’<sup>1–5</sup>. Evidence for the latter process is accumulating<sup>2,6</sup>: matings between relatives lead to decreased reproductive success, and the individual level of inbreeding—measured as average heterozygosity—is a strong fitness predictor<sup>7–13</sup>. Females should thus benefit from mating with genetically dissimilar males<sup>2,14</sup>. In many birds, social monogamy restricts mate choice, but females may circumvent this by pursuing extra-pair copulations<sup>15,16</sup>. Here we show that female blue tits, *Parus caeruleus*, increase the heterozygosity of their progeny through extra-pair matings. Females thereby produce offspring of higher reproductive value, because less inbred individuals have increased survival chances, a more elaborate

male secondary sexual trait (crown colour) and higher reproductive success. The cost of inbreeding may therefore be an important factor driving the evolution of female extra-pair mating.

Previous work suggests that blue tit females gain genetic benefits from mating with multiple males<sup>17,18</sup>: broods of socially monogamous pairs frequently contain extra-pair young, which are in better condition and are more likely to fledge than their within-pair nest mates<sup>18</sup>. So far, the genetic mechanism underlying this fitness advantage is unknown. Individual genetic diversity (heterozygosity) reflects the level of inbreeding and influences survival and fitness in various species<sup>7,9–13</sup>. High individual heterozygosity reduces the likelihood that recessive deleterious alleles are expressed, or increases the number of potentially useful gene products (for example, at major histocompatibility complex (MHC) genes)<sup>4</sup>. Therefore, mating with individuals carrying dissimilar alleles can be advantageous<sup>2,14</sup>. If restricted social mate choice leads to pairings of genetically similar individuals, extra-pair copulations provide a mechanism to counteract inbreeding depression by increasing the genetic diversity, and hence fitness, of offspring<sup>16</sup>. If female blue tits use this strategy, extra-pair young should be more heterozygous than within-pair young and individual heterozygosity should influence fitness.

We examined genetic parentage, breeding success, and offspring and adult survival in a population of individually marked blue tits breeding in nest boxes in the Viennese Forest, Austria (48°13' N, 16°20' E), from 1998 to 2001. Extra-pair young were more heterozygous than their maternal half-siblings sired by the social father (Fig. 1). This suggests that females are less related to extra-pair fathers than to their social mates. We tested this with all known extra-pair fathers but did not find the expected result (social male, mean relatedness ± standard error of the mean (s.e.m.) = -0.016 ± 0.016; extra-pair males, -0.029 ± 0.014, paired *t*-test, *t* = 0.62, *n* = 96 dyads, *P* = 0.54; 95% confidence interval for the difference in relatedness between female and social/extra-pair male (-0.029, 0.056)). However, 28% of all extra-pair young were sired by unknown males (not found breeding in the study area). Extra-pair young produced by these non-local fathers mainly accounted for the difference in heterozygosity (Fig. 1). Among known extra-pair fathers, close neighbours did not increase offspring heterozygosity, whereas local non-neighbours tended to sire more heterozygous extra-pair young (Fig. 1). Therefore, only non-neighbouring extra-pair males should be less related to the female than her social partner. We failed to find this, but our sample size of broods with known extra-pair fathers is small (local non-neighbours, *n* = 15; non-local males, *n* = 7, both *P* > 0.7). We then tested whether females were generally less related to more distantly breeding males. We measured the distance and calculated the relatedness between all breeding males and females in each of three years. We then averaged relatedness for each of 14 distance classes, ranging from 0 m (social partner) to 1,300 m. In each year, genetic similarity decreased with breeding distance (1998, *r<sub>p</sub>* = -0.48, *n* = 13, *P* = 0.096; 1999, *r<sub>p</sub>* = -0.63, *n* = 14, *P* = 0.015; 2000, *r<sub>p</sub>* = -0.67, *n* = 13, *P* = 0.017; Fisher's combined probability, *P* = 0.003). Thus, a genetic structure in this population enables females to obtain genetically less similar extra-pair partners by copulating with males that breed further away.

Our data show that females increase their offspring's heterozygosity by extra-pair copulations with non-neighbouring males, which accounts for 50% of all extra-pair young (*n* = 385). Extra-pair matings with close neighbours did not lead to increased offspring heterozygosity, but are nevertheless actively pursued by female blue tits (ref. 17 and our own unpublished data). Local extra-pair males were older, larger and sang longer strophes than the cuckolded males in a Belgian population<sup>17,18</sup>. Similarly, we found here that extra-pair males were older and larger than the social males if they were close neighbours (age in years: social male, mean ± s.e.m. = 1.6 ± 0.1; extra-pair male, 2.0 ± 0.1, Wilcoxon's