## Phylogenetic relationships among Palearctic–Oriental starlings and mynas (genera *Sturnus* and *Acridotheres*: Sturnidae)

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We used nuclear and mitochondrial genes to generate a phylogenetic hypothesis for the Palearctic–Oriental starlings and mynas (genera *Sturnus, Acridotheres, Leucopsar, Creatophora* and *Fregilupus*: Sturnidae). Our results indicate that the group has undergone a rapid diversification in Asia since the late Miocene. A reassessment of the morphological and ecological characters used in previous taxonomic revisions shows that all characters are highly homoplastic. We suggest that the elevated morphological and ecological plasticity favoured the adaptation of starlings and mynas to the local environment, the exploitation of all niches and their successful radiation in south-east Asia. Under the current limits the genera *Sturnus* and *Acridotheres* are not monophyletic, and we propose a revised phylogenetic taxonomy for the entire clade. We confirm that the extinct *Fregilupus varius* is a starling and it colonized Réunion Island (Mascarenes) by transoceanic dispersal from Asia.

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#### Introduction

The Palearctic–Oriental starlings and mynas of the genera *Sturnus* and *Acridotheres* form a rather homogeneous group of medium-large passerines with stocky built, short tail and strong bill. Although generic limits and species delimitations have been subject to various interpretations, their close relationship has never been questioned. Based on morphology, three other monotypic genera, *Leucopsar*, *Creatophora* and *Fregilupus*, were considered belonging to the same taxon (Amadon 1943, 1956). Indeed, recent molecular analyses support in part the morphological data, recovering all these taxa in a monophyletic clade, but also suggest that the traditional limits of genera are in need of a re-evaluation (Zuccon *et al.* 2006; Lovette & Rubenstein 2007).

Following the then current tendency to accept small genera, 19th century ornithologists used to recognize a large number of starling genera (e.g. Oates 1889; Sharpe 1890, 1909). The influential reviews of Amadon (1943, 1956) reversed the trend, and since then smaller species are lumped

in Sturnus, while larger, more terrestrial taxa with graduated tail are merged in Acridotheres. However, while a crest formed by the forehead feathers is the synapomorphy that defines Amadon's Acridotheres, no diagnostic characters are provided for Sturnus, and the latter seems to be a catch-all for the remaining species. The Sturnus-Acridotheres boundaries remain disputed, because three species (melanopterus, burmannicus and nigricollis) have been alternatively shifted from one genus to the other (e.g. Harrison 1963; Sibley & Monroe 1990; Dickinson 2003). A different treatment is provided by Feare & Craig (1998). They included melanopterus and burmannicus in Acridotheres, but split Sturnus, resurrecting the genera Pastor, Sturnia, Temenuchus and Gracupica. Feare & Craig (1998) advocated the use of narrow genera to point out that despite a general morphological uniformity, the smaller starlings differ in a number of behavioural and ecological ways.

Three morphologically more divergent species are retained in monotypic genera. *Creatophora cinerea* is a nomadic African starling, long suspected to be closer to the Asiatic species

than to the other African starlings. During the breeding season, males have prominent wattles covering most of the head, a character unique among African species. The Bali endemic Leucopsar rothshildi is a highly endangered species. Although about 1000 individuals are estimated to survive in captivity, a survey in March 2005 found only 24 in the wild (BirdLife International 2007). It is one of the few landbirds with an almost completely white plumage, but shares similar stocky body, bare skin patches around the eye and courtship display with the genus Acridotheres (Harrison 1963). Fregilupus varius is an extinct species from Réunion (Mascarene Islands), of which no more than 20 specimens are known (Violani et al. 1999). Almost nothing is known about its biology, except it was a tame, quite common bird and vanished before the middle of 19th century, presumably by a combination of hunting, competition with introduced species and habitat changes (Fuller 2001). Plumage, osteology and myology suggest an affinity with Sturnus, Acridotheres and Gracula (Murie 1874; Miller 1941), but Berger (1957) pointed out some similarities with prionopid shrikes (Prionopidae).

Two recent phylogenetic hypotheses of starling relationships (Zuccon *et al.* 2006; Lovette & Rubenstein 2007) included a limited selection of taxa, but suggested that all *Acridotheres* mynas form a monophyletic clade. In contrast, the genus *Sturmus* is clearly polyphyletic, with more complex phylogenetic relationships.

In the present study we generate a comprehensive phylogenetic hypothesis for starlings and mynas in the Palearctic–Oriental region by combining nuclear and mitochondrial data obtained from fresh and study-skin samples. Monophyly of this clade has previously been inferred from analyses of molecular data (Zuccon *et al.* 2006; Lovette & Rubenstein 2007). Our analysis comprises all recognized species, including also the extinct *Fregilupus varius*. The results provide the basis for a new phylogenetic classification and offer insights into the evolution of this group.

## **Materials and methods**

## Taxon sampling strategy

We obtained and sequenced at least one individual from all currently recognized species in the polytypic genera *Sturnus* and *Acridotheres*, as well as the monotypic genera *Leucopsar*, *Fregilupus* and *Creatophora*. Seven other starlings (see Table 1) were used as outgroup, following the results of Zuccon *et al.* (2006) and of Lovette & Rubenstein (2007). The samples used in this study include both fresh tissues and toe-pads from museum skins. All details, including subspecific taxonomy, collection localities and GenBank accession numbers of samples are reported in Table 1. The taxonomy follows Dickinson (2003).

We analysed two data sets. The first data set (mixed data set) includes nuclear and mitochondrial sequences of all species, with one individual per species (27 ingroup taxa). To investigate the intraspecific variability, we analysed a second data set of only mitochondrial sequences that includes 64 ingroup individuals. The sequences of some of the taxa included in the mitochondrial data set were retrieved from GenBank.

## DNA isolation and sequencing

Total genomic DNA was extracted from fresh samples (blood or muscles) using the DNeasy Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Toe-pad samples were extracted using either the DNeasy Tissue Kit or the DNeasy Micro Kit (Qiagen), but optimizing the manufacturer protocol for the digestion of bigger samples. More details on the procedure followed are available in Zuccon (2005) and Irestedt *et al.* (2006).

We analysed five genes: three nuclear loci, the intron 2 of the myoglobin gene, the introns 6 and 7 of the ornithine decarboxylase (ODC) gene, and the intron 11 of the glyceraldehyde-3-phosphodehydrogenase (GAPDH) gene, and the two mitochondrial genes, the NADH dehydrogenase II (ND2) gene and cytochrome oxydase II (CO2) gene. The introns and the ND2 gene were amplified and sequenced using standard primers and amplification profiles as described in Irestedt et al. (2002), Fjeldså et al. (2003), Allen & Omland (2003) and Zuccon et al. (2006). The CO2 gene was amplified and sequenced using the newly designed primers CO2-ExtF (5'-CAGGTGAAACCCCAGTACACCTC-3') and CO2-ExtR (5'-AGGCTAGCGCTGTTCCATAGCTTC-3'), with the amplification profile: initial denaturation 5 min at 94 °C, 40 cycles denaturation 40 s at 95 °C, annealing 60 s at 59 °C, extension 60 s at 72 °C, final extension at 72 °C for 8 min. PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen) and run on an ABI Prism 3100 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Norwalk, CT).

The toe-pad samples were amplified in short, overlapping fragments 200-300 bp long, using a large series of specific primers, designed using the fresh samples sequences as a guide. The primer sequences are available from the authors. PCR products quality was assessed by electrophoretic migration in a 1.5%  $0.5\times$  TBE agarose gel. In several cases the target fragment was co-amplified with other weaker bands of smaller size. The gel slice containing the target band was excised with a clean blade and the PCR product eluted with QIAquick Gel Extraction Kit (Qiagen). When the band obtained was too weak for a direct sequencing, it was reamplified with the same primers and reaction profile used in the first PCR.

Great care was taken to avoid contamination of toe-pad samples, during the extraction and amplification steps. All extractions of old material were made in a dedicated room, 

 Table 1
 Samples and sequences included in this study, with museum accession numbers and collection localities. For the toe-pad samples the collection year is added. Sequences published previously are listed together with their GenBank accession numbers and references.

Taxon	Accession numbers	Skin age	Myoglobin	ODC	GADPH	ND2	C02	Locality	
* Acridotheres albocinctus	MNHN 1898.1838	1886	EU551855	EU551905	EU551874	EU551936	EU551980	Burma	
Acridotheres cinereus	BMNH 1873.5.12.1871	1856				EU551937	EU551981	Sulawesi, Makassar	
Acridotheres cinereus	RMNH 144688	1897				EU551938	EU551982	Sulawesi, Makassar	
* Acridotheres cinereus	RMNH 144689	1897	EU551856	EU551906	EU551875	EU551939	EU551983	Sulawesi, Makassar	
* Acridotheres cristatellus brevipennis	NRM 20047102		EU551857	EU551907	EU551876	EU551940	EU551984	Vietnam, Hanoi bird market	
Acridotheres cristatellus cristatellus	NMNH B3778					EF468165 [4]	EF484295 [4]	Phillippines, Luzon Island, Cagayan Prov.	
Acridotheres cristatellus cristatellus	NRM 569476	1921				EU551941	EU551985	China, Anwhei Province, Kuei-Chih-Hsien	
Acridotheres fuscus fuscus	MNHN 1939.271	1914				EU551942	EU551986	India, Madhya Pradesh, Chilpi	
Acridotheres fuscus mahrattensis	BMNH 1949.Whi.1.15083	1940				EU551943	EU551987	India, Karnataka, Mysore	
* Acridotheres fuscus torquatus	AMNH PRS693		DQ466813 [1]	EU551908	EU551877	DQ466849 [1]	EU551988	Malaysia, near Kuala Lumpur	
* Acridotheres ginginianus	CLOFBP AKD04					EF468167 [4]	EF484297 [4]	Unknown (captivity)	
Acridotheres grandis	AMNH 9614					EF468168 [4]	EF484298 [4]	Malaysia, Kuala Lumpur	
* Acridotheres grandis	NRM 20026680		EU551858	EU551909	EU551878	EU551944	EU551989	Vietnam, Hanoi bird market	
* Acridotheres javanicus	Gelang2004		EU551859	EU551910	EU551879	EU551945	EU551990	Sumatra, Padang, bird market	
Acridotheres javanicus	NRM 569653	1920				EU551946	EU551991	Java, Karangbolang	
* Acridotheres melanopterus subsp. **	UMMZ 233261		EU551860	EU551911	EU551880	EU551947	EU551992	Unknown (captivity)	
Acridotheres melanopterus tertius	MNHN 1931.695	1930				EU551948	EU551993	Bali	
* Acridotheres tristis tristis	AMNH PRS701		DQ466814 [1]	EU551912	EU551881	DQ466850 [1]	EU551994	Singapore	
Acridotheres tristis tristis	MNHN 2005.1755	1914				EU551949	EU551995	India, Himachal Pradesh, Kulu, Bayaura	
Acridotheres tristis tristis	NRM 20046686					EU551950	EU551996	Iran, Khorasan	
Acridotheres tristis tristis	UWBM 42794					EF468170 [4]	EF484300 [4]	Cook Islands, Mangaia, Lake Tiriara	
Creatophora cinerea	NRM 551698	1964				EU551951	EU551997	Ethiopia, Lake Zwai	
* Creatophora cinerea	UWBM 70373		DQ466824 [1]	EU551913	EU551882	DQ466860 [1]	EU551998	South Africa, Free State, Springfontein	
* Fregilupus varius	NRM 523079	1830 ca.				EU551952	EU551999	Réunion Island	
* Leucopsar rothschildi	UWBM 1991-102		EU551861	EU551914	EU551883	EU551953	EU552000	Unknown (captivity)	
Leucopsar rothschildi	AMNH PRS760					EU551954	EU552001	Unknown (captivity)	
* Sturnus albofrontatus	NRM 569480	1928	EU551862	EU551915	EU551884	EU551955	EU552002	Sri Lanka	
Sturnus burmannicus leucocephalus	AMNH-PRS690					EU551956	EU552003	Malaysia, Kajang, near Kuala Lumpur	
* Sturnus burmannicus leucocephalus	NRM 569479	1935	EU551863	EU551916	EU551885	EU551957	EU552004	Vietnam, Bao Cat	
Sturnus cineraceus	UWBM 47190					EF468177 [4]	EF484306 [4]	Russia, Khabarovskiy Kray, Khurmuli	
* Sturnus cineraceus	UWBM 59925		DQ466843 [1]	EU551917	EU551886	DQ466881 [1]	EU552005	Mongolia, Nömrögiyn Gol, Dornnod Aymag	
* Sturnus contra floweri	NRM 569410	1938	EU551864	EU551918	EU551887	EU551958	EU552006	Thailand, Chieng Mai	
Sturnus contra jalla	NRM 569475	1920				EU551959	EU552007	Java, Karangbolang	
* Sturnus erythropygius andamanensis	MNHN 1999.558	1875	EU551865	EU551919	EU551888	EU551960	EU552008	Andaman Islands	
Sturnus malabaricus blythii	BMNH Vel.Cat.12.192	1939				EU551961	EU552009	India, Karnataka, Mysore	
Sturnus malabaricus malabaricus	NRM 569477	1926				EU551962	EU552010	India, North Bengal, Siliguri	
Sturnus malabaricus nemoricola	NMNH B5708					EF468178 [4]	EF484307 [4]	Burma, Sagaing Division, Kan Blu, Kyat Thin	
* Sturnus malabaricus nemoricola	NRM 20026160		EU551866	EU551920	EU551889	EU551963	EU552011	Unknown (captivity)	
Sturnus malabaricus nemoricola	NRM 569411	1938				EU551964	EU552012	Vietnam, Dran	
Sturnus nigricollis	NMNH B5709					EF468173 [4]	EF484303 [4]	Burma, Sagaing Division, Kan Blu, Kyat Thin	
* Sturnus nigricollis	NRM 20026676		DQ466844 [1]	EU551921	EU551890	DQ466882 [1]	EU552013	Vietnam, Hanoi bird market	
Sturnus pagodarum	LSUMNS B37263					EF468187 [4]	EF484313 [4]	Unknown (captivity)	

Table 1 Continued.

Taxon	Accession numbers	Skin age	Myoglobin	ODC	GADPH	ND2	C02	Locality	
* Sturnus pagodarum	MNHN 1970.1004	1960	EU551867	EU551922	EU551891	EU551965	EU552014	India, Maharashtra, 60 km east of Pune	
Sturnus philippensis	CLOFBP BTK4					EF468179 [4]	EF484308 [4]	Unknown (captivity)	
* Sturnus philippensis	NRM 569478	1930	EU551868	EU551923	EU551892	EU551966	EU552015	Kurile Islands, Shana, Yeterofu	
Sturnus roseus	UWBM 46226					EF468181 [4]	EF484309 [4]	Kazakhstan, Almaty Oblysy, Alma Ata	
* Sturnus roseus	ZMUC 123696		EU551869	EU551924	EU551893	EU551967	EU552016	Spain	
Sturnus sericeus	CLOFBP BTK1					EF468182 [4]	EF484310 [4]	Unknown (captivity)	
* Sturnus sericeus	NRM 20036878		EU551870	EU551925	EU551894	EU551968	EU552017	China, Bejing bird market	
* Sturnus sinensis	NRM 20036882		DQ466845 [1]	EU551926	EU551895	DQ466883 [1]	EU552018	China, Bejing bird market	
Sturnus sinensis	NRM 569654	1911				EU551969	EU552019	Thailand, Bangkok	
* Sturnus sturninus	NRM 556617	1936	EU551871	EU551927	EU551896	EU551970	EU552020	North Korea, Riuganpo	
Sturnus sturninus	NRM 896485	1984				EU551971	EU552021	Russia, Chitinoskaya oblast, Borzya, Ust Borzya	
Sturnus unicolor	CLOFBP 3251760					EF468185 [4]	EF484311 [4]	Spain, Madrid Prov., Collado Villalba	
Sturnus unicolor	MNHN 1970.642	1955				EU551972	EU552022	Morocco, Douan Zaara, Beni Snassere	
* Sturnus unicolor	ZMUC 119334		DQ466846 [1]	EU551928	EU551897	DQ466884 [1]	EU552023	Spain, Extremadura, Trujillo	
Sturnus vulgaris granti	MNHN 1987.652	1986				EU551973	EU552024	Azores, Sao Migue, Santa Ana	
Sturnus vulgaris porphyronotus	MNHN 1960.1730	1912				EU551974	EU552025	Kyrgyzstan, Naryn	
Sturnus vulgaris subsp.**	NRM 20046688					EU551975	EU552026	Iran, Khorasan	
Sturnus vulgaris vulgaris	CUMV 44167					EF468186 [4]	EF484312 [4]	USA, New York, Ithaca	
Sturnus vulgaris vulgaris	DZC 19930523-01					EU551976	EU552027	Italy, Piedmont	
Sturnus vulgaris vulgaris	DZC 19980829-01					EU551977	EU552028	Italy, Umbria	
* Sturnus vulgaris vulgaris	NRM 966615		AY228322 [3]	EF441253 [5]	EF441231 [5]	DQ146346 [3]	EU552029	Sweden, Stockholm	
Outgroup									
* Aplonis panayensis strigatus	AMNH PRS692		DQ466817 [1]	EU551929	EU551898	DQ466853 [1]	EU552030	Singapore	
* Cinnyricinclus leucogaster verreauxi	UWBM 72577		DQ466822 [1]	EU551930	EU551899	DQ466858 [1]	EU552031	Malawi	
* Gracula religiosa subsp.**	AMNH PRSL344		DQ466825 [1]	EU551931	EU551900	DQ466862 [1]	EU552032	Unknown (captivity)	
* Lamprotornis purpuropterus purpuropterus	ZMUC 124452		EU551872	EU551932	EU551901	EU551978	EU552033	Uganda, Queen Elizabeth National Park	
* Onychognathus walleri walleri	ZMUC 132489		EU551873	EU551933	EU551902	EU551979	EU552034	Kenya, Marsabit	
* Rhabdornis mystacalis mystacalis	ZMUC 119523		DQ466837 [1]	EU551934	EU551903	DQ466874 [1]	EU552035	Philippines, Luzon, Baliuag	
* Saroglossa aurata	FMNH384699		DQ466839 [1]	EU551935	EU551904	DQ466876 [1]	EU552036	Madagascar	

Museum acronyms: AMNH, American Museum of Natural History, New York; FMNH, Field Museum of Natural History, Chicago; MNHN, Muséum National d'Histoire Naturelle, Paris; NRM, Swedish Museum of Natural History, Stockholm; UMMZ, University of Michigan, Museum of Zoology, Ann Arbor; UWBM, Burke Museum, University of Washington; ZMUC, Zoological Museum, University of Copenhagen. [1]: Zuccon *et al.* (2006); [2]: Ericson & Johansson (2003); [3]: Fuchs *et al.* (2006); [4]: Lovette & Rubenstein (2007); [5]: Jønsson *et al.* (2007). \*samples included in the mixed data set. \*\*subspecies not identified.

with separate equipment. Tubes and equipment were sterilized for 30–60 min under UV light before the extraction, and a negative control extraction was used to assess for possible contamination during this phase. Negative controls were used also during the PCR amplification. The correctness of toe-pad samples sequences was checked controlling for the perfect fragment matching in the overlapping regions, and further corroboration comes from the very high similarity between sequences of the same species, obtained from toe-pad and fresh samples.

## Gene characterization and phylogenetic analyses

The DNA sequences were aligned with MegAlign<sup>TM</sup> (DNAStar, Madison, WI) and, for the intron alignments only, adjusted manually. The possible amplification of pseudogenes was checked translating the protein coding genes into amino acid sequences, but no unexpected stop codons or unusual amino acidic substitutions were observed. The base composition homogeneity for the five genes used in this study was assessed by a  $\chi^2$  analysis of base frequencies across taxa. For the mitochondrial coding genes (ND2 and CO2), each codon position was also analysed separately.

The two data sets were analysed under the parsimony and Bayesian criteria. Maximum parsimony (MP) analyses were conducted using PAUP\* 4.0b10 (Swofford 2003). Parsimony analysis of the mixed data set was performed applying an equal weight to all characters. Transition/transversion plots revealed that third codon positions in the mitochondrial data set are saturated (data not shown). We downweighted the transitions to transversions 1–5 in the third codon positions in the mitochondrial data set only. For both data sets the heuristic searches were executed using the tree-bisection and reconnection (TBR) branch-swapping algorithm and with 1000 random additions of taxa. Branch supports were estimated with 1000 bootstrap replicates, with 10 random sequence additions per bootstrap replicate.

Bayesian inference analyses were performed with MRBAYES 3.1 (Ronquist & Huelsenbeck 2003). Nucleotide substitution models for the Bayesian analyses were selected for each gene partition separately by using the Akaike Information Criterion (AIC, Akaike 1973) and with the software MRMODELTEST 2.0 (Nylander 2005) in conjunction with PAUP\*. For each data set, two independent runs with four incrementally heated chains were run for  $5 \times 10^6$  generations and sampled every 100th generation. The two runs, starting from different randomly chosen trees, ensured that the individual runs converged on the same target distribution. In the mixed data set we defined two partitions, corresponding to the nuclear introns and the mitochondrial genes. The two partitions differ significantly in base composition and substitution models (see below). For the nuclear-mitochondrial data set only, we ran a mixed-model Bayesian analysis, to allow the distribution parameters to vary independently between partitions. The trees sampled during the burn-in phase (i.e. before the chain had reached its apparent target distribution) were discarded, and after checking for convergence, final inference was made from the concatenated output from the two runs.

We used the software PATHD8 (Britton *et al.* 2006) to generate a linearized tree and to estimate the divergence time among the Palearctic–Oriental starlings. A reliable temporal calibration is critical to generate good estimates, but unfortunately no useful fossil of starlings exists. The few known fossils of *Sturnus* and *Acridotheres* refer to Pleistocene or late Pliocene deposits, and are of little use here (Mlíkovský 1996; Tyrberg 1998). Instead we calibrated the tree by using the calculated age for the separation of the *Sturnus vulgaris–S. unicolor* clade from the other Eurasian taxa, 9.85 mya (Zuccon *et al.* 2006).

A number of morphological, ecological and behavioural characters used to delimit the starling genera were retrieved from the literature (Cramp & Perrins 1994; Feare & Craig 1998; Rasmussen & Anderton 2005). Each character was mapped onto the Bayesian tree topology and the degree of homoplasy was calculated in PAUP\*.

## Results

## Gene properties

We obtained complete mitochondrial sequences for all fresh samples and all but one toe-pad samples. For *Fregilupus varius* we were not able to amplify 166 bp at the end of the ND2 sequence and 141 bp in the second half of the CO2 gene, respectively. Complete sequences for the nuclear introns were obtained from all fresh samples, and a selection of toe-pad samples (see Table 1). The sequence alignment was straightforward, thanks to the limited number of indels in the three introns. For the mitochondrial genes, only *Cinnyricinclus leucogaster* (in the outgroup) has a 3-bp insertion just before the stop codon, as already noticed by Lovette & Rubenstein (2007), whereas all the other sequences have the standard length of 1041 and 684 bp for ND2 and CO2 genes, respectively. The mitochondrial and the mixed data sets are 1728 and 3396 bp long, respectively.

Table 2 presents a summary of the molecular properties of each gene. The proportions of variable and potentially informative characters are very low in the two introns, but more substantial in the two mitochondrial genes. All genes show a slightly skewed base composition. However, a  $\chi^2$  analysis of base frequencies across taxa could not reject a null hypothesis of homogeneity in the five genes (*P* = 1.00 all comparisons). The nucleotide substitution model selected for the mitochondrial genes was GTR +  $\Gamma$  + I, whereas the three introns fit the simpler HKY +  $\Gamma$  evolutionary model better.

Table 2 Sequence characteristics of myoglobin intron 2, ODC introns 6–7, GADPH intron 11, ND2 and CO2 genes for the two data sets, mixed nuclear–mitochondrial and mitochondrial only.

Gene region	Myoglobin mixed data set	ODC mixed data set	GADPH mixed data set	ND2 mixed data set	CO2 mixed data set	ND2 mt data set	CO2 mt data set
Alignment length	727	671	279	1044	684	1044	684
Number of variable bases (%)	98 (13%)	115 (17%)	58 (21%)	489 (47%)	243 (36%)	494 (47%)	245 (36%)
Number of parsimony	32 (4%)	45 (7%)	16 (6%)	394 (38%)	184 (27%)	425 (41%)	213 (31%)
informative bases (%)							
% A nucleotides (range)	28.8 (28.2–29.0)	27.1 (26.5–28.4)	21.5 (20.7–22.8)	30.1 (29.0–31.2)	29.0 (27.0–30.0)	30.0 (28.9–31.4)	29.0 (27.0–30.0)
% C (range)	21.0 (20.4–22.5)	17.1 (16.7–17.4)	20.4 (19.7–22.2)	34.8 (32.9–35.6)	33.0 (32.2–34.8)	34.7 (32.9–36.7)	33.0 (32.2–34.8)
% G (range)	23.3 (22.8–23.8)	20.4 (19.9–20.9)	32.6 (31.9–33.3)	12.4 (11.0–13.8)	15.9 (14.0–17.1)	12.6 (11.0–13.9)	16.0 (14.0–17.1)
% T (range)	26.9 (26.0–27.4)	35.4 (34.1–36.0)	25.6 (24.4–26.3)	22.6 (21.5–23.7)	22.1 (21.2–23.2)	22.7 (21.1–25.0)	22.1 (21.2–23.2)
$\chi^2$ (d.f. = 156)	4.025 (P = 1.00)	3.308 (P = 1.00)	2.200 (P = 1.00)	34.402 (P = 1.00)	13.593 (P = 1.00)	59.063 (P = 1.00)	22.682 (P = 1.00)
Selected substitution model	$HKY + \Gamma$	$HKY + \Gamma$	$HKY + \Gamma$	$GTR + \Gamma + I$	$GTR + \Gamma + I$	$GTR + \Gamma + I$	$GTR + \Gamma + I$

#### **Phylogenetic results**

The phylogenetic hypotheses recovered from the two data sets are highly congruent, differing only in few basal nodes lacking statistical support.

In the Bayesian tree obtained from the mixed data set (Fig. 1), a deep split divides the species pair *Sturnus vulgaris*– *S. unicolor* from the other starling taxa. The remaining species form four major lineages. Two species, *Creatophora cinerea* and *S. roseus*, are isolated taxa, emerging as separated lineages from the basal node. One well-defined clade includes two species pairs, the large *S. nigricollis*–*S. contra* and the small *S. sturninus*–*S. philippensis*. Within the other major clade, the large *Acridotheres* taxa and *S. burmannicus* form a well-defined lineage, sister to the species pair *S. sericeus*–*S. cineraceus*. In the last lineage, the species pair *S. albofrontatus*–*Leucopsar rotbshildi* and *Fregilupus varius* branch off successively and are sister to a supported clade of the four small *S. sinensis*, *S. pagodarum*, *S. malabaricus* and *S. erythopygius*. The parsimony analysis recovers a similar consensus tree, differing only in unsupported nodes.

The results from the mitochondrial data set agree with the topology described above although it differs in three nodes (Fig. 2): (i) *Creatophora cinerea* here takes a more basal position, just above the clade containing *Sturnus vulgaris–S. unicolor*; (ii) *S. albofrontatus* is basal to, not sister of *Leucopsar rothshildi*; (iii) *Acridotheres javanicus* is sister of *A. cinereus*, not of *A. fuscus*, although the node receives no support. Most species represented by two or more samples are monophyletic. The species pair *S. vulgaris–S. unicolor* is a notable exception, where three specimens of *S. unicolor* are nested among the seven *S. vulgaris* samples. Intraspecific variability is generally low, with all combined ND2–CO2 uncorrected distances in the range 0–1.3%. Only the two samples of *S. contra* have a slight higher genetic distance (1.7%)

The character mapping onto the Bayesian topology recovered from the analysis of the mixed data set reveals that all selected characters are highly homoplastic (homoplasy indices between 0.60 and 0.78, Fig. 3).

## Discussion

Our results indicate that the Palearctic-Oriental starlings and mynas belong to a relatively recent radiation in which most taxa (above the Sturnus vulgaris-S. unicolor branch) diverged within the last 6 my (Fig. 1). The period between 8 and 6 mya saw global changes in climate and vegetation, with a shift from a predominant C<sub>3</sub> plants community to one that was dominated by C4 plants (Cerling et al. 1997; Jacobs et al. 1999). This change in vegetation, which may be described as an increase of more open habitats, was a worldwide phenomenon that occurred simultaneously in all continents although it was more marked at tropical and subtropical latitudes (Cerling et al. 1997; Jacobs et al. 1999). Its causes are not yet fully understood (e.g. Hill 1987; Molnar 2005), but it is clear that the changes in vegetation also affected many faunal communities. One example comes from the fossil record of mammals in Pakistan where many woodland adapted mammals were replaced by more open habitat species between 8 and 7 mya (Barry et al. 1985). Our data suggest that the starlings radiated at the end of the climatic transition, when the Asian climate became more arid (Hoorn et al. 2000; Molnar 2005). According to our chronogram, all major lineages were established in a rather short interval at the end of the Miocene (6.3-5.1 mya). Most extant starling species prefer open habitats and it is likely that the starlings as a group were favoured by the expansion of open habitats at the expense of woodlands.

All recent taxonomic revisions of starlings and mynas have been guided by morphology and overall similarities (Amadon 1962; Wolters 1982; Sibley & Monroe 1990; Feare & Craig 1998; Dickinson 2003). However, the molecular data do not support any of these arrangements. When mapped onto the molecular tree, all morphological, ecological and behavioural characters used to define generic limits in the past show to be highly homoplastic (Fig. 3). For instance, a great taxonomic value was associated with the jaw muscles (Beecher 1978). In



**Fig. 1** Chronogram (calibrated ultrametric tree with branch lengths proportional to time) for the *Sturnus-Acridotheres* radiation estimated from the majority rule consensus tree recovered from the Bayesian analyses of the mixed data set. Posterior probability equal or higher than 0.95 are indicated at the node. The calibration point (split between *S. vulgaris–S. unicolor* and the remaining species) is indicated with the arrow. The grey area indicates the climatic and vegetational shift that occurred at 6-8 mya. Note that *S. albofrontatus* and *L. leucogaster* are recovered as a clade in the Bayesian analysis, although the branch at the base of the clade is very short. The smoothing operated by PATHD8 on the Bayesian topology resulted in the collapse of that branch.

most birds the abductor muscles that close the bill are more powerful than the protractor muscles for opening it. In some starlings this condition is reversed. Many starlings feeding on the ground use a peculiar technique called prying: the bird inserts the closed bill into the substrate and then spreads it energetically, to reveal concealed prey. The prying adaptation is lacking in the African and Oriental starlings sister of the *Sturnus–Acridotheres* clade. Presumably prying emerged early in the evolution of the entire *Sturnus–Acridotheres* clade, and is thus plesiomorphic to this group. In all dissected species the protractor muscles are hypertrophic (Beecher 1978), but their maximum development in three unrelated ground feeders (*S. vulgaris, S. unicolor* and *S. cineraceus*) indicates the adaptive nature of the prying. We suggest that the elevated morphological and ecological plasticity favoured the adaptation of starlings and mynas to the local environment, the exploitation of all niches and their successful radiation in south-east Asia.



Fig. 2 The majority rule consensus tree obtained from the Bayesian analyses of the mitochondrial data set. Posterior probability equal or higher than 0.95 are indicated at the node.



**Fig. 3** The character states of selected morphological, ecological and behavioural characters have been mapped onto the Bayesian topology (see Fig. 1, outgroup not shown). The homoplasy indices of each character have been estimated using PAUP\*. ? denotes unknown character states. See the Appendix for full details on characters and character states.

Previous molecular studies focusing on genus level relationships in starlings have identified a strongly supported, monophyletic clade including both *Sturmus* and *Acridotheres* (Zuccon *et al.* 2006; Lovette & Rubenstein 2007). However, they have been unable to disentangle the finer relationships at the species level due to the lack of several critical taxa. Although some basal nodes lack statistical support, a congruent pattern emerges from the two data sets analysed herein.

Despite a remarkable superficial similarity with other starlings (e.g. S. cineraceus), S. vulgaris and S. unicolor belong to an isolated lineage at the base of the entire clade. These two taxa have a parapatric distribution, with S. unicolor restricted to the Iberian peninsula, Morocco, Algeria, Sardinia, Corsica and Sicily, while S. vulgaris inhabits the rest of Europe, Russia, the Middle East and Central Asia east to Pakistan. *Sturmus unicolor* has extended its breeding range eastwards from the 1950s and now the two species overlap in a restricted area between Spain and France (Cramp & Perrins 1994). Clear morphological differences and limited evidence of hybridization (Motis 1992), make them two distinct species under all species concepts. However, in the mitochondrial tree the three *S. unicolor* samples are nested within the seven *S. vulgaris*. The low genetic distances, with an average of 0.54% in mtDNA, are more similar to those observed between subspecies than between full species. It seems likely that *S. vulgaris* and *S. unicolor* are allospecies, which originated by the division of the ancestor species into two populations isolated in separate southern refugia during the Pleistocene. The incomplete lineage sorting may reflect this recent speciation, but our sampling is too sparse to be conclusive. An alternative scenario involving a single semispecies cannot be ruled out.

Some species (Sturnus roseus, Creatophora cinerea, Fregilupus varius, Leucopsar rothschildi) belong to isolated lineages, with no close relatives. Acridotheres melanopterus has occasionally been placed in the genus Leucopsar, together with L. rothschildi and S. burmannicus, mainly for its black and white plumage (Wolters 1982). However, the similarities between the two species (elongated nape feathers, body size and proportions, bare patches around the eyes and courtship display) appear to be just due to convergence, and A. melanopterus is indeed part of the Acridotheres clade, sister of S. burmannicus. Within Acridotheres, the three black (A. cristatellus, A. grandis, A. albocinctus) and the three brown-grey (A. fuscus, A. javanicus, A. cinereus) species cluster in well-supported, distinct clades. The taxonomy of the brown-grey forms has been debated. Traditionally they were lumped in the single, polytypic A. fuscus (e.g. Amadon 1956, 1962), but the parapatric distribution and some plumage differences support the separation into three species (e.g. Sibley & Monroe 1990; Feare & Craig 1998). In an alternative arrangement, Inskipp et al. (1996) merged the insular populations (corresponding to A. javanicus and A. cinereus) in A. grandis, restricting A. fuscus to the mainland form. Our mitochondrial tree rules out any close relationship with A. grandis, and it supports the recognition of three distinct taxa. The south Indian subspecies A. fuscus mahrattensis has been suggested to deserve a full species status, on the ground of modest plumage differences and a disjunct distribution (Feare & Craig 1998; Rasmussen & Anderton 2005). The mahrattensis haplotype is nested within the clade containing A. fuscus fuscus and A. fuscus torquatus, and it is better retained in the same species.

The six species allocated in the genus Sturnia by Feare & Craig (1998) constitute another case of morphological convergence involving three clades. Despite a similar plumage pattern, S. sturninus and S. philippensis are not related to S. sinensis. The latter belong to a clade together with S. malabaricus, S. erythropygius and S. pagodarum. The last species in this genus, the Sri Lankan endemic S. albofrontatus, represents a more basal lineage. The purported synapomorphies (small size, pointed wings and more arboreal lifestyle) seem to be adaptive characters, easily responding to ecological selection. The subspecies S. malabaricus blythii has a plumage markedly different from the conspecific taxa and has a disjunct distribution in southern India. Rasmussen & Anderton (2005) recognized the taxon as a good species. Although in our tree S. malabaricus blythii is sister of S. malabaricus malabaricus (northern India) and S. malabaricus nemoricola (Burma and Vietnam), the genetic divergence is so small (between 0.2% and 0.8%) that all taxa are better retained in a single species.

In agreement with most morphological studies (Murie 1874; Miller 1941), the molecular data demonstrate unambiguously that the extinct, Réunion endemic Fregilupus varius is indeed a starling. The Mascarene Islands, to whom Réunion belongs, are much closer to Madagascar than to India and south-east Asia (800 vs. 4200 km), but several island chains connect India to the Mascarenes, and these were likely used as stepping stones by the Fregilupus ancestor to cross the Indian Ocean. No less than five other landbirds colonized the Mascarenes from Asian ancestors using the same dispersal route (Raphus cucullatus, Pezophaps rodericanus, Psittacula eques, Hypsipetes borbonicus, H. olivaceus; Shapiro et al. 2002; Groombridge et al. 2004; Warren et al. 2005). The tempo of colonization remains an open question. Geological evidence suggests that although the Réunion volcanic hot-spot has an estimate age of 5.1 mya, the island emerged much later, at about 2.1 mya (McDougall 1971), an age considerably younger that our estimate of 4.0 mya for the separation of the Fregilupus lineage. The lineage leading to Fregilupus likely spread along the island chains (now partly submerged) that include also the Maldives and the Mascarenes, while Réunion was finally colonized at a later stage.

The results of our study show that the genera *Sturnus* and *Acridotheres*, as traditionally defined, are not monophyletic. We here propose a revised generic classification that better reflects the phylogenetic relationships among starlings and mynas.

Genus Sturnus Linnaeus, 1758

(type species *Sturnus vulgaris* Linnaeus, 1758; gender masculine) *Sturnus vulgaris* Linnaeus, 1758 *Sturnus unicolor* Temminck, 1820

Genus Creatophora Lesson, 1847

(type species *Gracula carunculata* Gmelin, 1789 = *Rallus cinereus* Meuschen, 1787; gender feminine)

Creatophora cinerea (Meuschen, 1787)

Genus Gracupica Lesson, 1831

(type species *Gracula melanoptera* Daudin, 1800 = *Gracula nigricollis* Paykull, 1807; gender feminine)

*Gracupica contra* (Linnaeus, 1758) *Gracupica nigricollis* (Paykull, 1807)

Genus Agropsar Oates, 1889

(type species *Gracula sturnina* Pallas, 1776; gender masculine) *Agropsar sturninus* (Pallas, 1776) *Agropsar philippensis* (J. R. Forster, 1781)

Genus Pastor Temminck, 1815

(type species *Turdus roseus* Linnaeus, 1758; gender masculine) *Pastor roseus* (Linnaeus, 1758) Genus Sturnornis Legge, 1879 (type species Heterornis albofrontata E.L. Layard, 1854; gender masculine) Sturnornis albofrontatus (E.L. Layard, 1854)

Genus Leucopsar Stresemann, 1912 (type species Leucopsar rothschildi Stresemann 1912; gender masculine) Leucopsar rothschildi Stresemann, 1912

Genus Fregilupus Lesson, 1830 (type species Upupa capensis Gmelin, 1789 = Upupa varia Boddaert, 1783; gender masculine) Fregilupus varius (Boddaert, 1783)

Genus Sturnia Lesson, 1837 (type species Pastor elegans Lesson, 1834 = Oriolus sinensis Gmelin, 1788; gender feminine) Sturnia sinensis (Gmelin, 1788) Sturnia pagodarum (Gmelin, 1789) Sturnia erythropygia (Blyth, 1846) Sturnia malabarica (Gmelin, 1789)

Genus Spodiopsar Sharpe, 1889

(type species *Sturnus sericeus* Gmelin, 1789; gender masculine) *Spodiopsar sericeus* (Gmelin, 1789) *Spodiopsar cineraceus* (Temminck, 1835)

Genus Acridotheres Vieillot, 1816

(type species Paradisea tristis Linnaeus, 1766; gender masculine) Acridotheres tristis (Linnaeus, 1766) Acridotheres ginginianus (Latham, 1790) Acridotheres burmannicus (Jerdon, 1862) Acridotheres melanopterus (Daudin, 1800) Acridotheres javanicus Cabanis, 1851 Acridotheres cinereus Bonaparte, 1850 Acridotheres fuscus (Wagler, 1827) Acridotheres albocinctus Godwin-Austen & Walden, 1875 Acridotheres grandis F. Moore, 1858 Acridotheres cristatellus (Linnaeus, 1758)

## Nomenclatural note on the genus Sturnornis Legge, 1879

The genus name *Sturnornis* was introduced by Legge (1879) as a monotypic genus for the endemic Sri Lankan species then known as *Heterornis senex* Bonaparte, 1851. Consequently, *Heterornis senex* Bonaparte, 1851 became the type species by monotypy of *Sturnornis* Legge, 1879. Mees (1997) demonstrated that the holotype of *Heterornis senex* Bonaparte, 1851 is actually a specimen of the Chinese taxon now known as *Sturnus sinensis* (Gmelin, 1789), of which it becomes a junior subjective synonym, and the valid name for the Sri Lankan

species is *Sturnus albonotatus* (Layard, 1854). *Heterornis senex* Bonaparte, 1851 *sensu* Legge, 1879 is a clear misidentification, since the Chinese species has never been recorded in Sri Lanka (Phillips 1978). In full agreement with the provisions of the International Code of Zoological Nomenclature (ICZN 1999) in cases of misidentification of the type species, and in order to best serve the nomenclatural stability, the type species of the genus name *Sturnornis* Legge, 1879 is now fixed (under Article 70.3 of the Code) as *Heterornis albofrontata* E.L. Layard, 1854, misidentified as *Heterornis senex* Bonaparte, 1851 in the original designation by Legge (1879).

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## Appendix

# State of selected morphological, ecological and behavioural characters

The characters states have been scored according to standard reference books (e.g. Cramp & Perrins 1994; Feare & Craig 1998; Rasmussen & Anderton 2005). The morphological characters 1, 2 and 4–6 have also been checked using the bird skins in the collections of the Naturhistoriska Riksmuseet, Stockholm, and the Muséum National d'Histoire Naturelle, Paris.

1. Size. Body weight data are available only for a limited number of starlings. We used the wing length as a proxy for the body size. Small: wing length < 111 mm (white square); medium: wing length 111–130 mm (black square); large: wing length > 130 mm (grey square).

2. Sex dimorphism. Absent (white square), weak (only in the hand, black square), or present (sexes easily identified at distance in the field, grey square).

3. Prying adaptation. Moderate development of the protractor muscles (white square), or strong development of the protractor muscles (black square); data from Beecher (1978).

4. Forehead crest. Presence (black square) or absence (white square) of stiff, erected feathers on the forehead.

5. Elongated feathers. Presence (white square) or absence (black square) of elongated feathers on the crown and nape.

6. Bare eye patch. Presence (black square) or absence (white square) of areas of bare skin around the eyes, often brightly coloured.

7. Reproduction. Most pairs breeding in (loose) colonies (white square) or solitary breeders (black square).

8. Habitat. Open habitats with limited arboreal cover (white square), open woodland (black square), or woodland areas (grey square).

9. Feeding. Predominantly ground feeders (white square), arboreal feeders (black square), or feeding on both habitats (grey square).

10. Food. Insectivorous (diet predominantly composed by insects, white square), omnivorous (including both insects and fruits, black square), frugivorous (mostly or only fruits and berries, grey square).

11. Migration. Migratory (breeding areas completely vacated during winter, white square), partially migratory (some populations sedentary, black square), or sedentary (grey square).

## Note added in proof

After the submission of our manuscript, a paper analyzing the phylogenetic relationships of the same taxa become available (Lovette et al., in press, Molecular Phylogenetics and Evolution). Although the loci analyzed in the two papers are in part different, the topologies recovered from the two datasets are highly congruent. However, Lovette et al. proposed a different taxonomic arrangement compared to ours. In particular, Lovette et al. suggested to assign the species Sturnus sinensis, S. pagodarum, S. erythropygium and S. malabaricus to the genus name Temenuchus "Cabanis 1815". This is a clear slip, because the genus name Temenuchus was published in the first volume of Museum Heineanum. Verzeichniss der ornithologischen Sammlung des Oberamtmann Ferdinand Heine, with the imprint date "1850-1851". Hence the valid genus name is Sturnia Lesson, 1837, which has 14 years of priority over Temenuchus. The other point of disagreement is the genus name used for the species S. sericeus and S. cineraceus. Lovette et al. used the genus name Poliopsar Sharpe, 1888, but it is invalid due to the homonymy with Poliopsar Cassin, 1867 (currently a junior subjective synonym of Icterus Brisson, 1760). When Sharpe became aware of the synonymy, he proposed the substitute name Spodiopsar Sharpe, 1890, which is the valid name for that clade.