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The butterfly subfamily Pseudopontiinae is not monobasic: marked genetic diversity and morphology reveal three new species of *Pseudopontia* (Lepidoptera: Pieridae)

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Abstract. The Afrotropical butterfly subfamily Pseudopontiinae (Pieridae) was traditionally thought to comprise one species, with two subspecies (*Pseudopontia paradoxa paradoxa* Felder & Felder and *Pseudopontia paradoxa australis* Dixey) differing in a single detail of a hindwing vein. The two subspecies also differ in their known geographic distributions (mainly north of versus south of the equator). Unlike most butterflies, *Pseudopontia* is white with no visible wing or body markings. We now report that males of *P. paradoxa australis* have an area of ultraviolet-reflecting scales along the anal vein of the forewing, whereas males of *P. paradoxa paradoxa* and all females do not. A total of 21 individuals of the northern subspecies, which were collected in three localities south of the equator, were found in the collection of the Royal Museum for Central Africa, indicating sympatry of the two traditional subspecies in the Congo River basin. To determine if additional cryptic species might be present, we sequenced three nuclear genes (*CAD*, *DDC* and *wingless*) as well as *cytochrome oxidase I* (*COI*), examined amplified fragment-length polymorphisms, and re-examined wing and genitalic morphology, using recently collected specimens from several regions of Africa. Phylogenetic analyses of the *COI* sequences and amplified fragment-length polymorphism data concur, and indicate the existence of at least five monophyletic, non-interbreeding populations, with a particularly deep divergence between three populations of *P. paradoxa paradoxa* and two of *P. paradoxa australis*. Despite the slow rate of evolution of the nuclear genes studied, individual gene trees and a concatenated three-gene tree demonstrate, with high bootstrap support, clear divergence among the five populations of *Pseudopontia*. In addition, consistent variations in details of wing vein stalks were found among four of the genetically distinct populations, which supports the hypothesis of multiple species.

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Division of *Pseudopontia* into five phylogenetic species is proposed, including the elevation of ssp. *australis* to species rank and the description of *Pseudopontia mabira*, *Pseudopontia gola* and *Pseudopontia zambezi*

Introduction

The wholly African butterfly subfamily Pseudopontiinae (Pieridae) comprises the single genus and species *Pseudopontia paradoxa* (Felder & Felder, 1869), which occurs in tropical forests across a vast expanse of sub-Saharan Africa. Its wing venation is distinctive among butterflies and, for some time in the 19th and early 20th centuries, this led to disagreements as to its family placement in the Lepidoptera (discussed in Dixey, 1923; Ackery *et al.,* 1998). The type locality is Calabar, Nigeria, near the Atlantic coast, at about 5°N latitude, although it occurs as far north and west as Sierra Leone, south as far as Gabon and northern parts of the Congo River basin, and eastwards to central Uganda. A second subspecies (*Pseudopontia paradoxa australis* Dixey) was described on the basis of a single morphological difference, a detail of hindwing venation, which was not considered sufficient grounds to define a new species. In particular, male genitalia were examined and found not to differ (Dixey, 1923; confirmed by A.Y. Kawahara, present work). This form is found south of the equator in many parts of the Democratic Republic of Congo (henceforth 'DRC'), and in gallery forests in the central African plateau in the DRC, north-western Zambia and Angola (Larsen, 2005).

The rather appropriate vernacular name is 'the Ghost' because of its unmarked, translucent, nearly white wings and very slow, erratic flight in the darkness under the canopy of evergreen rainforest. It frequents wet forest near the understory plants, and populations appear to be sedentary. Very little is known about larval host plants or other aspects of the biology of the genus. Heath (1977) recorded development from larva to adulthood on *Pentarhopalopilia marquesii* (Opiliaceae) for *Pseudopontia paradoxa australis* (correction of the plant name by D.C. Lees, personal communication). Another species in the Opiliaceae has been reported as a host plant for the nominate subspecies in Cameroon (Ackery *et al*., 2002; D.C. Lees, personal communication). Oviposition was reported on an unrelated plant (*Pseuderanthemum tunicatum*, Acanthaceae) in Sierra Leone (Owen, 1971), but the suitability of these species as larval host plants remains unconfirmed.

In the course of an ongoing study of the molecular phylogeny of lepidopteran families (see http://www.leptree.net), one of us (K.T. Mitter) observed that the *cytochrome oxidase I* (*COI*) sequence of a specimen of the nominate subspecies differs from that of the southern subspecies by 15% of nucleotide positions. This large difference in *COI* sequence suggested that these were two long-divergent species, and led us to search for further variation in molecular sequences in specimens recently collected from across the distribution of *Pseudopontia* (Plötz, 1870). Utilizing both molecular and morphological data, we have attempted to determine whether marked population structure or separate species might be detectable in *Pseudopontia*, despite the historical absence of species distinctions in this well-studied and commonly collected, but almost colourless, butterfly.

We examined the ultraviolet-reflecting and ultraviolet-fluorescent patches of the wings. Pierid wing scales that reflect the ultraviolet component of daylight have been examined with scanning electron microscopy, and have been shown to have fine structure that differs from other wing scales. This characteristic is not dependent on pigmentation, and its presence or absence may vary among species in a single genus (see, e.g., Silberglied & Taylor, 1973, 1978; Rutowski, 1977; review in Silberglied, 1984; Kemp *et al*., 2005).

Wing vein morphology was closely examined because many specimens collected in Mayumbe Forest in DRC have longer wing vein stalks compared with those collected in other regions. Morphological differences are slight but support the phylogenetic analyses of molecular data, which indicate the existence of multiple species of *Pseudopontia*. The *australis* subspecies is therefore elevated to full species rank, and three new species are described.

Material and methods

Specimens and localities

Specimens for molecular studies were collected in six regions plus three subregions, all in equatorial Africa. Collection sites are shown in Fig. 1, as are the inferred geographic ranges, with regions marked by numbers 1–6 with or without a letter suffix denoting a country name (see also Table 1). The designation of different regions was adapted from Larsen (2005). Proposed species names for the taxon in each region are also indicated on the map. Region 3 includes the type locality for *P. paradoxa*, whereas the type locality for *P. australis* (indicated by a 'T' in Fig. 1) lies in central DRC, midway between our collecting regions 5 and 6.

Most specimens for molecular studies were placed in 100% ethanol either upon arrival in Maryland, U.S.A., within a few months of collection, or in the field, immediately after collection. A smaller number were kept dry in private collections for up to 10 years. Two were parts of pinned specimens (one head and one partial abdomen, collected by T.B. Larsen) that had been in the museum collection at the University of Copenhagen since 1969. These were used because *Pseudopontia* no longer

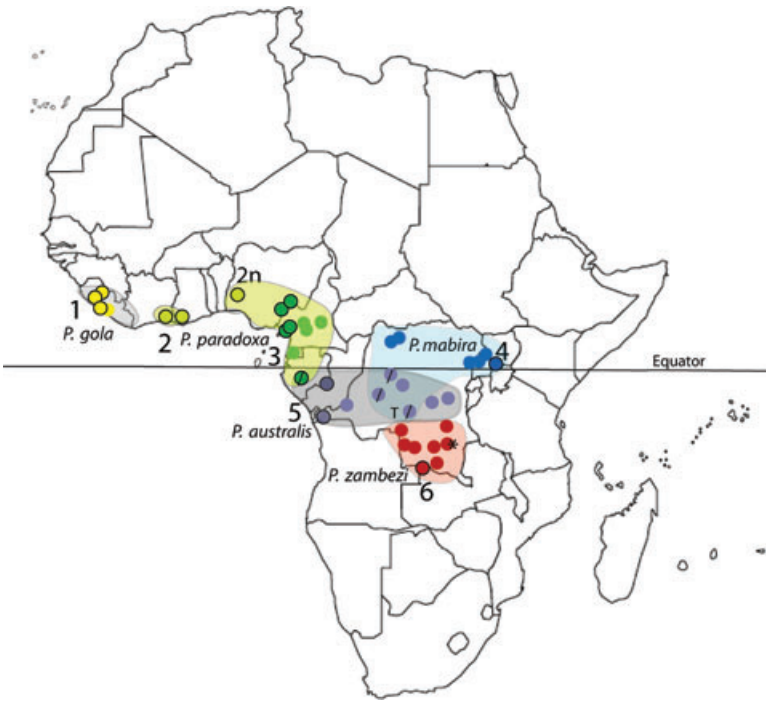

Fig. 1. Map showing areas of collection for recent specimens used in genetic and morphology studies (dots with black outlines), as well as approximate areas for museum specimens used for wing morphology only (dots without outlines). The 'T' in central DRC marks the type locality of *Pseudopontia australis* Dixey. Region 1, eastern Sierra Leone (Gola Forest) and Liberia; 2, eastern Cote d'Ivoire and western Ghana, plus western Nigeria (labelled 2n); 3, eastern Nigeria and Cameroon, plus central Gabon (labelled 3g); 4, Uganda and Democratic Republic of Congo (DRC) north of the equator; 5g, Batéké Plateau in eastern Gabon; 5, Mayumbe Forest in western DRC; 6, north-western Zambia (Ikelenge). Dots with a slash (/) indicate provinces where specimens of two different species were collected. Asterisk marks the location of Upemba National Park.

occurs at that locality (Gambari Forest, Nigeria, designated here as locality 2n) because of urbanization and the consequent widespread destruction of habitat. Localities 3g and 5g are located in Gabon in different habitats, i.e. rainforest versus riverine forest on sandy soil (King, 2008), about 345 km apart, and differ in the hindwing-vein phenotype of *Pseudopontia* found there. Tissue samples of all specimens are stored in ethanol at −80°C in the University of Maryland–Smithsonian Institution frozen Lepidoptera collection (UM-SI).

Table 2 lists 330 museum specimens, collected between 1869 and 1965, that were studied for wing morphology only. Depositories of specimens are listed as follows: ABRI, African Butterfly Research Institute, Nairobi, Kenya; BMNH, the Natural History Museum, London, U.K.; CMNH, Carnegie Museum of Natural History, Pittsburgh, PA, U.S.A.; HMO, Hope Museum, Oxford, U.K.; RMCA, Royal Museum for Central Africa, Tervuren, Belgium; USNM, United States National Museum of Natural History (Smithsonian Institution), Washington, D.C., U.S.A.

The spelling of localities follows the Times Comprehensive Atlas of the World, 11th edition. Elevations were estimated using approximate geographic coordinates in the programme GOOGLE EARTH (downloaded from http://earth.google.com). Approximate distances between localities were calculated from coordinates using the website http://www.ig.utexas.edu/outreach/googleearth/latlong.html.

Morphology

The type specimens of *Pseudopontia*, including the holotype of *Pseudopontia paradoxa* (Felder & Felder 1869; Fig. 2A) and Dixey's *Pseudopontia paradoxa australis* lectotype (Dixey 1923; Fig. 2C), are remarkably similar in appearance. Although several of the images in this figure are shown with increased contrast, the distinguishing details of wing venation are nevertheless not evident without magnification (some of these are shown in insets, and all are described in the Results).

Dissections of genitalia were conducted on a total of 15 male and eight female specimens from the six regions, all collected recently. No differences in genitalic structures were noted; none were found when the *australis* subspecies was described (Dixey, 1923).

Wing vein stalk lengths were measured in 72 recently collected specimens using wings mounted in coin holders and scanned at 600 pixels per inch against a white background. Images were enlarged four- to five-fold and printed with enhanced contrast. Measured lengths from the base of the wing were expressed as a fraction of the wing length in the same image. Two hundred and ninety additional specimens (listed in Table 2) in the Royal Museum for Central Africa, collected between 1870 and 1959, were measured directly under 10× magnification. Specimens from the collections of the U.S. National Museum ($N = 12$) and the Carnegie Museum of Natural History ($N = 11$), the African Butterfly Research Institute ($N = 10$) and the Hope Museum ($N = 7$) were measured using photographs (Table 2). Data were first analysed by sex (where known) within each region. Although there were slight differences by sex (approximately 10%) in mean forewing length, there were no differences at all between the sexes in mean values for measurements expressed as a fraction of wing length. Data for the two sexes were therefore combined. Population differences were evaluated using Student's *t*-tests assuming unequal variances, with the

Table 1. Recent specimens used for genetic studies.

Region	Country, locality	Latitude	Longitude	Collector[a], month/year	Specimen ID	DNA solution	*COI*	Nuclear genes	AFLP data	Gen. diss.
	paradoxa species group									
1	Sierra Leone, Gola North	7.65°	−10.90°	ABRI, 11/2008	SC-08-SL01	SL1	Yes	—	—	♀
1	Sierra Leone, Gola North	7.65°	−10.90°	ABRI, 11/2008	SC-08-SL02	SL2	Yes	—	Yes	—
1	Sierra Leone, Gola South	7.37799°	−11.25295°	SzS, 11/2008	SS-08-SL03	SL3	Yes	Yes	Yes	—
1	Sierra Leone, Gola South	7.37799°	−11.25295°	SzS, 11/2008	SS-08-SL04	SL4	Yes	—	Yes	♂
1	Sierra Leone, Gola North	7.6696°	−10.94823°	SzS, 11/2008	SS-08-SL05	SL5	Yes	Yes	Yes	—
1	Sierra Leone, Gola North	7.6047°	−11.0392°	SzS, 3/2009	SS-08-SL11	SL11	Yes	—	Yes	—
1	Sierra Leone, Gola North	7.6047°	−11.0392°	SzS, 3/2009	SS-08-SL12	SL12	Yes	—	Yes	—
1	Liberia, 'Cape Mount Ca'	6.717°	−11.35°	1953[b]	—	USLib4	Yes	—	—	—
2	Ghana, Ankasa National Park	5.2210°	−2.6472°	TBL, 11/2008	TBL-08-1005	GH5	Yes	—	Yes	—
2	Ghana, Ankasa National Park	5.2210°	−2.6472°	SzS, 11/2008	SS-08-GH06	GH6	Yes	Yes	Yes	♀
2	Cote d'Ivoire, Yapo Forest	5.73°	−4.030°	HWG, 1/1999	TBL-08-1003	GH3	Yes	—	Yes	♀
2	Cote d'Ivoire, Alepe Forest	5.15°	−2.783°	HWG, 1/2000	TBL-08-1004	GH4	Yes	—	Yes	—
2n	Nigeria, Gambari forest	7.4947°	3.8883°	TBL, 1969	TL-69-001	TL1	—	—	Yes	—
2n	Nigeria, Gambari forest	7.4947°	3.8883°	TBL, 1969	TL-69-003	TL3	—	—	Yes	—
3	Cameroon, Mount Etinde	4.043°	9.1218°	GH, 1/1992	AV-92-0002	AV	Yes	Yes	Yes	—
3	Cameroon, NV Edea	3.8°	10.403°	ABRI, 3/2008	SC-08-cam	CAM	Yes	Yes	Yes	♀
3	Nigeria, Cross River	6.250°	9.033°	DK, 11/2004	DK-04-5001	DK1	Yes	—	Yes	♂
3	Nigeria, Oban Hills	5.817°	8.683°	DK, 11/2004	DK-04-5002	DK2	Yes	—	Yes	♀
3g	Gabon, Waka National Park	−1.12°	11.13°	GVW, 3/2007	GVW-09-10012	GN12	Yes	—	Yes	—
3g	Gabon, Waka National Park	−1.12°	11.13°	GVW, 2/2008	GVW-09-14634	GN34	Yes	—	Yes	—
3g	Gabon, Waka National Park	−1.12°	11.13°	GVW, 2008	GVW-09-12558	GN58	Yes	—	Yes	—
3g	Gabon, Waka National Park	−1.12°	11.13°	GVW, 11/2005	GVW-09-6671	GN71	—	—	Yes	♀
3g	Gabon, Waka National Park	−1.12°	11.13°	GVW, 3/2008	GVW-09-9975	GN75	Yes	—	Yes	—
3g	Gabon, Waka National Park	−1.12°	11.13°	GVW, 11/2005	GVW-09-6888	GN88	Yes	—	Yes	♂
3g	Gabon, Waka National Park	−1.12°	11.13°	GVW, 12/2007	GVW-09-13698	GN98	Yes	—	—	—
4	Uganda, Mabira Forest	0.38908°	33.011°	ABRI, 12/2008	SC-08-UG07	UG7	Yes	Yes	Yes	♂
4	Uganda, Mabira Forest	0.3848°	33.0151°	ABRI, 4/2009	SC-09-UG08	UG8	Yes	—	Yes	—
4	Uganda, Mabira Forest	0.38908°	33.011°	ABRI, 7/2009	SC-09-UG09	UG9	Yes	—	Yes	—
4	Uganda, Mabira Forest	0.38908°	33.011°	ABRI, 4/2010	SS-10-UG21	UG21	Yes	—	—	—
4	Uganda, Mabira Forest	0.38908°	33.011°	ABRI, 4/2010	SS-10-UG22	UG22	Yes	—	—	—
4	Uganda, Mabira Forest	0.389°	33.01°	ABRI, 4/2010	SS-10-UG23	UG23	Yes	—	—	—
4	Uganda, Mabira Forest	0.389°	33.01°	ABRI, 4/2010	SS-10-UG24	UG24	Yes	—	—	—
4	Uganda, Mabira Forest	0.389°	33.01°	ABRI, 4/2010	SS-10-UG25	UG25	Yes	—	—	—
	australis species group									
3g	Gabon, Waka National Park	−1.12°	11.13°	GVW, 11/2005	GVW-09-6564	GN64	Yes	—	Yes	♂
5g	Gabon, Batéké Plateau	−2.113°	14.068°	GVW, 4/2007	GVW-09-11315	GS15	Yes	—	Yes	—
5g	Gabon, Batéké Plateau	−2.113°	14.068°	GVW, 5/2007	GVW-09-13424	GS24	Yes	—	Yes	—

Table 1. Continued

Region	Country, locality	Latitude	Longitude	Collector[a], month/year	Specimen ID	DNA solution	*COI*	Nuclear genes	AFLP data	Gen. diss.
5g	Gabon, Batéké Plateau	−2.113°	14.068°	GVW, 5/2007	GVW-09-13384	GS84	Yes	—	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	AYK, 5/2007	AYK-07-9241	Co41	Yes	Yes	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	AYK, 5/2007	AYK-07-9242	Co42	Yes	—	—	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	AYK, 5/2007	AYK-07-9243	Co43	Yes	—	—	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	AYK, 5/2007	AYK-07-9244	Co44	Yes	—	—	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	AYK, 5/2007	AYK-07-9248	Co48	Yes	—	—	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8164	C64	Yes	—	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8165	C65	Yes	—	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8166	C66	—	Yes	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8167	C67	Yes	—	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8168	C68	Yes	—	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8169	C69	Yes	—	Yes	♂
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8170	C70	Yes	—	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8171	C71	Yes	—	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8172	C72	Yes	—	Yes	—
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008	JDP-08-8173	C73	Yes	—	Yes	♂
5	Democratic Republic of Congo, Mayumbe Forest	−5.6212°	13.0891°	WJDP, 11/2008[c]	JDP-08-8174	C74	Yes	Yes	Yes	—
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T383	Z83	Yes	—	—	—
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T384	Z84	Yes	—	Yes	♀
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T385	Z85	Yes	Yes	Yes	—
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T386	Z86	Yes	Yes	Yes	♂
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T387	Z87	Yes	—	Yes	—
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T388	Z88	Yes	—	Yes	—

Table 1. Continued

Region	Country, locality	Latitude	Longitude	Collector[a], month/year	Specimen ID	DNA solution	*COI*	Nuclear genes	AFLP data	Gen. diss.
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T389	Z89	Yes	—	Yes	—
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T391	Z91	Yes	—	Yes	♂
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T392	Z92	Yes	—	—	♂
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T393	Z93	Yes	—	Yes	—
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T394	Z94	Yes	—	Yes	—
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T395	Z95	Yes	—	Yes	♂
6	Zambia, Ikelenge	−11.233°	24.267°	ABRI, 3/2001	SC-01-T396	Z96	Yes	—	—	♂

[a]Collectors: ABRI, African Butterfly Research Institute; AYK, Akito Y. Kawahara; DK, D. Knoop; GH, Gustavo Hormiga; GVW, Gaël vande Weghe; HWG, Haydon Warren-Gash; SzS, Szabolz Safian; TBL, Torben B. Larsen; WJDP, Willy and Jurate De Prins.

[b]This is a museum specimen in the U.S. National Museum in Washington, D.C., collected by 'C.C.B. & J.M.', from which a partial *cytochrome oxidase I* (*COI*) sequence was obtained.

[c]Not shown are six additional specimens collected in Mayumbe Forest, Democratic Republic of Congo in 2008, of which three males and one female were dissected.

null hypothesis of no difference being rejected at $P < 0.01$ or $P < 0.001$.

Ultraviolet (UV) reflectance was examined by viewing wings reflecting light from a hand-held near-UV source (not shown), and also for wings of recently collected specimens using a near-UV (365 nm) transilluminator and camera (AlphaImager HP; Alpha Innotech) with a 6-s exposure. This was facilitated by the absence of pigmentation in the untreated wings.

DNA sequencing

One or two legs were removed from specimens and sent to the Canadian Centre for DNA Barcoding at the University of Guelph, Ontario, Canada, for sequencing of the 658-bp *COI* barcoding region (Ratnasingham & Hebert, 2007). *COI* sequences, GenBank accession numbers, specimen photographs and associated data are available at the Barcode of Life Data Systems website (http://www.boldsystems.org).

Portions of the coding regions of the nuclear genes *CAD* (pyrimidine biosynthesis, 2194 bp; Moulton & Wiegmann, 2003), *DDC* (dopa decarboxylase, 1051 bp; Fang *et al.*, 1997), and *wingless* (403 bp; Brower & DeSalle, 1998) were sequenced at the University of Maryland from an extract of total nucleic acids from abdomens. Protocols and primers for sequencing these genes follow Regier *et al.* (2008). Outgroup taxa were sequenced as part of the Lepidoptera Tree of Life project at the University of Maryland (http://www.leptree.net). Sequences are deposited in GenBank under numbers GQ283565, GQ283572, GQ283644, GQ283651, GQ283879, GQ283886, HQ174317-HQ174332, and HQ186781-HQ196799.

Mitochondrial and nuclear gene sequences were analysed in a parsimony and a maximum-likelihood (ML) framework. Parsimony analyses were conducted using PAUP* v4.0b10 (Swofford, 2002) with tree–bisection–reconnection branch swapping, and without topological constraints. Bootstrap analyses were carried out for 1000 pseudoreplicates. ML searches and bootstrap analyses were conducted using GARLI 1.0 (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006) in parallel with grid computing via the Lattice Project (Bazinet & Cummings, 2009). We selected the best-fitting substitution model (GTR + I + Γ) under the Akaike information criterion, using JMODELTEST (Posada, 2008).

Amplified fragment length polymorphisms

For amplified fragment length polymorphism (AFLP) analysis (Vos *et al.*, 1995), a separate DNA extraction of heads or anterior halves of abdomens was made using DNEasy kits (Qiagen). DNA was digested with two six-base restriction enzymes, *Eco*R1 and *Pst*1, in the presence of standard adapter oligonucleotides and DNA ligase, to produce DNA constructs as previously described (Ahern *et al.*, 2009). After heat inactivation of the enzymes, the DNA constructs were

Table 2. Museum specimens examined for wing morphology.

Museum	Region/country	Province, area	Number of specimens ♂♂	♀♀	Total	Latitude	Longitude	Elevation (m)	Distance from PN Upemba (km)
paradoxa species group									
RMCA	1/Sierra Leone	No data	3	0	3	No data	—	<500	—
RMCA	1/Liberia	Kaouyeke	3	2	5	No data	—	<500	—
USNM	1/Sierra Leone	No data	—	—	1	No data	—	<500	—
USNM	1/Liberia	Mount Coffee	—	—	2	6.49°N	10.6°W	<500	—
CMNH	1/Liberia	'Holland Collection'	—	—	3	No data	—	<500	—
BMNH	3/Nigeria (type)	Calabar	—	—	1	5.0°N	8.4°E	<100	—
RMCA	3/Cameroon	Various	7	5	12	Various	—	200–700	—
CMNH	3/Cameroon	Lolodorf	—	—	6	3.2°N	10.7°E	400	—
RMCA	3/Eq. Guinea	Various	1	3	4	No data	—	<500	—
USNM	3/Eq. Guinea	Monte Bata	—	—	1	1.9°N	9.9°E	<100	—
RMCA	4/Uganda	Budongo Forest	2	1	3	1.7°N	31.7°E	1050	—
RMCA	4/Uganda	Msisi	2	0	2	1.0°N	30.5°E	750	—
CMNH	4/Uganda	Budongo Forest; Jackson Kibale Forest	—	—	2	1.7°N	31.7°E	1100	—
ABRI	4/Uganda (type)	Mabira Forest	—	—	6	0.4°N	33.0°E	1300	—
HMO	4/Uganda	Semliki	—	—	3	0.8°N	30.1°E	920	—
HMO	4/Uganda	Ankole	—	—	2	—	—	—	—
RMCA	DRC	Ituri, P.N. Albert	4	0	4	1.2°N	29.8°E	950	—
RMCA	DRC	Ituri, P.N. Albert II	6	11	17	0.7°N	29.6°E	750	—
RMCA	DRC	Equateur, Abumombazi	16	8	25	3.7°N	22.2°E	400	—
RMCA	DRC	Equateur, Yakoma	21	10	31	4.1°N	22.4°E	500	—
RMCA	DRC	Tshuapa	2	1	3	0.2°S	20.7°E	345	—
RMCA	DRC	Kasai Occidental	0	1	1	3.8°S	18.8°E	340	—
RMCA	DRC	Sankuru	17	0	17	5.0°S	23.4°E	530	—
australis species group									
RMCA	DRC	Tshuapa	6	1	7	0.2°S	20.7°E	345	1170
RMCA	DRC	Sankuru	5	0	5	2.0°S	21.4°E	430	967
RMCA	DRC	Maniema	7	3	10	3.0°S	25.9°E	500	655
RMCA	DRC	Sankuru	7	8	15	3.4°S	24.4°E	500	680
RMCA	DRC	Kasai Occidental	1	6	7	3.8°S	18.8°E	340	1035
RMCA	DRC	Kinshasa	7	4	11	4.3°S	15.3°E	300	1365
RMCA	DRC	Sankuru	9	5	14	5.0–5.5°S	23.4°E	530	565
USNM	5/DRC	Bas-Congo, Landana	—	—	1	5.6°S	13.1°E	300	1550
RMCA	5/DRC	Bas-Congo, Mayumbe	2	2	4	5.5°S	13.1°E	320	1550
HMO	5/DRC (type)	Kasai, Luebo	—	—	1	5.3°S	21.3°E	400	710
RMCA	DRC	Haut-Lomami, Katompe	3	5	8	6.2°S	26.3°E	800	300
RMCA	DRC	Haut-Katanga, P.N. Upemba	20	9	29	8.8°S	26.8°E	1320	0
RMCA	DRC	Lualaba, Kapanga	2	1	3	8.3°S	22.6°E	850	465
RMCA	DRC	Lualaba, Kafakumba	9	8	17	9.7°S	23.7°E	980	350
RMCA	DRC	Lualaba, Kinda	1	3	4	9.3°S	25.1°E	830	195
RMCA	DRC	Lualaba, Sandoa	18	8	26	9.7°S	22.9°E	900	440
RMCA	DRC	Lualaba	2	1	3	10.3°S	Various	>900	—
USNM	6/DRC	Lualaba, Musokatanda	—	—	6	10.9°S	25.1°E	1350	300
USNM	6/Zambia	Mwinilunga	—	—	1	11.7°S	24.4°E	1300	415
ABRI	6/Zambia (type)	Ikelenge	—	—	4	11.2°S	24.3°E	1400	390
HMO	6/DRC	Upper Lubudi River	—	—	1	10.7°S	23.9°E	1100	390

diluted 50-fold into a pre-amplification mixture containing adapter-based primers with one-base extensions (A), and were then subjected to 22 cycles of polymerase chain reaction (PCR). The resulting products were diluted 200-fold into five selective amplification mixtures for 30 cycles of PCR (Ahern *et al*., 2009). The selective amplifications contained the *Eco*R1-based primer labelled with fluorescent 6-carboxyl fluorescin (6-FAM), and having the three-base extension AGT, and one of five different *Pst*1 primers with extensions AT, AGC, AAC, ACG or ACC. Products were analysed on an ABI 3730 (Applied Biosystems) using 1000-bp internal standards (Genescan 1000-ROX; Applied Biosystems).

For the two 40-year-old specimens, this procedure yielded very few and weak AFLP bands, despite total DNA

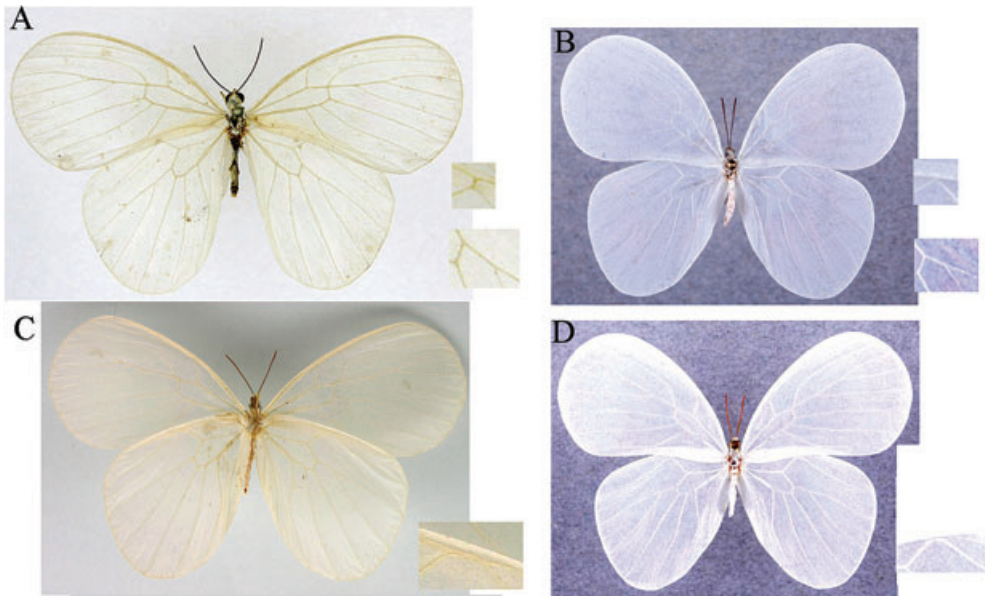

Fig. 2. Habitus of four *Pseudopontia* type specimens. A, *Pseudopontia paradoxa* holotype; B, *Pseudopontia mabira* **sp.n.** holotype; C, *Pseudopontia australis* lectotype (verso, showing hindwing details); D, *Pseudopontia zambezi* **sp.n.** holotype. Insets show two-fold enlargements of critical hindwing vein details: in panels A and B, costal–subcostal hindwing vein fusion (upper inset, from verso) and length of m1/m2 stalk (lower inset); in panels C and D, near contact of costal and subcostal veins.

concentrations in the extracts (measured by absorbance) that were comparable with fresh specimens. In order to compensate for the degraded state of the DNA, the following modifications were made to the pre-amplification step. The dilution of these two constructs into the pre-amplification was reduced to five-fold, the total reaction volume was increased by five-fold and the PCR cycles were increased to 30. These modifications resulted in a 50-fold increase in the probability of observing any particular, very-low-copy band, as well as a 70–700-fold increase in the level of product for each band (1.7-fold increase per cycle, raised to the eighth power, and possibly multiplied by ten for the decreased dilution). Three different dilutions of these products in the selective amplifications were tried, but in general gave similar results. AFLP bands for these specimens were scored in bins devised for the other specimens, and also in bins unique to but shared by these two specimens, using GENEMAPPER v3.7 (Applied Biosystems).

Two hundred and seventeen bands were used for analysis, ranging in size from 80 to 862 bp in length. Duplicate amplifications with each primer pair were carried out for 32 specimens. Bands with more than one discordant duplicate were eliminated from the dataset, as were any bands that occurred in blank reactions with no added DNA, bands occurring in only one specimen and the small number of bands, occurring in more than 75% of specimens, that were excluded as being uninformative. In the set of bands analysed, the mean number of specimens with discordant data was 0.33 per band, giving an average of 1.04% discordance or 99% accuracy.

The AFLP data were analysed twice using PAUP* with the criteria set as distance and as parsimony. Branch swapping of unrooted trees was conducted with tree–bisection–reconnection, without topological constraints. Bootstrap analysis was carried out for 1000 pseudoreplicates. AFLP data were also analysed using STRUCTURE 2.3.1, with admixture among populations (Pritchard *et al*., 2000; Evanno *et al*., 2005). There were 600 000 iterations after a burn-in of 200 000 iterations, inferring a separate alpha and independent allele frequencies for each population. Increasing the number of iterations to 2 million after a burn-in of 1 million had no effect on the results. The analysis was repeated between six and ten times for each K value, with similar results; bar graphs show the results with the highest posterior probability. The evaluation of differences in posterior probabilities followed that of Evanno *et al.* (2005).

Results

COI sequences

The *COI* sequence data was obtained from 61 recent specimens and one museum specimen. These sequences formed six clades in two major lineages when analyzed either by ML (Fig. 3) or parsimony (not shown). The two lineages correspond exactly to the former subspecies, whereas the six clades correspond almost exactly to the six numbered collecting regions, with the few exceptions noted below. All specimens in the *paradoxa*-group lineage had *COI* sequences that diverged 15–16% (K2P distance) from those of specimens in the *australis* lineage. ML mean branch length sums between *paradoxa* and *australis* species groups ranged from

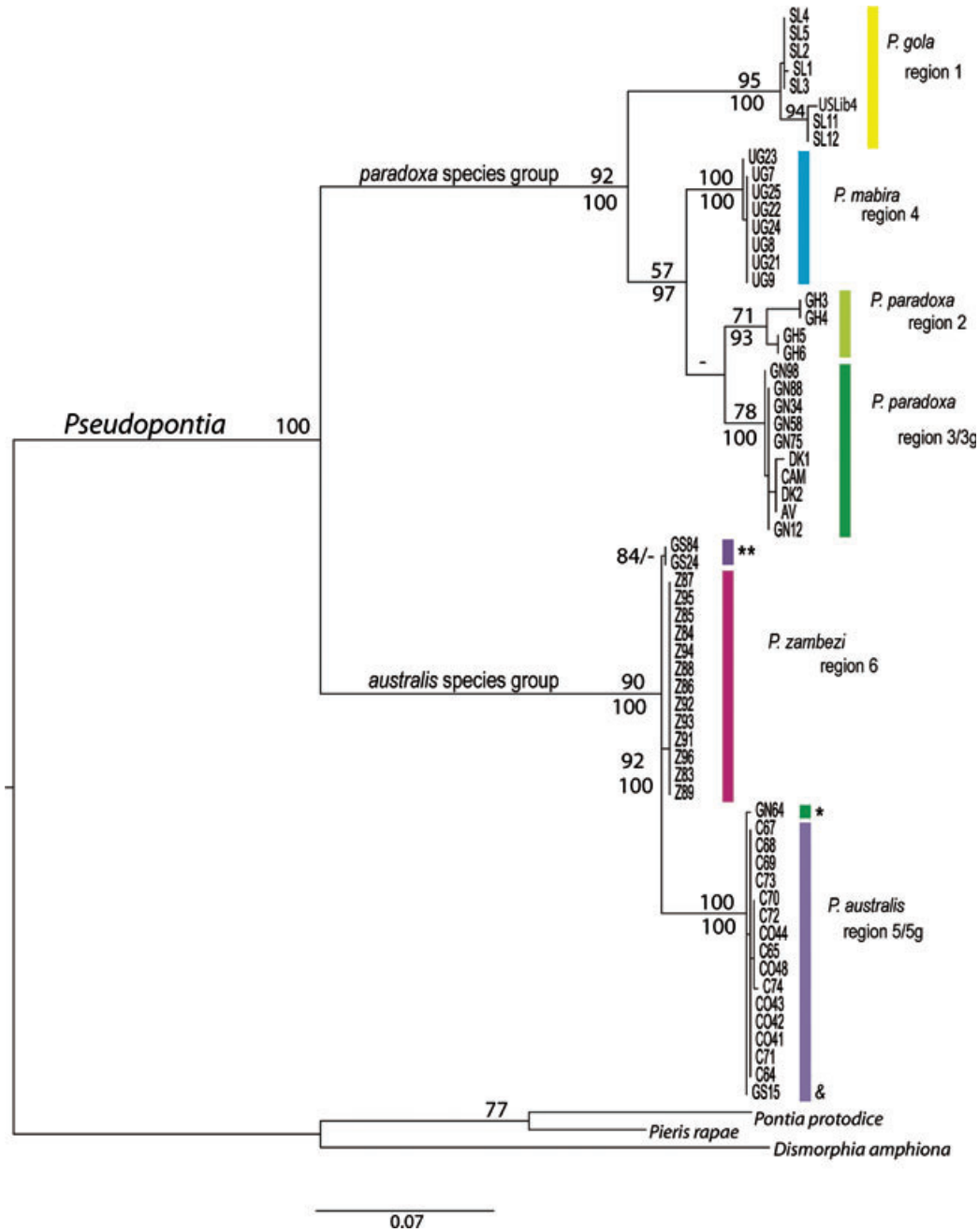

Fig. 3. Maximum-likelihood (ML) tree for *cytochrome oxidase I* (*COI*) sequences. Bootstrap values for ML are shown above branches (percentages of 1000 pseudoreplicates) and below branches for parsimony analysis. Topology was identical for the single maximum parsimony tree. Branch lengths are proportional to distances. Of 658 total nucleotides, 510 were constant, one was uninformative and 147 were parsimony informative (excluding out-groups). Tip labels refer to DNA solutions in Table 1. Coloured bars correspond to collecting regions in Fig. 1. Asterisk marks data for the *australis* specimen collected in region 3g.

0.287 to 0.3418 substitutions per site (Table S1). Within the *paradoxa* lineage, pairwise *COI* sequence differences among regions ranged from 3.21 to 9.17% (K2P distance or 21–60 steps by parsimony; Table S1) or 0.0324–0.1089 substitutions per site by ML. *COI* sequences differ between the two main *australis*-group clades (regions 5 and 6) by at least 3.06% of nucleotides or 0.0368 substitutions per site. Within-region variation (maximum K2P distance) was low in regions 3, 4, 5 and 6, ranging from 0 to 0.47%. Specimens from regions 1 and 2 showed higher maximum variation in *COI* sequences, comprising 1.38 and 1.69% of nucleotides, respectively.

Bootstrap analyses using parsimony and ML criteria resulted in high support (97–100%, parsimony) or moderate to high support (71–100%, ML) for each of the six clades (Fig. 3). Specimens from regions 3 (Cameroon, eastern Nigeria and central Gabon rainforests) and 2 (coastal Ghana and Cote d'Ivoire) formed sister groups. However, intermediate nodes indicating relationships among the *paradoxa*-group populations were unsupported or only weakly supported in ML analyses.

A few specimens gave unexpected results. One specimen collected in *paradoxa*-group region 3g has the *australis*-group hindwing vein character (not fused), unlike the other specimens collected there. Consistent with this, the specimen has a *COI* sequence that clusters with *australis*-group region 5 (Fig. 3, single asterisk). In addition, the three specimens collected in locality 5g have the *australis*-group hindwing vein character, and one has a *COI* sequence that aligns with the region-5 clade (ampersand in Fig. 3), whereas the other two align with region 6 (Fig. 3, double asterisk). This result was reconfirmed using replicate extractions and sequencing. In the interim, all three specimens were assigned to region 5 or population 5.

Wing morphology compared with molecular clades

Wings from recently collected specimens of the two historical subspecies and three additional, proposed species are shown in Fig. 4. Small insets show enlargements of the hindwing vein character that formerly distinguished the subspecies, and now distinguish two species groups: the *paradoxa* group (Fig. 4A–C), with costal and subcostal veins secondarily fused or bridged, as in the original description (Felder & Felder, 1869), found in regions 1–4; and the *australis* group (Fig. 4D, E), with costal and subcostal veins closely aligned for a short distance, but not fused (Dixey, 1923), found in regions 5 and 6. There are no detectable differences in wing structure between *P. paradoxa* (Fig. 4A) and *Pseudopontia gola* **sp.n.** (Fig. 4B), whereas *Pseudopontia*

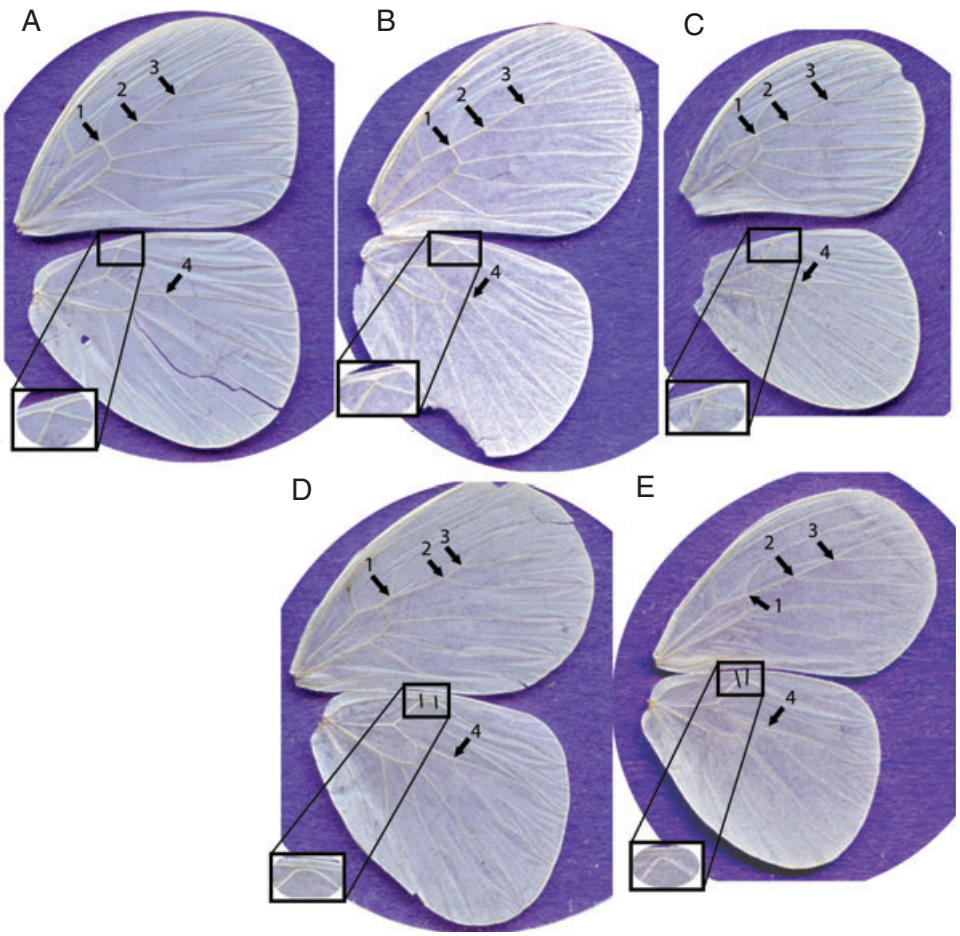

Fig. 4. Representative wings of five *Pseudopontia* species. A, *Pseudopontia paradoxa* Felder & Felder; B, *Pseudopontia gola* **sp.n.**; C, *Pseudopontia mabira* **sp.n.**; D, *Pseudopontia australis* Dixey; E, *Pseudopontia zambezi* **sp.n**. Insets show two-fold enlargements of hindwing vein fusions (A–C) or near contact (D, E). Arrows indicate the points where the following veins emerge from stalks: 1, Rs1; 2, M2; 3, M1; 4, M2 in hindwing. Two short lines across costal and subcostal veins of hindwings in (D) and (E) indicate the extent of near contact between those veins. The ends of this near-contact zone were estimated as the points where the distance between the veins exceeds 1.5 times the nearest approach.

mabira **sp.n.** has a shorter M1/M2 stalk in the hindwing (Fig. 4C, node at arrow 4). As compared with all three of the preceding taxa and *Pseudopontia zambezi* **sp.n.** (Fig. 4E), *P. australis* (Fig. 4D) has longer Rs1 and M2 stalks in the forewing (arrows 1 and 2). *Pseudopontia australis* also has a longer zone of near contact between the hindwing costal and subcostal veins than does *P. zambezi* **sp.n.**

To test for the reliability of these slight morphological differences, measurements of wing lengths and wing vein branches or stalks (measured from the base of the wing to each node) were made on all recently collected as well as 330 museum specimens (listed in Table 2). In specimens collected in region 4 (Uganda) as well as the adjacent Parcs nationaux Albert in DRC (now Virunga National Park) and Equateur province in north-western DRC, the hindwing M1–M2 vein branch is slightly more proximal as compared with *paradoxa*-group specimens from other regions (mean values in Fig. 5A, $P < 0.01$; see Fig. 2A versus 2B insets and Fig. 4A versus 4C). This small morphological difference, as well as the difference from *P. paradoxa* in overall size (forewing length 18–23 mm versus 22–28 mm, $P < 0.01$) supports the recognition of *P. mabira* **sp.n.** as a separate species in Uganda and other areas of central Africa close to the equator.

Forewings from region 5/5g (*P. australis*; Fig. 4D) have a more distal branch, where forewing vein M2 separates from the common stalk with M1 + Rs2 + 3 + 4 (mean values in Fig. 5B; $P < 0.01$). In addition, the mean length of the Rs1 stalk (length from the end of the discal cell) is longer in region 5/5g than in region 6 (*P. zambezi* **sp.n.**; mean values of 0.0365 ± 0.0034 versus 0.0073 ± 0.0024, as fraction of forewing length, $P < 0.001$). There is no significant difference between regions 5 and 6 in the stalk lengths for veins R or M1, or in the length of the discal cell (data not shown). In *paradoxa*-group specimens (148 museum and 30 recently collected), these two forewing vein characters (more distal M2 branch and long RS1 stalk) were found at very low frequencies (0.052 and 0.029, respectively), whereas each of these was present in 86% of recent specimens from region 5/5g.

In addition, the length of near contact between the costal and subcostal veins of the hindwing was measured in *australis*-group specimens (see insets in Figs 4D, E and 2C, D). In specimens from region 5/5g (*P. australis*), this length had a broad distribution, with longer values (and a flattened appearance of the subcostal vein) being most common, whereas in region 6 (*P. zambezi* **sp.n.**), all recent specimens had shorter lengths of contact (mean values of 0.0430 ± 0.0024 for region 5 versus 0.0289 ± 0.0010, $P < 0.001$).

A preliminary assessment based on wing vein morphology of the geographic ranges of several taxa (and their possible overlap in the DRC) was made using museum specimens, principally from the RMCA, as well as recent specimens. First, in the RMCA collection we found 21 specimens of *Pseudopontia* with fused hindwing veins (*paradoxa* species group) that were collected up to 5.5° south of the equator. These localities are indicated in Fig. 1 as purple dots

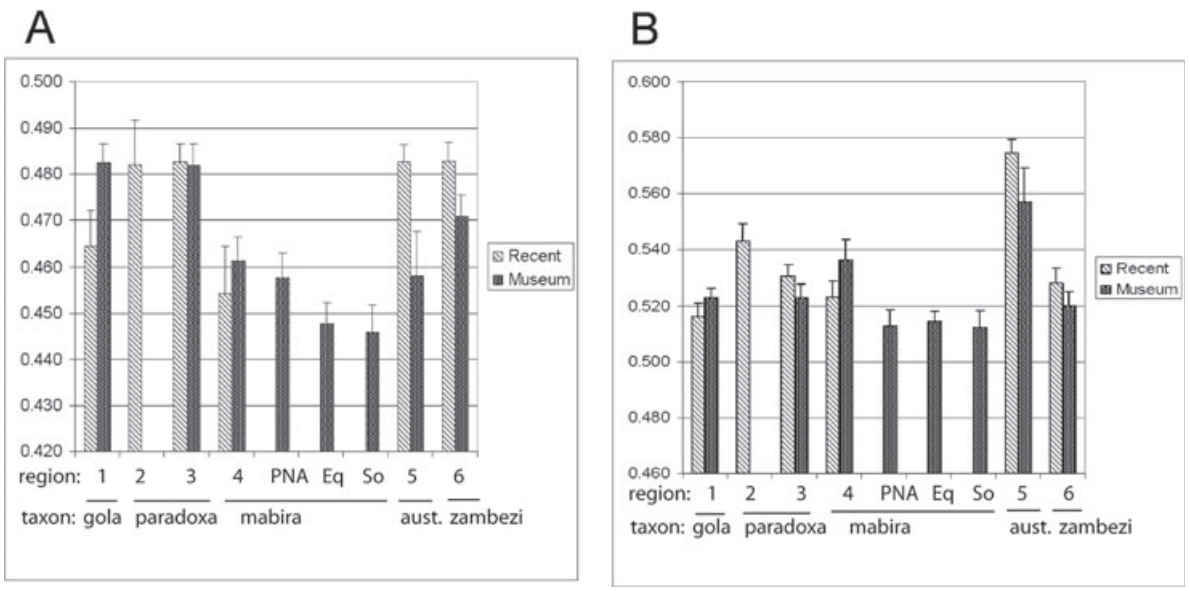

Fig. 5. Wing vein characters that distinguish *Pseudopontia mabira* **sp.n.** and *Pseudopontia australis* **stat.n.** from other *Pseudopontia* taxa (mean values + SEM). A, hindwing M1/M2 stalk length; B, forewing M2 stalk length. Data for *australis*-group taxa in regions 5 and 6 are shown on the right. All other specimens have the *paradoxa*-group hindwing vein fusion. Distance in millimetres from base of wing to the indicated branch was divided by the wing length in the same image. Numbers of specimens measured were: region 1, seven recent and 15 museum; region 2, four recent; region 3/3g, ten recent and 23 museum; region 4, eight recent and 19 museum; region 5/5g, 22 recent and six museum; region 6, 13 recent and 12 museum; Parcs nationaux Albert (PNA), 21 museum specimens; Equateur Province (Eq), 55 museum; southern Democratic Republic of Congo ('So', see text), 21 museum. For regions 5 and 6, only museum specimens from localities within 100 km of our recent collecting areas were included in this analysis.

marked with a slash (/). The specimens resemble those from Uganda (*P. mabira* **sp.n.**) in having a more proximal hindwing M1–M2 vein branch, with mean values shown in Fig. 5A (data labelled 'So'). Other specimens collected in the same areas were identified as *P. australis*.

Second, data for wing vein characters was plotted against geographic location to determine whether the distributions of *P. australis* and *P. zambezi* **sp.n.** might be different. In Fig. 6, each point represents data for an individual butterfly plotted against distance from Upemba National Park, a densely forested park in south-eastern DRC (8.8°S, 26.8°E, 1350 m a.s.l.; see Fig. 1, asterisk), in which the *Pseudopontia* have several distinctive wing vein measurements. Localities within 500 km of Upemba are also in the southern highlands at elevations of over 750 m. Localities farther away are at lower elevations, and at latitudes between 0.2°S and 5.6°S. Although there is overlap in the range of values for the forewing M2 branch measurement (Fig. 6A), the data clearly separate into three groups: (i) specimens from Upemba National Park have the lowest values; (ii) those from the remaining highlands at 200–500 km away (including our region 6, *P. zambezi* **sp.n.**, at 390 km) have intermediate values; (iii) those from the lowland areas have the highest values, including our region 5 (*P. australis*) at 1550 km away. Data for the hindwing vein near-contact length (Fig. 6B) also exhibits distribution differences among these three areas. The distinctive set of values in Upemba National Park for the two characters shown and others not shown suggest the possible existence of another taxon in this location.

When UV fluorescence/reflectance was examined, one area of the wings was found to be intensely fluorescent in some specimens (Fig. 7). Male specimens of *P. australis* and *P. zambezi* **sp.n.** (regions 5 and 6; Fig. 7A, B, respectively) have strong UV reflectance/fluorescence in a streak surrounding the proximal half of the anal vein of the forewing. Females (Fig. 7D) do not; nor do any *paradoxa*-group specimens (e.g. Fig. 7C). Viewed in a light microscope, this area of the wing has a dense array of overlapping scales in both UV-reflecting and non-reflecting wings, in contrast to the orderly array of apparently non-overlapping scales in other areas of the wing (not shown). The ultrastructure of the scales was not examined.

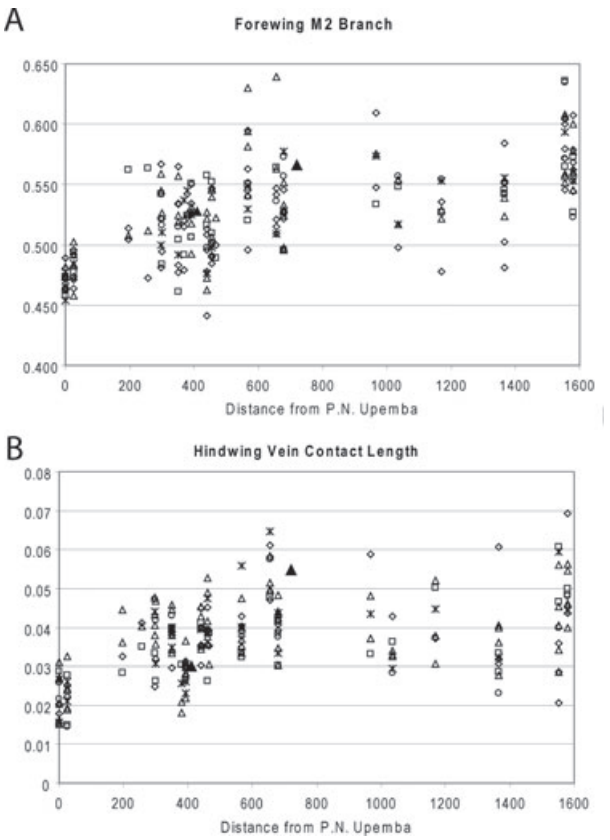

Fig. 6. Wing vein character values for individual specimens of *australis*-group taxa as a function of distance in km from Upemba National Park. A, forewing vein M2 node (length from base of wing, as fraction of forewing length); B, hindwing costal-subcostal vein near-contact length, as fraction of hindwing length. Measurements for the *Pseudopontia australis* lectotype and the *Pseudopontia zambezi* **sp.n.** holotype are shown as larger, filled triangles at 720 and 390 km, respectively. Symbols for some specimens overlap or coincide. In order to improve clarity, some symbols are displaced slightly along the horizontal axis. Number of specimens: 212, including 38 recent, 161 from the Royal Museum for Central Africa, four from African Butterfly Research Institute, two from the Hope Museum and seven from the U.S. National Museum of Natural History. Twenty-nine specimens were collected from Upemba National Park, 83 were from 200–500 km away and 100 were from 550–1600 km away.

DNA sequence data: nuclear genes

Segments of *CAD*, *DDC* and *wingless* totalling 3648 bp were sequenced from between one and three exemplars from each of the six regions. Each gene was first analysed separately to examine whether there was strong conflicting signal among genes. The three resulting ML trees were not identical, but did not strongly conflict because different parts of each were unsupported or poorly supported in bootstrap analysis (Fig. 8A). Single-gene ML trees for *CAD* and *wingless* each separately resulted in a monophyletic *paradoxa* species group, with 81 and 99% bootstrap support, respectively, and also showed moderate-to-high (76–99%) bootstrap support within the species group. The two *australis*-group species, *P. australis* and *P. zambezi* **sp.n.**, did not form a clade in the single-gene trees for *CAD* and *wingless*, and were separated by very short internal branches with low bootstrap support. The arrangement of terminal branches among the *paradoxa*-group taxa differed somewhat, although *P. paradoxa* regions 2 and 3 were more derived sister groups in both trees. In contrast, in the ML tree for DDC, the *australis* species group was monophyletic, with moderate bootstrap support (78%), whereas the *paradoxa* species group was paraphyletic, with very short internal branches and low bootstrap support.

Nuclear gene sequence data for duplicate specimens within each region or taxon differed by 0 (in most cases) to 0.1% of nucleotides (one instance; Table S2). Pairwise distances

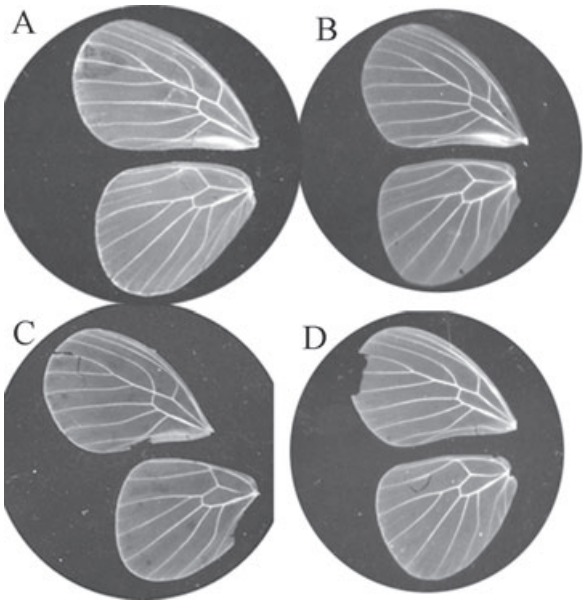

Fig. 7. *Pseudopontia* wings viewed in near-ultraviolet light. A, *Pseudopontia australis* (region 5), ♂; B, *Pseudopontia zambezi* **sp.n.** (region 6), ♂; C, *Pseudopontia mabira* **sp.n.** (region 4), ♂; D, *Pseudopontia zambezi* **sp.n.** (region 6), ♀.

between populations were between four- and 12-fold smaller than for *COI* sequences.

Because the individual gene trees showed no signs of strong conflict, the three nuclear gene sequences were concatenated into a single dataset. The ML tree for the three-gene concatenated dataset (Fig. 8B) demonstrates strong divergence between *australis*- and *paradoxa*-group lineages, and smaller but clear divergence among the six geographic regions, with bootstrap support between 88 and 100% for most nodes. The topology of this tree is somewhat different from that for *COI* sequences: the *australis* clade is not recovered as monophyletic, as in two of the individual gene trees (Fig. 8A). Regions 2 and 3 are sister groups, as in the *COI* tree. Bootstrap support for relationships among *paradoxa* taxa is strong in this tree (91–100%), in contrast to the lack of support for these nodes in the *COI* data (Fig. 3).

As there was little conflict between the *COI* and the concatenated nuclear gene trees because of low bootstrap support in different parts of each, the *COI* and nuclear gene sequences were concatenated into a four-gene dataset. The resulting ML tree (Fig. 6C) was again poorly supported at one of two intermediate nodes in the *paradoxa* lineage, as in the *COI* tree (Fig. 3), but monophyly of both the *paradoxa*- and *australis*-group clades were strongly supported (bootstraps of 99–100%), as found for *COI* alone. There was high support (100%) for the node linking regions 2 and 3 as sister groups, as found with the three-gene dataset.

Translation of *COI* and nuclear gene sequences showed that some of the variation among *Pseudopontia* taxa is non-synonymous. For comparison, there are 11 fixed amino acid residues in the *COI* of all *Pseudopontia* specimens that distinguish them from six other Pieridae of various subfamilies. As summarized in Table S3, there are eight fixed amino acid differences between all *paradoxa*-group species and the two *australis*-group species, three fixed amino acid changes that distinguish *P. australis* specimens (region 5) from *P. zambezi* **sp.n.** (region 6), one amino acid position with a fixed, unique substitution in region 3, and two fixed substitutions in *P. gola* **sp.n.** (region 1). Similarly, there were several fixed amino acid differences among the regions in nuclear genes, also summarized in Table S3.

Amplified fragment length polymorphisms (AFLPs)

The most inclusive form of genetic analysis in this study used AFLP data, which samples parts of the sequences of many unlinked genes scattered throughout the genome. Fifty-one specimens were analysed for 217 AFLP loci, and the results were subjected to two different kinds of analysis. First, phylogenetic analysis using the distance criterion resulted in six clades by geographic region, with all three specimens from locality 5g and the one *australis*-group specimen from locality 3g being included in the region-5 clade (Fig. 9A). The two *australis*-group populations (regions 5/5g and 6) are strongly divergent from each other and from all of the *paradoxa*-group populations. Bootstrap support for the main nodes is moderate to high (75–100%) in distance analysis, indicating distinct populations from each broad region. The same major branches were obtained in the parsimony strict consensus tree (not shown). The two specimens from locality 2n (western Nigeria) are joined very weakly with the region-2 clade in the distance analysis (Fig. 9A), but not in the parsimony analysis. The large number of loci unique to locality 2n is reflected in the long branch length; the size of this divergence suggests that the Gambari forest may have housed a separate population in 1969, which no longer exists there.

The six-region AFLP tree (Fig. 9A) was not very informative concerning relationships among the *paradoxa*-species group, as there was only weak bootstrap support for the intermediate branches. However, this was found to be an artifact because of the 'sharing' of the absence of many bands that are found in the *australis*-group specimens but not in any of the *paradoxa*-group specimens. When the data for regions 1–4 were analysed again after excluding the bands present only in the *australis*-group specimens (162 bands instead of 217), bootstrap support for relationships among populations 1–4 was high (86–99%; Fig. 9B), with regions 2 and 3 as sister groups with 86% bootstrap support. In this tree, locality 2n is part of the region-2 clade with 83% bootstrap support, whereas region 1 (*P. gola* **sp.n.**) is the most ancestral of the *paradoxa*-group populations, as in the *COI* tree and the six-region tree (Fig. 9A), with 99% support.

Pairwise genetic distances between regions for the six-region AFLP dataset (Table S4A) show that genetic distances among regions 1–3 are somewhat smaller than among regions 4–6, or between the latter regions and regions 1–3. This disparity is considerably less evident in the four-region dataset (Table S4B).

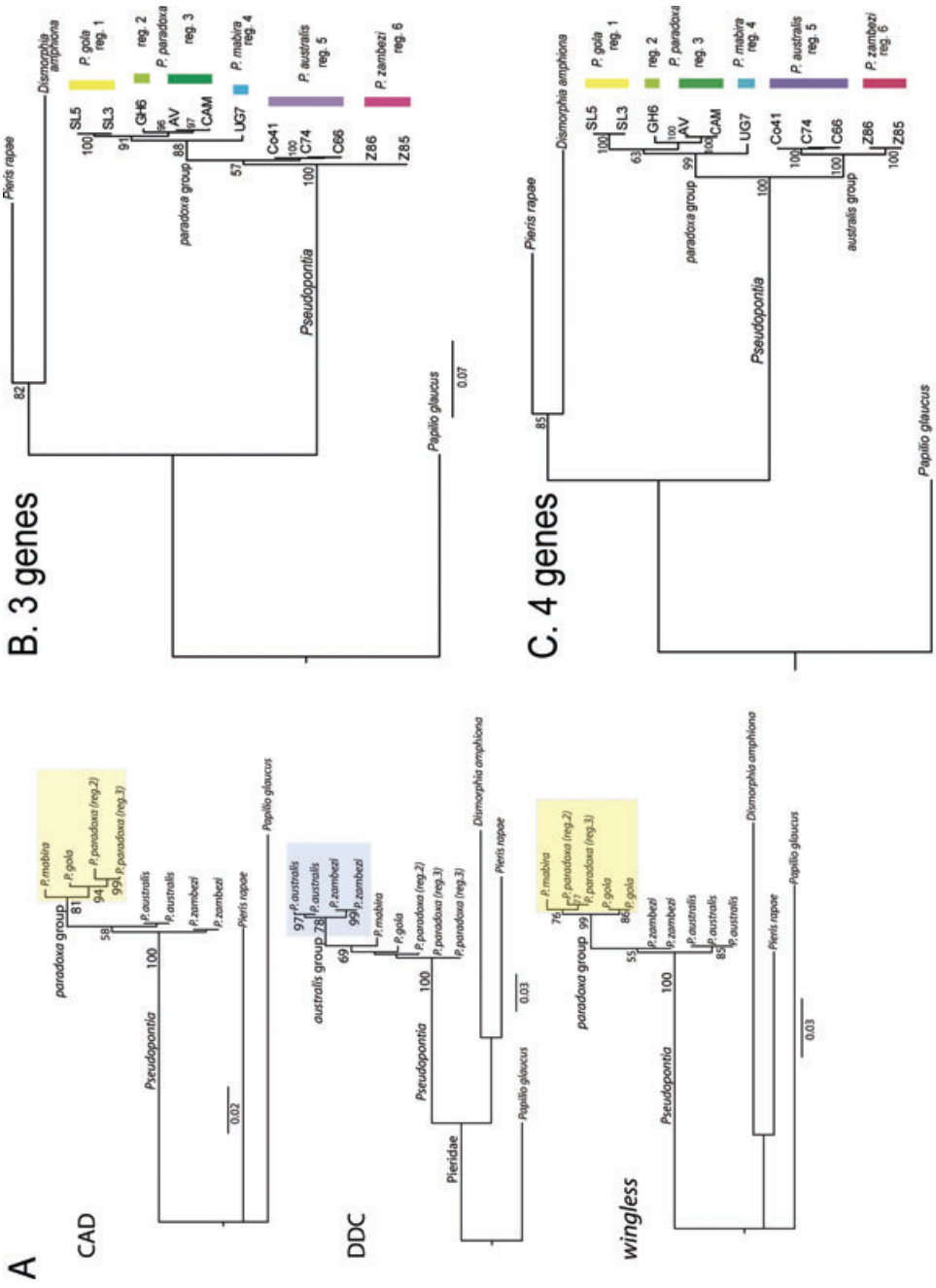

Fig. 8. Maximum likelihood trees for nuclear genes. A, individual gene trees with bootstrap values (percentages of 200 pseudoreplicates). B, three nuclear gene sequences concatenated. C, *cytochrome oxidase I* (*COI*) plus three nuclear gene sequences concatenated. The tip labels in (B) and (C) refer to DNA solutions in Table 1. Branch lengths are proportional to distances; bootstrap values for major nodes are shown [percentage of 1000 pseudoreplicates in (B) and (C)]. In nuclear gene sequences, there were 3647 total nucleotide sites, of which 3510 were constant, 53 were uninformative and 84 were parsimony informative (excluding out-groups).

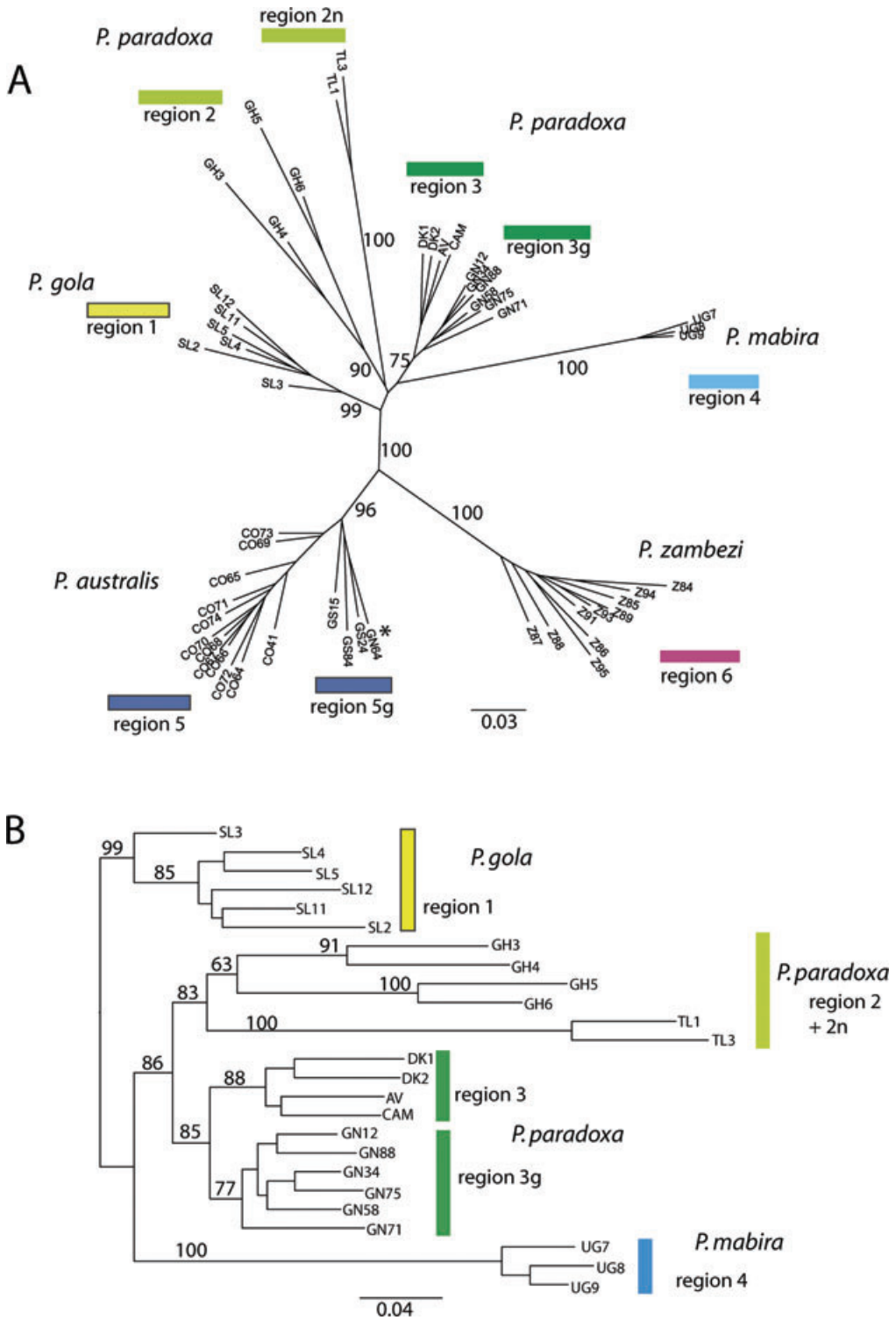

Fig. 9. Distance analyses of amplified fragment length polymorphism (AFLP) data (minimum evolution, single best tree, unrooted). A, data for six populations with bootstrap values (percentage of 1000 pseudoreplicates) for major nodes. Branch lengths are proportional to distances. In parsimony analysis, the strict consensus tree had the same topology of the main nodes. Tip labels refer to DNA solutions in Table 1. Asterisk marks data for the *Pseudopontia australis* specimen collected in region 3g. B, analysis of data for four *paradoxa*-group populations only, excluding bands found only in *australis*-group populations, with bootstrap values (percentages of 1000 pseudoreplicates) for major nodes.

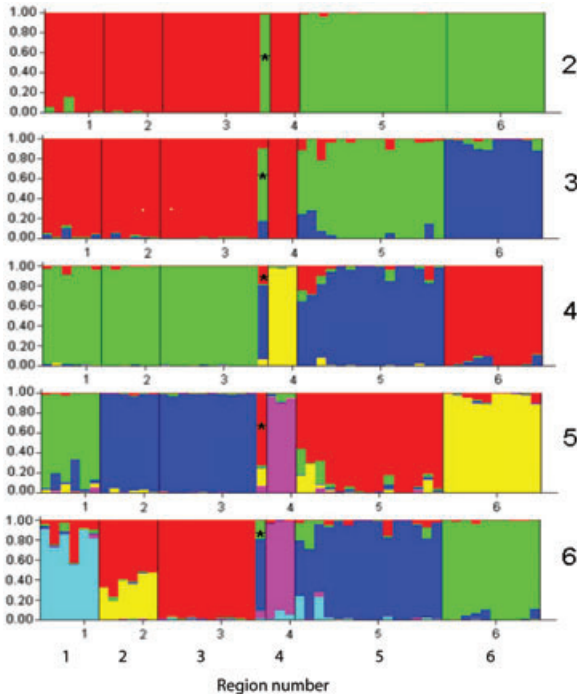

Fig. 10. Structure analysis of amplified fragment length polymorphism (AFLP) data. K values, which represent the putative number of different populations posited in each analysis, are shown on the right. Colours are assigned arbitrarily and represent putative populations of origin. Values on the x-axis are the regions from which specimens were collected. The scale on the y-axis represents the proportion of each individual's genetic variation assigned to each ancestral population. *Data for specimen GN64, a *Pseudopontia australis* specimen found among *Pseudopontia paradoxa* in the same region (3g). Numbers of individuals analysed in each region are: region 1, six; region 2 (including 2n), six; region 3 (including 3g), 11; region 4, three; region 5 (including 5g), 15; region 6, ten. At $K = 2$, ln likelihood is −3382; at $K = 3$, −2795; at $K = 4$, −2567; at $K = 5$, −2334; at $K = 6$, −2142; at $K = 7$ (not shown), −2278.

Each of the six regions and subregion 2n had many high-frequency AFLP bands (Table S5), as well as several high-frequency private bands (not found elsewhere). Except for region 3, each region had some private bands that appeared to be fixed, occurring in all specimens from that region only. In addition, there were 13 bands occurring at high frequency in multiple populations of each larger clade, but not the other. However, this number of common bands was considerably less in every case than the total number of high-frequency private bands in each region, which averaged 34 (range: 22–51; Table S5).

Analyses of AFLP data using STRUCTURE resulted in the highest posterior probability for six populations (Fig. 10), each of which was consistent with and predicted by geographic location, with the exception of one specimen. The *australis*-group specimen collected in locality 3g (Fig. 10, asterisk) was always grouped with *australis* region 5/5g (having the same predominant colour in the figure), in agreement with the phylogenetic results shown in Fig. 9. At $K = 2$, the two *australis*-group populations (regions 5 and 6) were identified as different from the *paradoxa*-group populations (regions 1–4); at $K = 3$, the two *australis*-group populations also differed from each other; at $K = 4$, region 4 was identified as differing from the other *paradoxa*-group populations; at $K = 5$, region 1 also differed; and at $K = 6$, each of the six regions was identified as housing a separate population, although region 2 had considerable admixture with the ancestral population of region 3 (but not vice versa).

Systematic account

We propose three new species and elevate the subspecies *australis* to species rank.

Pseudopontia Plötz

Pseudopontia Plötz, 1870: 348.
Type species Pseudopontia calabarica Plötz, 1870. By monotypy.
(Plea for addition to Official List of Generic Names in Zoology; see Hemming, 1965: 104.)

Globiceps Felder & Felder,1869.
Junior homonym of *Globiceps* Lepeletier & Serville, 1825 (Heteroptera).
Type species Globiceps paradoxa Felder & Felder, 1869. By monotypy.

Gonophlebia Felder, 1870: 95.
Type species Globiceps paradoxa Felder & Felder, 1869. By monotypy.
Junior subjective synonym of *Pseudopontia* Plötz, 1870. Formally synonymised by Kirby (1871), as already implied by Hewitson (1870).
(Plea for suppression, see Hemming, 1964: 174; Hemming, 1965: 104; Hemming, 1967: 384; for settlement see Cowan, 1983: 41.)

***Pseudopontia paradoxa* (Felder & Felder, 1869)**
(Figs 2A; 4A)

'*Globiceps Paradoxa*' Felder & Felder, 1869.
(Plea for addition to Official List of Specific Names in Zoology, see Hemming, 1965: 104.)

Pseudopontia calabarica Plötz, 1870.
'*Pseudopontia Calabarica* n. gen. et n. sp.' Plötz, 1870: 348, pl. 2, fig. 1a–f
Junior subjective synonym of *P. paradoxa.* Formally synonymised by Kirby (1871), as already implied by Hewitson (1870).
Type locality NIGERIA: 'Old Calabar, Guinea'.
Type material Holotype, (not examined) in Pogge Collection, Greifswald University, Germany; illustrations in Plötz (1870, pl. 2, fig. 1a–f) exactly resemble the holotype of *P. paradoxa*.

Pseudopontia cepheus [*sic*] Ehrmann, 1894
'*Pseudopontia cepheus, sp. nov*'. Ehrmann, 1894: 77 (not illustrated).

Type locality LIBERIA: 'Grand Sess, West Africa'.
This is a misnomer: the description includes black markings on the wing margins, and therefore it is not *Pseudopontia* (see also discussion in Dixey, 1923: lxiii).

Type Holotype ♂, NIGERIA, Calabar, no date (Felder) (BMNH). Labels (4): 'BMNH # 135705', 'paradoxa n.', 'Old Calabar', 'Felder Colln' (examined).

Other material examined. CAMEROON: one specimen, Mount Etinde, January 1992 (G. Hormiga) (UM-SI); one ♀, NV Edea, March 2008 (Collins) (UM-SI); four ♀, three ♂, 'Mukonje near Kumba', 1 August–1 October 1930 (Rosenfeld) (RMCA); one ♂, one ♀, 'Mbio–Mamfe', 1 June 1977 (Ph. Darge) (RMCA); one ♂, 'Johann-Albrechts-Hohe', 1898 (L. Conradt) (RMCA); one ♂, Yaounde, 6 December 1938 (L. Berger) (RMCA); one ♂, Akoefin, 1914 (S.G. Tessmann) (RMCA); four specimens, Lolodorf, October–December 1922 (A.I. Good) (CMNH); one specimen, Lolodorf, November 1918 (J.A. Reis Jr) (CMNH); # 4366, Lolodorf, March 1911 (A.I. Good) (CMNH). COTE D'IVÔIRE: one ♀, Yapo Forest, January 1999 (Warren-Gash) (UM-SI); one specimen, Alepe Forest, January 2000 (Warren-Gash) (UM-SI). EQUATORIAL GUINEA: one ♂, Nkolentangan, 8 January 1906 (S.G. Tessmann) (RMCA); one ♀, Benitogebiet, 6 March 1906 (S.G. Tessmann) (RMCA); one ♀, Makomo Campogebiet, 1–17 February 1906 (S.G. Tessmann) (RMCA); one ♀, Makomo Mtungebiet, 27 April 1906 (S.G. Tessmann) (RMCA); one specimen, Monte Bata, 9 April 1965 (no collector) (USNM). GABON: one ♂, one ♀, Waka National Park, November 2005 (Vande Weghe) (UM-SI); one specimen, Waka National Park, March 2007 (Vande Weghe) (UM-SI); four specimens, Waka National Park, December 2007–March 2008 (Vande Weghe) (UM-SI).

GHANA: one specimen, Ankasa National Park, 4 November 2008 (Larsen) (UM-SI); one ♀, Ankasa National Park, November 2008 (Sáfián) (UM-SI). NIGERIA: one ♂, Cross River, November 2004 (Knoop) (UM-SI); one ♀, Oban Hills, November 2004 (Knoop) (UM-SI).

Redescription (adapted from Clench, 1955; Ackery *et al*., 1998). Forewing length 22–28 mm. Wings rounded, unmarked, translucent, nearly white, slightly greenish. Body white, eyes green, legs green. Antennae unisulcate. Most wing scales small, highly specialized, bifid, non-overlapping. Forewing has a dense area of overlapping scales along the basal one-third of the anal vein (not ultraviolet-reflective). Forewing veins M1 and M2 stalked with Rs2 + 3 + 4. Forewing vein Rs1 emerges from the discal cell slightly proximal to or at its distal end. Hindwing vein Sc + R secondarily fused with Rs before middle of the wing. Hindwing vein M2 stalked with M1. Lamella of metadiscrimen runs straight into the furca. Hind tibia without spurs.

Genitalia. No differences from *P. australis* (Dixey, 1923): harpes fused ventrally; uncus two-lobed.

Distribution. Wet forests in Cameroon, Cote d'Ivoire, Equatorial Guinea, west-central Gabon (Waka National Park), western Ghana, Nigeria.

***Pseudopontia mabira* Mitter & Collins sp.n.**
(Figs 2B; 4C; 7C)

Types. Holotype ♂, UGANDA: Mabira Forest, 0.3848°N, 33.01513°E, 1290 m a.s.l, 5 July 2009 (P.R.F. Ward) (ABRI). Label (1): 'UGANDA Bulkwe Rd, Mabira Forest, 00°23.088′N, 033°00.908′E, 5 July 2009, P.R.F. Ward'. Paratypes, UGANDA: six specimens, Mabira Forest, March–July 2009 (P.R.F. Ward) (ABRI); one ♀, two ♂, Mabira Forest, 28 February 2008, 19 June 2009 and 5 July 2009 *(Collins)* (UM-SI); one specimen, Budongo Forest, iv.1954 *(P. Barton-Eckett)* (CMNH); one specimen, Jackson Kibale Forest, ii.1951 *(P. Barton-Eckett)* (CMNH); 2 ♂, Msisi, no dates *(Bayer)* (RMCA); 2 ♂, 1 ♀, Unyoro-Budongo, iv.1958, *(T.H.E. Jackson)* (RMCA); 3♂, 'Semliki District, Buamba valley', 21–27.vii.1921 *(Carpenter)* (HMO); two specimens, Ankole, viii.1946 *(T.H. Jackson)* (HMO).

Diagnosis. Differs from *P. paradoxa* in the following: stalk joining hindwing veins M1 and M2 is usually shorter in *P. mabira sp.n.* Forewing length is smaller than *P. paradoxa*. Also differs in nucleotide sequences of COI and nuclear genes *wingless* and CAD, and in AFLP data.

Differs from *P. australis* and *P. zambezi sp.n.* in the following: secondary fusion of hindwing veins Sc + R with Rs, which is not found in *P. australis* and *P. zambezi sp.n.*; in males, forewing scales surrounding part of anal vein are not UV-reflective; as well as in COI sequence ('barcode'), nucleotide sequences of *wingless*, DDC, and CAD, and in AFLP data.

Etymology. The specific epithet refers to the Mabira forest reserve in Uganda. The specific name is a noun in apposition.

Description (adapted from Clench, 1955; Ackery *et al.,* 1998). Forewing length 18–23 mm. Wings rounded, unmarked, translucent, nearly-white, slightly greenish. Body white, eyes green, legs green. Antennae unisulcate. Most of wing-scales highly specialized, bifid, non-overlapping. There is a dense area of overlapping wing scales along the anal vein of the forewing. Wing venation similar to that of *P. paradoxa*, with forewing veins M1 and M2 stalked with Rs2 + 3 + 4. Forewing vein Rs1 emerges from the discal cell slightly proximal to or at its distal end. Hindwing vein Sc + R secondarily fused with Rs before middle of the wing. Hindwing vein M2 in a short stalk with M1. Hind tibia without spurs.

Genitalia. No differences from *P. australis* (Dixey, 1923): harpes fused ventrally; uncus bilobed.

Distribution. DRC north of the equator, lowlands of DRC south of the equator, and Uganda.

***Pseudopontia gola* Sáfián & Mitter sp.n.**
(Fig. 4B)

Types. Holotype ♂, SIERRA LEONE, Eastern Province, Gola North Forest Reserve, 7.6047°N, 11.0392°W, 26 March 2009 (Sáfián) (UM-SI). Preserved as mounted wings and frozen tissue #SS-08-SL11. Paratypes, LIBERIA: two specimens, Mount Coffee, 31 March 1897 and 13 April 1897 (R.P.Currie) (USNM); one specimen, Cape Mount, 1953 ('CCB & JM') (USNM); three specimens, Holland Collection, no dates, no collectors (CMNH); one ♂, no date, no collector (RMCA); two ♂, two ♀, Kaouyeke, 21 March 1948–1 April 1948 (B. Holas) (RMCA). SIERRA LEONE: one ♀, one ♂, and four additional specimens, Gola Forest, November–December 2008 (Sáfián, ABRI) (UM-SI); one ♀, no date (E.T. Owen) (USNM); three ♂, no dates, no collectors (RMCA).

Diagnosis. Differs from *P. paradoxa* only in nucleotide sequences of *COI* and nuclear genes *wingless* and *CAD*, and in AFLP data. No morphological differences have been detected. Forewing length 22–25 mm.

Differs from *P. mabira* **sp.n.** in larger size, and in that stalk joining hindwing veins M1 and M2 is usually shorter in *P. mabira* **sp.n.** Differs also in nucleotide sequences of *COI* and nuclear genes *wingless* and *CAD*, and in AFLP data.

Differs from *P. australis* and *P. zambezi* **sp.n.** in the following: secondary fusion of hindwing veins Sc + R with Rs, which is not found in *P. australis* and *P. zambezi* **sp.n.**; in males, forewing scales surrounding part of anal vein are not UV-reflective, as well as in nucleotide sequences of *COI*, *wingless*, *DDC* and *CAD*, and in AFLP data.

Etymology. The specific epithet refers to the Gola Forest Reserve of the type locality. The specific name is a noun in apposition.

Distribution. Rainforests of Sierra Leone and Liberia.

***Pseudopontia australis* Dixey, 1923, stat.n.**
(Figs 2C, 4D, 7A)

'*Pseudopontia paradoxa australis* subsp. nov.' Dixey, 1923: lxi–lxvii, pl. B, fig. 6.

Types. Lectotype ♂, here designated: DEMOCRATIC REPUBLIC OF CONGO: Kasai Province, Luebo, prior to 1906 (Paul Landbeck) (HMO). Labels (3): 'Congo State, SW of, 1,345 ft, Kassai, R. Luebo District, Forest, Paul Landbeck, coll. pres.1906 Zool Mus Tring', '1906–3328', 'Selected as Lectotype, T.B. Larsen det. 2009'. Paralectotypes, nine specimens of both sexes, with identical labels: DEMOCRATIC REPUBLIC OF CONGO: Kasai Province, Luebo, prior to 1906 (Paul Landbeck) (HMO). Labels (2): 'Congo State, SW of, 1,345 ft, Kassai, R. Luebo District, Forest, Paul Landbeck, coll. pres.1906 Zool Mus Tring', '1906–3328'.

Other material examined. DEMOCRATIC REPUBLIC OF CONGO: one ♂, Bas-Congo, Mayumbe forest, 17–28 May 2007 (Kawahara) (UM-SI); eight ♂, nine ♀, Bas-Congo, Mayumbe, 26 November 2008 (De Prins & De Prins) (UM-SI); one ♀, Bas-Congo, Mayumbe forest, 25 February 1908 (Waelbroeck) (RMCA); one ♀, Bas-Congo, Mayumbe forest, 16 January 1918, (R. Mayné) (RMCA); seven specimens, Bas-Congo, Mayumbe, 17–28 May 2007 (Kawahara) (McGuire Center for Lepidoptera and Biodiversity, Robert F. Denno collection). GABON: two ♂, one ♀, Batéké plateau, April–May 2007 (Vande Weghe) (UM-SI).

Diagnosis. 'Differs from typical *P. paradoxa* . . . only in the fact that the costal and subcostal veins in the hind-wing are separated by a distinct interval, running parallel with each other for a short distance, but never joining' (Dixey, 1923, p. lxiv). Also differs from *P. paradoxa* in the following: forewing length is slightly smaller (20–25 mm); forewing vein M2 emerges from the common stalk two-thirds to three-fourths of the way from the end of the cell to the M1 branch, rather than at most halfway between those points; forewing vein Rs1 is stalked for 0.5–3 mm with Rs2 + 3 + 4 + M1 + M2, rather than emerging from the discal cell proximal to or at its distal end. In males only, the dense area of overlapping wing scales along the anal vein of the forewing is ultraviolet-reflective.

Differs from *P. mabira* **sp.n.** and *P. gola* **sp.n.** in all of the same characters except size; *P. mabira* **sp.n.** is slightly smaller than *P. australis*.

Differs from *P. zambezi* **sp.n.**, which has a shorter length of near contact (<0.8 mm) between costal and subcostal hindwing veins, and usually does not have forewing vein Rs1 stalked together with M1 + M2 + Rs2 + 3 + 4. In *P. zambezi* **sp.n.**, M2 vein emerges from the common stalk halfway between the end of the discal cell and the M1 branch.

Also differs from each of the other species in nucleotide sequences of *COI* and nuclear genes *wingless*, *DDC* and *CAD*, and in AFLP data.

Genitalia illustrated and described in Dixey, 1923; lxvi–lxvii, pl. B, Figs 1–4.

Distribution. Central Africa south of the equator, at elevations less than 800 m; DRC, Republic of Congo (Brazzaville) and eastern Gabon (Mayumbe forest and Batéké plateau).

***Pseudopontia zambezi* Mitter & W. De Prins sp.n.**
(Figs 2D, 4E, 7B, 7D)

forma ***siccana*** Pinhey, 1962: 885 (not illustrated). (As dry season form of *Pseudopontia paradoxa australis*; infrasubspecific name not available.)

Types. Holotype ♂, ZAMBIA: Northwest Province, Mwinilunga district, Ikelenge, Isombu stream, 11.11°S, 24.267°E, 1260 m a.s.l., 2 October 1973 (A. Heath) (ABRI). Labels (2): '2:X:1973, Isombu, Ikelenge, N.W.Prov', 'A. Heath Collection'. Paratypes: DEMOCRATIC REPUBLIC OF CONGO:

two ♂, four ♀, Katanga Province, Musokatanda, 10.9°S, 25.1°E, 1350 m a.s.l., 10 May 1963 and June–August 1965 (V. Allard) (USNM). ZAMBIA: ten ♂, three ♀, Ikelenge, Zambezi Bridge, 11.233°S, 24.267°E, 1300 m a.s.l., January 2000 (Collins) (UM-SI); one specimen, as previous (Museum of Comparative Zoology, Harvard University); two specimens, Northwest Province, Ikelenge, Isombu, 12 June 1974 and 2 October 1973, (A. Heath) (ABRI); one specimen, as previous, 12 October 2002 (‘*TCEC/SCC*’) (ABRI); one specimen, Mwinilunga, 11.72°S, 24.42°E, 1300 m a.s.l., 28 April 1963 (V. Allard) (USNM).

We have not examined the two specimens in Zimbabwe National Museum, Bulawayo.

Diagnosis. Differs from *P. paradoxa*, *P. gola* **sp.n.** and *P. mabira* **sp.n.** in that hindwing vein Rs nearly touches but does not fuse with Sc + R before middle of the wing, then separates; also, in males the dense area of wing scales along the basal one-third of the anal vein of the forewing is ultraviolet-reflective. Differs from *P. australis* in the following: very short near contact (<0.8 mm) between Sc + R (costal) and Rs (subcostal) hindwing veins; more proximal forewing M2 vein branch, which lies at most halfway between the M1 branch and the end of the cell in *P. zambezi* **sp.n.**; forewing vein Rs1 emerges from the discal cell at the end of the cell or only slightly beyond it in a very short stalk with M1 + M2 + Rs2 + 3 + 4. Forewing length 2 mm smaller on average.

Differs from all four other species in nucleotide sequences of *COI* and nuclear genes *wingless*, *DDC* and *CAD*, and in AFLP data.

Etymology. The specific epithet refers to the Zambezi River, the headwaters of which arise near the type locality. The specific name is a noun in apposition.

Description (adapted from Clench, 1955; Ackery *et al*., 1998). Forewing length 19–23 mm. Wings rounded, unmarked, translucent, nearly white, slightly greenish. Body white, eyes green, legs green. Antennae unisulcate. Most wing-scales highly specialized, small, bifid, non-overlapping. A dense area of overlapping scales on the forewing surrounds the basal one-third of the anal vein; these are ultraviolet-reflecting in males. Forewing veins similar to those of *P. paradoxa*, with M1 and M2 stalked with Rs2 + 3 + 4. M2 emerges from the common stalk less than halfway from the discal cell to the M1 branch. Forewing vein Rs1 emerges from the discal cell at the distal end of the cell or in a very short stalk with M1 + M2 + Rs2 + 3 + 4. Hindwing vein Rs very briefly runs near to but does not fuse with Sc + R before middle of the wing. Hindwing vein M2 stalked with M1. Hind tibia without spurs. Genitalia: no differences from *P. australis* (Dixey, 1923): harpes fused ventrally; uncus two-lobed.

Distribution. Central Africa south of the equator; most common at elevations over 800 m; Angola, southern DRC, Zambia.

Discussion

Summary of evidence for multiple species of Pseudopontia

In the present study, *COI* sequences are deeply divergent between specimens identified traditionally as *P. paradoxa* Felder & Felder and *P. paradoxa australis* Dixey. By itself, this would suggest that each subspecies may represent either a species or a species group, despite differing by only one previously reported morphological character (Dixey, 1923). Here, we describe another structural difference, an area of ultraviolet-reflecting scales on the forewing of males of the *australis* species group. Somewhat smaller but still considerable divergences in *COI* sequences among geographic areas, ranging between 3 and 8% of nucleotide positions, identify six populations within the two subspecies or, as here defined, the two species groups. As is summarized in Table 3, this study provides two additional kinds of genetic data, including multi-locus AFLP data and slowly evolving nuclear gene sequences, which separate *Pseudopontia* into six genetically monophyletic populations.

Taken together, the data indicate that subspecies *P. paradoxa australis* merits elevation to full species rank as *P. australis* Dixey. In addition, two new species, *P. mabira* **sp.n.** and *P. zambezi* **sp.n.**, are proposed on the basis of marked genetic differentiation from *P. paradoxa* and *P. australis*, respectively, combined with small but recognizable differences in wing vein stalk lengths and apparent sympatry or parapatry of each with *P. australis* in the Congo River basin. The two new species also differ from their congeners in their ecology, that is, in their toleration of less humid climates (and lower oxygen pressure) at relatively high elevations (800–1400 m) and the resulting intermittent forest cover, which is found along river courses in the central African plateau or in scattered valleys in Uganda. *Pseudopontia mabira* **sp.n.** is smaller in overall size than *P. paradoxa*, and female wing size is an important determinant of male mating preference in some other Pieridae (Silberglied & Taylor, 1978). A third new species, *P. gola* **sp.n.** is proposed solely on the basis of marked genetic divergence from the other species, together with the shape of the phylogenetic trees for multiple genetic markers, which would make *P. paradoxa* paraphyletic if *P. gola* **sp.n.** were included within it (Figs 3, 9). Two populations of *P. paradoxa* are left as subpopulations in West Africa, as these are sister groups that are less distinct based on the genetic data used in this study.

Variations in wing structure of *Pseudopontia* have long been held to be uninformative of population structure, possibly because of sympatry of several species with only slightly different morphology in some localities. This is true, especially in central areas of DRC where at least two (*P. australis* and *P. mabira* **sp.n.**), probably three (including *P. zambezi* **sp.n.**), and possibly four different taxa may coexist (if the form in P.N. Upemba can be shown to be a distinct taxon). We note that the type locality for *P. australis* Dixey lies in the lowlands of central DRC (approximate coordinates 5.3°S, 21.3°E, elevation 410 m) (Dixey, 1923). The known occurrence in nearby areas (Fig. 1) and possible occurrence at the type

Table 3. Summary of evidence for species distinctions.

	Genetic evidence			Wing morphology			Sympatry	
Region	COI divergence, monophyly	Nuclear gene divergence	AFLP monophyly, private bands	Costal-subcostal HW vein fusion	Metric variations from type	Ultraviolet reflectant scales on male forewing	Co-occurs with another taxon	Taxon names
paradoxa species group								
1	Yes	Yes	Yes	Yes	No	No	??	*Pseudopontia gola* **sp.n.**
2	Yes	Yes	Yes	Yes	No	No	—	*Pseudopontia paradoxa* Felder & Felder
3	Yes	Yes	Yes	Yes	(Type)	No	Yes	*Pseudopontia paradoxa* Felder & Felder
4	Yes	Yes	Yes	Yes	Yes	No	Yes	*Pseudopontia mabira* **sp.n.**
australis species group								
5	Yes	Yes	Yes	No	Yes	Yes	Yes	*Pseudopontia australis* Dixey
6	Yes	Yes	Yes	No	No	Yes	??	*Pseudopontia zambezi* **sp.n.**

locality of the *paradoxa*-group species *P. mabira* **sp.n.** and of *P. zambezi* **sp.n.**, both of which are distinguished most readily from *P. australis* using modern genetic tools, may have been factors that persuaded Dixey (1923) to name *P. australis* as a subspecies rather than a new species.

Furthermore, we note that one specimen of *P. australis*, identified by hindwing vein morphology (costal/subcostal veins not fused) as well as *COI* sequence and AFLP data, was found among several *P. paradoxa* in central Gabon (region 3g), again suggesting the sympatry of two taxa.

Stability of population divergences across multiple collections and up to 140 years of time is shown by the agreement between recent and museum specimens in the morphological differences cited in the present study (Fig. 5). The stability of the genetic data is reflected in the monophyly of data (Figs 3, 9) from specimens collected in multiple years and/or places within each of five regions (Table 1; Fig. 1); only in region 6 were recent specimens collected at one time and place.

Sequencing studies

The three nuclear genes chosen for sequencing in the present study are known to evolve more slowly in Lepidoptera than *COI*, and have been used to study the phylogeny of Lepidopteran families and subfamilies (Regier *et al*., 2009). Maximum divergence among taxa (regions) in nuclear gene sequences (summed for the three genes) was 4.7–12-fold less than that in *COI* (Tables S1, S2), which is comparable with the 12-fold difference between *COI* and *EF1-α* divergence rates reported elsewhere for genera of Pieridae (Braby *et al*., 2006). Nevertheless, sequences of the three nuclear gene coding regions demonstrate significant divergences among the six *Pseudopontia* populations, with high bootstrap support among the more divergent populations for each gene (Fig. 8A). The divergence in slowly evolving genes among populations within *Pseudopontia*, together with very little or no divergence among individuals from any single population, suggests that the specimens studied (and therefore the populations) do not belong to a single species.

Furthermore, the differing individual ML trees among the four genes (including *COI*) do not represent conflicting information, but rather different information. Each nuclear gene differs more among some of the species than among others, the latter of which have low bootstrap support and occupy more ancestral positions in the tree, implying that those sequences are less derived (note several very short, deep branches in Fig. 8A). Thus, for example, *CAD* and *wingless* sequences provide more phylogenetically useful information about relationships within the *paradoxa*-species group, but less about the *australis*-species group, whereas the reverse is true for *DDC*.

The lack of complete congruence in the branching pattern of the single-gene trees (Figs 3, 8A) is not unexpected, as gene lineages may differ among genes and, in particular, may differ from the true species lineage (e.g. Pamilo & Nei, 1988; Degnan & Rosenberg, 2006). This emphasizes the limited utility of using one or few genes for inferring the phylogeny and relationships of closely related taxa (Shaw, 2002; Galtier *et al*., 2009; Zakharov *et al*., 2009).

AFLP studies

Unlike sequences of one or a few genes, our AFLP data represent a random sampling of more than 200 independent,

most likely unlinked loci drawn from the entire, mainly nuclear genome (Vos *et al*., 1995). Our data clearly show a different 'fingerprint' for each of the geographic areas sampled (Figs 9, 10), although regions 2 and 3 in West Africa (both designated here as *P. paradoxa*) are related more closely to each other than to the other populations. The genetic distinctness of the six populations is reflected in the fact that many AFLP loci were present at high frequency within each population (Table S5), whereas a considerable number (81 total) were present at high frequency in only one of the six populations. Furthermore, a few bands were fixed, i.e. present in all the specimens studied from a particular region, in all regions except one. These may represent fixed alleles within regions.

Other studies of AFLP data in insects have demonstrated that some neighbouring populations (e.g. on different host plants) show evidence of extensive genetic exchange. This is instantly recognizable: phylogenetic trees constructed from such data show complete intermingling of tree branches, representing individuals of putative host-associated or geographic races, whereas STRUCTURE analyses show that all such specimens share mixed ancestry. In contrast, other populations or taxa in the same studies are shown to be monophyletic (e.g. Parsons & Shaw, 2001; Ahern *et al*., 2009). In the present study, the AFLP phylogeny concurs with phylogenies obtained using *COI* and nuclear gene sequences: all six populations are divergent and reciprocally monophyletic (Fig. 9A, B), although two populations are related more closely than the others. These data strongly support the hypothesis of five separate species of *Pseudopontia*.

Introgression

The two *australis*-group specimens with discordant AFLP and *COI* data may represent introgression of the mitochondrial gene from locality 6 (*P. zambezi* **sp.n.**) into locality 5g (***P. australis***), i.e. hybridization between individuals from the two populations that occurred at some time in the past. Introgression does not necessarily mean there is only one species involved, as the maintenance of extensive genetic differences between the populations, and of strong genotype similarities within each population, e.g. monophyly expressed in AFLP data (Fig. 9), indicates that selection is operating differently on the nuclear genome in the two populations (Shaw, 2002; Zakharov *et al*., 2009). In wild populations any measurable decrease in the fitness of hybrids is expected to result in the long-term maintenance of different genetic characters in each species, even if hybridization may occur, even repeatedly (e.g. Descimon & Mallet, 2009). Although our recent collecting localities 5g and 6 are separated geographically by over 1500 km, *australis*-group butterflies also occur at multiple intervening localities in the DRC, as is reflected in the RMCA museum collection (Fig. 1; Table 2). Those almost certainly include individuals of both *P. australis* and *P. zambezi* **sp.n.**; no recent specimens from the intervening areas were available for genetic studies to determine the frequency of introgression.

An alternative hypothesis, that an ancestral *COI* haplotype matching region 6 has persisted in some individuals in locality 5g, is possible, as regions 5/5g and 6 also share a few AFLP loci at high frequency (Table S5). However, even the two specimens with the *COI* sequences matching region 6 diverge from region 6 at many other AFLP loci (Figs 9, 10; Tables S4, S5).

Wing morphology suggests different geographic ranges for australis-group taxa

When morphological data are plotted against geographic location, discontinuities in the distribution of sizes of two wing vein characters are apparent (Fig. 6). The data suggest that *P. zambezi* **sp.n.** is common (and *P. australis* is not) in the southern highlands of DRC (with the possible exception of Upemba National Park, which may house a different, endemic species); *P. zambezi* **sp.n.** may also occur in lowland areas further north. Meanwhile, localities closer to the equator and at lower elevations are populated mainly but perhaps not exclusively by *P. australis* (and *P. mabira* **sp.n.**).

There are no obvious geographic barriers or sudden discontinuities of terrain between our collecting regions 5 and 6. However, the increasing distance from the equator combined with slowly increasing elevation is accompanied by lower annual rainfall (Le Houerou, 2008). As a result, forested areas in the southern parts of the DRC above 800 m elevation are considerably less extensive, although gallery forests along the rivers still supply suitable habitat for some rainforest-associated Lepidoptera. Although *P. australis* and *P. zambezi* **sp.n.** probably overlap in their distributions to some extent, a loosely defined and approximate boundary between the two ranges lies between 5.6°S and 6.2°S, at an elevation of about 800 m. This is slightly beyond the southern limit of the RMCA collections of *paradoxa*-group specimens (here proposed as *P. mabira* **sp.n.**); it is possible that all three taxa occur in some lowland areas.

Divergence times and climate changes

To assign tentative dates for the genetic divergences identified in the present work, rates of 1.25 or 0.76% of nucleotide substitutions in *COI* per lineage per million years may be used (Zakharov *et al*., 2004; Braby *et al*., 2006). Using these two rate estimates, we calculate that the divergence between *paradoxa*-group and *australis*-group lineages occurred either 12 or 18.7 Mya, using ML branch lengths (Table S1). Within the *P. paradoxa* species group, the divergence time of *P. mabira* **sp.n.** is calculated to be 3.8 or 6.3 Mya, and that of *P. gola* **sp.n.** is calculated to be 4.9 or 8.2 Mya, by ML branch lengths. We note that two separate multispecies radiations within Lepidoptera have been dated similarly, to 10 and 4 Mya (Megens *et al*., 2004; Kandul *et al*., 2004, respectively). Even shorter species-level divergences have been reported in other taxa: within 2–2.5 Mya in horned lizards (Leaché *et al*., 2009) and trap-door spiders (Bond & Stockman, 2008), and less than 0.5 Mya in crickets in

the Hawaiian Islands (Mendelson *et al*., 2004), from which divergence can be dated confidently because of the island orogenesis. Similarly, in *Pseudopontia*, the two most recent divergences (between *paradoxa*-group populations in regions 2 and 3 and between *P. australis* and *P. zambezi* **sp.n.**) fall within the Pleistocene at 1.2 or 2 Mya.

The slight morphological differences among taxa in the present study, despite considerable genetic diversity in the genes studied and in AFLP data, and despite the long-branch position of *Pseudopontia* in the phylogeny of Pieridae (Braby *et al*., 2006), may reflect limited diversity in morphological potential as a result of a previous bottleneck and/or massive extinction events, which may have been caused by the widespread loss of suitable habitat. In this regard, we note that during the Pleistocene era, global glacial maxima were accompanied by the marked drying of coastal (and probably continental) African climates, with the consequent severe losses of and fragmentation of rainforest areas (Leal, 2004). Other lengthy episodes of aridification occurred earlier, in the Tertiary period (Couvreur *et al*., 2008). Several of our collecting regions are centered near various wet-forest refugia postulated to have existed during the last glacial maximum, as well as during other maxima in more distant geological times (Douglass & Miller, 2003; Leal, 2004). The presence of several extant, geographically separated and genetically distinct populations of *Pseudopontia*, a genus found only in very humid habitats, may result from ancient and recurring rainforest fragmentation.

Our genetic data appear to reflect in part the vicariance effects of the Dahomey Gap (which lies between regions 2 and 2n), a 300–km section of the West African coast that currently has no rainforest because of limited rainfall (Fermon *et al*., 2001; Larsen, 2005), which has persisted since about 4000 years before the present (Vincens *et al*., 1999; Salzmann & Hoelzmann, 2005; Le Houerou, 2008), and probably existed for long intervals in the more ancient past, and also of the Niger River delta, which lies between regions 2n and 3 (Larsen, 2005; van Velzen *et al*., 2009; Larsen *et al*., 2010). The specimens collected in the Gambari forest in western Nigeria (locality 2n) are of special interest. Although this forest is located east of the Dahomey Gap, AFLP data relate these specimens most closely with those collected to the west of the gap (Figs 9B, 10). This and our failure to detect anatomic differences between specimens from regions 2 and 3 suggests relatively recent (and/or recurring) gene flow among these populations in West Africa, such as might have occurred during times of hyper-humid climates, when the coastal rainforest would have been continuous. The most recent such period occurred between 10 000 and 4000 years before the present (Le Houerou, 2008), and there may have been others during interglacial periods in the more distant past.

Biogeography

The agreement between the molecular and morphological data compares well with current ideas on the biogeography of African butterflies. Coastal Africa west of the Dahomey Gap constitutes a biogeographical subregion of its own, divided into a Liberian and a Ghanese subregion separated at the Bandama River in Cote d'Ivoire. This separation is mirrored in our molecular findings. Western Nigeria is a curious area, with elements from both west and east of the Dahomey Gap: here, the western Nigerian population is allied with the Ghanese subregion and more loosely with region 3 (these issues are discussed by Larsen, 2005).

Our region 3 covers eastern Nigeria, Cameroon, Gabon, the Central African Republic and northern parts of the DRC, and has many endemic butterflies, as well as species from both west, east and south. It is the richest in Africa, with as many as two-thirds of all African butterflies. Our region 4 is centered on Uganda and the Kivu Province of the DRC, and has a high degree of endemism, some of it strictly eastern, in other cases meeting its western counterparts in the Equateur Province of the DRC, which fits well our new species *P. mabira* **sp.n.** The forest areas of regions 3 and 4 were at least once, and probably more than once, separated from each other by a tongue of Kalahari sands, with savannah vegetation. There are remnants of these habitats in Gabon (Batéké Plateau) and parts of southern Congo-Brazzaville.

Our region 6 (Katanga, Shaba and Zambia) is distinctive, and has much fauna that is related to south-eastern Africa; many parallels with the distribution of *P. zambezi* **sp.n.** can be found, although most have wider distribution as they can survive under more arid conditions than any *Pseudopontia*. The central parts of the DRC that comprise the bulk of our region 5 are characterized by a rather limited degree of endemism and the presence of elements from many parts of Africa, its ecology being such that even south-eastern savannah elements penetrate far into the present, mainly forest habitat. Thus, it is not surprising to find more than one species of *Pseudopontia* there.

Conclusions

This is one of several recent papers describing new species of butterflies that are difficult if not impossible to distinguish on the basis of adult morphology. In several cases, the demonstration of genetic differences among members of complexes of cryptic species has led to the discovery of differences in larval morphology, habitat preferences and host plants, among other characters (e.g. Hebert *et al*., 2004; Burns *et al*., 2008; McBride *et al*., 2009; van Velzen *et al*., 2009). We anticipate that this study may encourage comparative investigations of the natural history and ecology of *Pseudopontia* species in several regions of Africa, recognizing that the enormous distances, the tropical climates and political instability in many areas may hinder such investigations. At present, variations in wing vein structure in *Pseudopontia* are useful for identifications in most cases, but the most reliable and fixed characters that diagnose the proposed species are genetic. Under circumstances such as these, lack of gene flow among putative taxa, i.e. monophyly involving many genes (such as we have demonstrated here) has been advocated as a suitable means of defining phylogenetic species (Ferguson, 2002).

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2010.00549.x

Table S1. Pairwise sums of branch lengths for *COI*.

Table S2. Pairwise sums of branch lengths for three nuclear genes.

Table S3. Number of fixed amino acid differences among populations.

Table S4. Pairwise genetic distances based on amplified fragment length polymorphism (AFLP) data.

Table S5. Summary of amplified fragment length polymorphism (AFLP) bands with high frequencies.

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Author contributions

K.T.M. conceived and organized this study, carried out AFLP studies, analysed the data, and wrote and edited the manuscript; T.B.L., S.C., S.S., G.V.W., J.D.P., W.D.P. and A.Y.K. provided recently collected specimens, photographs and biogeographic data; T.B.L. facilitated contributions by additional collectors, examined the type specimens in the U.K. and made many other essential contributions; W.D.P. examined and measured RMCA museum specimens; E.V.Z. carried out *COI* sequencing and suggested examining for ultraviolet reflectance; D.J.H. provided materials, equipment and guidance for AFLP studies, and obtained preliminary data; J.C.R. supervised nuclear gene sequencing; A.Y.K. performed genitalia dissections and phylogenetic analyses; all authors helped to edit and approve the final article.

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