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The *Pseudagrion* split: molecular phylogeny confirms the morphological and ecological dichotomy of Africa's most diverse genus of Odonata (Coenagrionidae)

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Abstract

The continental African representatives of the genus *Pseudagrion* fall into two groups (A and B) based on their ecology and larval and adult morphology. While the B-group species are found in generally warmer habitats, which are more sunny, lentic or low-lying, the A-group representatives occur more in cooler habitats. We compared molecular genetic and ecological data of twelve species representing both groups. Mitochondrial DNA sequence analyses strongly support their segregation into two major clades and suggest the monophyly of each. High bootstrap support confirms the deep phylogenetic split. Overall, only a minority of species have been studied for each group. However, genetic distances of the species within each clade indicate that they are significantly more closely related to each other than to species of the opposite clade. We conclude that the observed ecological and morphological similarities are due to common ancestry, suggesting two independent radiations within the continental African *Pseudagrion* species. The biogeographic and palaeoecological history of the two clades remains unresolved.

Introduction

Pseudagrion Selys, 1876 is the largest genus of Odonata in Africa and one of the largest in the world: almost 100 species occur in Africa and Madagascar, another 40 range across southern Asia into Australia. The genus has occupied all freshwater habitats in tropical Africa, dominating zygopteran communities from pools in the Kalahari to alpine streams on the Kilimanjaro. Diverse assemblages inhabit equatorial rainforests, while relict populations survive in the Saharan mountains, Morocco and the Levant (Dijkstra & Clausnitzer 2006).

The most comprehensive revision of the Afrotropical species was made by Pinhey (1964a), who subsequently published numerous addenda (e.g. Pinhey 1971, 1973, 1978). However, over one-fifth of Afrotropical species was described since Pinhey's revision (e.g. Balinsky 1964, 1971; Aguesse 1968; Dumont 1978; Legrand 1987; Carletti & Terzani 1997; Terzani & Carletti 2001; Terzani & Marconi 2004). The ecological literature of the genus is comparatively extensive for its largely tropical distribution, e.g. for southern Africa information was published by Balinsky (1957), Chutter (1961), Meskin (1985, 1986, 1989) and Reinhardt (1999).

In continental Africa, the genus is subdivided into an A-group of 41 species and a B-group of 24, based on morphology as well as ecology (Table 1). Following more complex groupings of earlier authors, the morphological dichotomy was first observed in females by Balinsky (1957), confirmed in larvae by Chutter (1961) and firmly established in males by Pinhey (1964a) (Table 2). However, two western African species described by Pinhey (1973) cannot be assigned to any group and may not be congeneric (Dijkstra 2003). 31 species of forest streams in Madagascar and the Comoros probably form a third, separate group (K.-D.B. Dijkstra unpubl.).

The ecological segregation between the two continental groups is mostly clear-cut. For example, in Ethiopia Clausnitzer & Dijkstra (2005) found five B-group species restricted to swampy borders of the Rift Valley lakes, while five A-group species occupied streams and rivers flowing off the highlands. Dijkstra & Lempert (2003) found three A-group species confined to the upper courses of a stream-size gradient in Upper Guinean rainforest and five B-group species occurring only on exposed rivers.

The morphological and ecological traits lead to the hypothesis that the separation of two distinct groups is based on close genealogical relationships of the species within each group. To determine whether the morphological segregation of continental African *Pseudagrion* is based on common ancestry, twelve species (six of each group) were subjected to phylogenetic analysis using a mtDNA sequence comprising of three partial gene fragments.

Material and methods

Taxon survey and sampling

A total of 214 localities was surveyed in Ethiopia, Kenya, Tanzania and Uganda in different seasons from 1994 to 2005, resulting in over 2,500 field records, of which 284 were of the twelve studied species. Two habitat categories were scored for each locality; one pertaining to the type of water body (stream, river, pool, or lake) and the other to the prevailing vegetation cover (forest, gallery/secondary forest, bush, or open land). For each species the preferred habitat and the altitudinal and geographical range were determined (Table 3). For phylogenetic analyses 23 individuals belonging to twelve *Pseudagrion* species were collected during October 2001 through September 2002 in Namibia, Kenya and Tanzania (Table 4). Most specimens were sampled non-invasively (Fincke & Hadrys 2001), although voucher specimens were retained from most localities. Samples were stored in 70% or 98% ethanol prior to DNA-extraction.

Table 1. Morphological and ecological traits separating the continental African *Pseudagrion* species into two groups (A and B).

	A-group	**B-group**
Diversity		
	41 species; exclusively continental, with many small highland and rainforest ranges	24 species; mainly large open land ranges on the continent, two species in Madagascar, Comoros and Mascarenes
Apex S10 ♂		
	Without denticles	With distinct denticles
Cerci ♂		
	Generally with a strongly developed lower branch	With a weakly developed lower branch, or without one
Apex of penis		
	With two funnel-shaped lobes; shape rather uniform between species	Rounded to bilobed, these lobes rounded to pointed, but not funnel-shaped; diverse
Mesokatepisternum ♀		
	With epaulette and/or bristle pad	Without adornments
Mesostigmal lamina ♀		
	Simple	Well-developed
Mature coloration		
	Predominantly black with pale markings, some species with extensive pruinosity; limited variation within species	Predominantly pale with narrow black markings, rarely pruinose; extreme variation within species, may even be largely black
Habitat		
	Generally cooler; mostly running waters, often shaded and/or at high altitude	Generally warmer; running but also standing waters, often exposed, mostly at low altitudes

Table 2. Taxonomic history of continental African *Pseudagrion*.

	A-group	**B-group**
Selys (1876)	*angolense* *furcigerum* *melanicterum* *praetextatum* (= *kersteni*)	*glaucescens* *nubicum* *torridum*
Ris (1936)	*bicoerulans*-group *caffrum*-group *melanicterum*-group *spernatum*-group	*glaucescens*-group *punctum*-group *torridum*-group
Pinhey (1951)	Group a Group b Group d (part)	Group c Group d (part)
Balinsky (1957)	*salisburyense*-group *gigas* (by itself)	*massaicum*-group
Chutter (1961, 1962)	Group A	Group B
Pinhey (1964a, b)	Group A (*caffrum*-group)	Group B (*punctum-glaucescens*-group)

Table 3: Occurrence and habitat of studied *Pseudagrion* species — provided are the observed geographical and altitudinal range [m a.s.l.] and habitat parameters (percentage of recorded localities assigned to each category). These categories are exclusive for water body type (str[eam], riv[er], pool or lake) and prevailing vegetation (for[est], gall[ery/secondary forest], bush or open[land]). E: Ethiopia, K: Kenya, T: Tanzania, U: Uganda.

	Range		**Type of water body**				**Prevailing vegetation**			
	geogr.	altitude	str	riv	pool	lake	for	gall	bush	open
A-group										
P. bicoerulans	KTU	2,000-3,000	100	0	0	0	16.5	67	16.5	0
P. gamblesi	EKTU	561-1,000	50	50	0	0	0	33	50	17
P. hageni	EKTU	35-1,875	94	6	0	0	56	38	6	0
P. kersteni	EKTU	0-2,100	89	11	0	0	11	58	31	0
P. salisburyense	KT	1,500-1,850	86	14	0	0	0	29	57	14
P. spernatum	EKT	520-2,223	86	14	0	0	17	63	0	20
B-group										
P. acaciae	T	64-561	33	67	0	0	0	50	25	25
P. commoniae	KT	20-1,200	66.5	26.5	0	7	0	40	53	7
P. lindicum	KT	20-120	17	50	0	33	0	20	80	0
P. massaicum	EKTU	0-900*	55	25	10	10	0	53	26	21
P. nubicum	EK	738-1,700	0	0	0	100	0	0	25	75
P. sjoestedti	K	720	100	0	0	0	0	100	0	0

* isolated record on Mt Marsabit at 1,350 m.

DNA extraction, amplification and sequencing

The tissue samples were freeze-dried using liquid nitrogen to allow for better homogenisation and DNA was extracted following a slightly modified standard protocol (Hadrys et al. 1992). An approximately 610bp long mitochondrial DNA sequence, containing a partial fragment of the 16S rRNA gene, the intervening $tRNA^{Leu}$ and the NADH dehydrogenase region 1 (ND1), was amplified using the primers 5'>TTC AAA CCG GTG TAA GCC AGG<3' and 5'>TAG AAT TAG AAG ATC AAC CAG C<3' (Weller et al. 1994; Abraham et al. 2001). Two individual sequences from each *Pseudagrion* species were generated, except for *P. nubicum* (Table 4). All reactions were carried out in a 25 µl reaction mix, containing 1x amplification buffer [20 mM tris-HCl, pH 8.4; 50 mM KCl; Invitrogen], 2.5 mM $MgCl_2$, 0.05 mM dNTPs, 0.5 pmol/µl each primer, and 0.03 U/µl taq DNA polymerase (Invitrogen). Amplification was accomplished on a Gene Amp PCR System 9700 (Applied Biosystems). PCR-profiles were as follows: 2 min initial denaturation at 95°C, followed by 30 cycles of 94°C 30 s, 48°C 30 s, 72°C 1 min and 6 min extension at 72°C. All PCR-products were purified with Microcon-PCR Centrifugal Filter Devices (Millipore) following manufacturer's instructions. The sequencing reaction was carried out using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and products were subsequently purified over Sephadex columns (Sigma). Sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Table 4: Species identification — localities with geographical coordinates and GenBank accession numbers (GBAN) for sampled species.

Pseudagrion	**Locality**	**Coordinates**	**GBAN**
A-group			
P. bicoerulans Martin, 1907	Kilimanjaro, Tanzania	03°10'S, 37°14'E	EF221857
	Mt Elgon, Uganda	01°02'N, 34°46'E	EF221856
P. gamblesi Pinhey, 1978	Pangani River, Tanzania	04°37'S, 38°00'E	EF221858
			EF221859
P. hageni Karsch, 1893	Kiboko River, Kenya	02°15'S, 37°32'E	EF221860
			EF221861
P. kersteni (Gerstäcker, 1869)	Ongongo, Namibia	19°08'S, 13°49'E	EF221862
	Pemba River, Kenya	04°11'S, 39°24'E	EF221863
P. salisburyense Ris, 1921	Athi River, Kenya	01°24'S, 36°54'E	EF221864
			EF221865
P. spernatum Selys, 1881	W Usambara Mts, Tanzania	04°50'S, 38°40'E	EF221866
			EF221867
B-group			
P. acaciae Förster, 1906	Pangani River, Tanzania	04°37' S, 38°00'E	EF221868
			EF221869
P. commoniae (Förster, 1902)	Pemba River, Kenya	04°11' S, 39°24'E	EF221870
			EF221871
P. lindicum Grünberg, 1902	Rufiji, Ikwiriri, Tanzania	07°56' S, 38°58'E	EF221872
			EF221873
P. massaicum Sjöstedt, 1909	Kiboko River, Kenya	02°15' S, 37°32'E	EF221875
	Kuiseb River, Namibia	22°40' S, 16°37'E	EF221874
P. nubicum Selys, 1876	Lake Jipe, Kenya	03°36' S, 37°46'E	EF221876
P. sjoestedti Förster, 1906	Mzima Springs, Kenya	02°58' S, 38°01'E	EF221877
			EF221878
Ceriagrion			
C. glabrum (Burmeister, 1839)	E Usambara Mts, Tanzania	01°08' N, 23°36'E	EF221879

Genetic data analyses

Sequences were edited manually using SeqManII (DNASTAR) and aligned with SeaView (Galtier et al. 1996). Pair-wise genetic distances were calculated using the Simple Matching Coefficient (or uncorrected Hamming distance) as implemented in PAUP* 4.0 Beta 10 (Swofford 2002). Phylogenetic trees were estimated using PAUP* under the framework of Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbour Joining (NJ). Where appropriate, a model of evolution was assumed, as calculated by the hierarchical Likelihood Ratio Test in Modeltest 3.7 (Posada & Crandall 1998). Starting trees were obtained using random stepwise addition with 100 replicates. The branch-swapping algorithm employed was tree-bisection-reconnection (TBR). Characters were unordered and not weighted. The trees were rooted using *Ceriagrion glabrum* (Coenagrionidae). The results were tested for robustness by bootstrap analyses with 1,000 pseudo-replicates under the optimality criterion of parsimony (heuristic search).

Table 5. Genetic similarity of the twelve *Pseudagrion* species — figures represent percentages of the average interspecific genetic distance (uncorrected "*p*") based on the mtDNA fragment: lower values represent greater similarity. A-group species and distances between them are italic, B-group ones bold.

	ga	*ha*	*sa*	*sp*	*bi*	*ke*	**co**	**li**	**ac**	**nu**	**sj**
gamblesi											
hageni	*6.5*										
salisburyense	*5.4*	*8.0*									
spernatum	*4.9*	*7.8*	*7.3*								
bicoerulans	*8.9*	*11.0*	*10.1*	*9.3*							
kersteni	*3.9*	*6.7*	*5.2*	*3.8*	*9.6*						
commoniae	13.8	16.2	14.9	14.4	15.7	14.5					
lindicum	13.4	14.4	14.6	14.6	15.0	14.8	**11.2**				
acaciae	12.8	15.9	15.2	13.9	15.1	13.5	**10.3**	**12.0**			
nubicum	11.6	14.2	14.7	11.9	14.3	12.2	**10.3**	**8.0**	**10.4**		
sjoestedti	14.5	15.1	15.3	14.7	14.8	14.5	**11.9**	**8.5**	**11.1**	**9.2**	
massaicum	14.8	16.9	15.6	15.7	16.2	15.9	**4.6**	**11.4**	**10.8**	**10.3**	**12.4**

Results

The two groups showed clear differences in habitat requirements. Whereas A-group species were only found in running water, B-group representatives inhabited all types of water bodies. B-group species were never observed at truly forested sites, but four of the six A-group species were present in this category (Table 3).

The phylogenetic analyses based on the mtDNA sequence data and the genetic distance measures both supported the division of the genus *Pseudagrion* into two distinct clades (Fig. 1; Table 5). All three tree-building methods used (NJ, ML and MP) yielded trees with two clades, which coincide with the morphological classification. Of the 154 variable characters, 130 were parsimony-informative. A heuristic search under the framework of MP resulted in nine most parsimonious trees, whose topologies only differed in the species relationships within the A-group but showed no differences within the B-group. The ML tree (*-lnL* = 2042.94304) calculated under the TrN+I+G model (Tamura & Nei 1993) is shown (Fig. 1). Although only 15% and 25% of the known A- and B-group species were studied, the deep split into two clades was supported by MP bootstrap values of 80 and 100 respectively, suggesting the monophyly of the two clades.

Nucleotide diversity between the mtDNA sequences of the species showed a similarly clear pattern. The genetic distance values were significantly higher between species of the two groups than among species of the same group ($p = 0.00$; *t*-test; two-tailed). Ranging from 4% to 11% (mean 7.22 ± 2.28) within the A-group, from 5% to 12% within B (mean 10.16 ± 1.99) and from 12% to 17% between the groups (mean 14.6 ± 1.17) (Table 5).

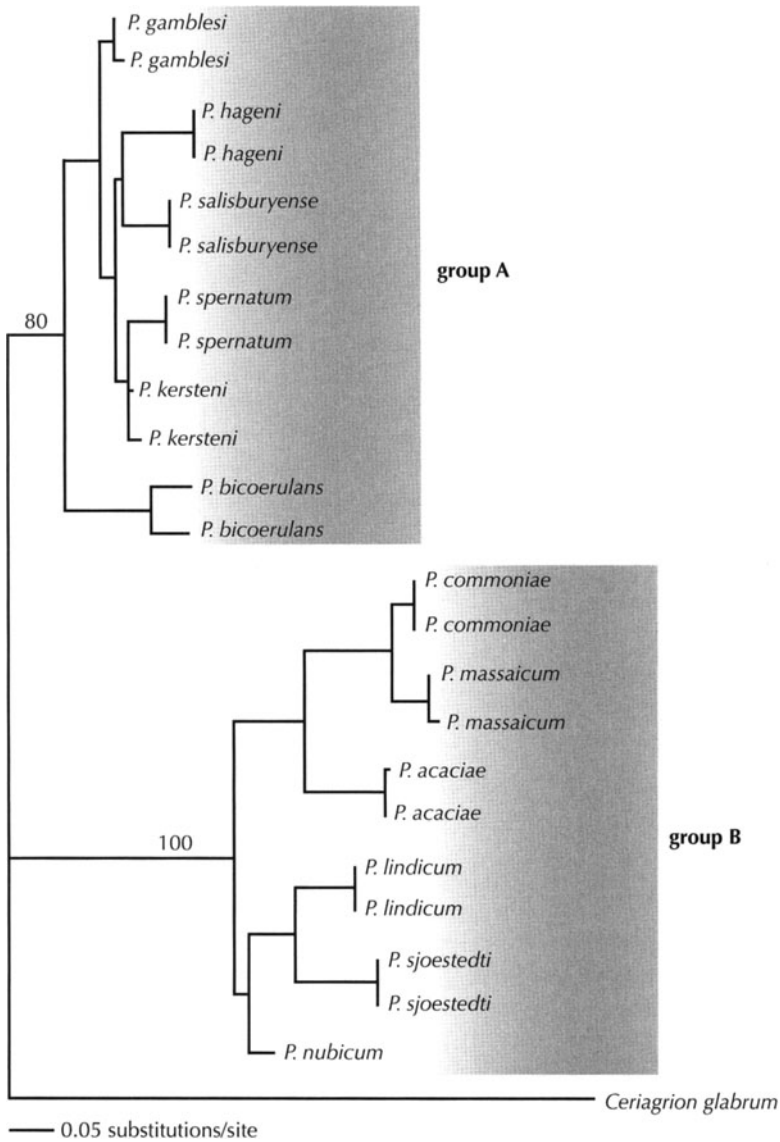

Figure 1: Maximum Likelihood phylogram of Afrotropical *Pseudagrion* spp. based on three partial mtDNA gene fragments (see text), with *Ceriagrion glabrum* as outgroup. The numbers above the branches indicate MP bootstrap support.

Discussion

Although all of the selected A-group species are confined to running waters, they are rather 'atypical' in having adapted to some degree to open habitats. No true rainforest species were, for instance, included. The six studied species are mainly distributed across eastern Africa. One of the selected species, *Pseudagrion kersteni* is abundant throughout tropical Africa on most streams outside dense forests. In contrast, *P. bicoerulans* is endemic to high-altitude forest streams in Kenya and adjacent Uganda and Tanzania. Regardless of their more 'borderline' ecological position, the genetic patterns are clear-cut. The basal phylogenetic split separates all species into the A- and B-groups, in concordance with their morphological differences.

The deep phylogenetic split of the A- and B-groups is a strong indication of independent radiations. Considering the concordance of the split with ecological patterns, differences in radiation events may be related to different habitat adaptations of A- and B-group species. In a comparative population genetic study of two A-group (*P. bicoerulans* and *P. kersteni*) and one B-group species (*P. massaicum*), differences in habitat and distribution are strongly reflected in the genetic structure of the species (Hadrys et al. 2006). Analyses of the same mtDNA fragment used in this study showed that mean genetic diversity and genetic isolation increased with habitat specificity and restricted distribution of the species. The two widespread species (*P. kersteni* and *P. massaicum*) displayed similarly low genetic diversities (ranging from 0.0% to 1.9%). The localized *P. bicoerulans* (see above) showed a much higher intraspecific genetic diversity (6.7%) and complete genetic isolation between populations. Comparison with the genetic distances of species in Table 5 suggests that speciation is in progress within this species (compare the two mtDNA-haplotypes from Mt Elgon and Kilimanjaro in Fig. 1). This pattern of divergence is neither correlated with geographic distance nor with differences in morphology. Therefore the results provide a good example of how genetic data can provide information about cryptic speciation. Since the A-group includes many species with relatively small and fragmented ranges (e.g. in highland and rainforest), subsequently faster radiation within the group may be expected.

Although our study shows that the basal split into two clades is deep and well supported, their evolutionary history remains unknown. Dijkstra & Clausnitzer (2006) postulated that forest streams are the ancestral habitat of *Pseudagrion* and that the A- and B-groups diversified separately in non-forest habitats. Reflective pruinosity is considered an adaptation to increased insolation (Corbet 1999: 282). Perhaps A-group species with pruinosity evolved in cool but sunny highland habitats and were thus better suited to invade open lowland habitats. Such habitats presently dominate the African continent and *P. kersteni*, which is the most strongly pruinose A-group species, is also the most widespread one there. The B-group species share morphological similarities with Asian species and possibly arrived later, radiating into warmer habitats left unoccupied by A-group members.

The inclusion of more A- and B-group species and Malagasy and Asian species will shed more light into the evolution of the two clades. However, it was not the purpose of this study to analyse the history of the two clades, nor to verify or falsify Dijkstra & Clausnitzer's (2006) hypothesis of *Pseudagrion* radiation in continental

Africa. Further tests of the hypothesis demand additional biogeographical data and a more complex, fine-scale phylogenetic analysis. This may help identify the closest non-African relatives, thus determining the phylogeographic history of the genus. Ultimately a more fine-scale reconstruction of the ecological and distributional history of Afrotropical *Pseudagrion* could be mapped onto to the climatological and geological history of the continent. Such reconstruction may clarify evolutionary pathways within the clades, e.g. whether the pruinose A-group species of more open habitats evolved from forest species or vice versa, and whether this happened once or repeatedly, e.g. under influence of orogenesis or forest regression.

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