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THE MORPHOLOGICAL 'FORMS' OF *PALPOPLEURA LUCIA* (DRURY) ARE SEPARATE SPECIES AS EVIDENCED BY DNA SEQUENCING (ANISOPTERA: LIBELLULIDAE)

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P. lucia is a widespread African sp. with a checkered taxonomic history. Currently 2 'forms' or subspecies, *P. l. lucia* and *P. l. portia* are recognized, although debate over the taxonomic status of these taxa has hardly let up over the last 230 years. The 2 'forms' show distinctive wing pattern differences although other aspects of their morphology are very similar. They can occur highly sympatrically at some localities in southern Africa, as well as elsewhere, thus raising the question of whether they are two species or one perhaps with balanced polymorphism. DNA sequence data from the ITS2 and COI genes were collected from specimens of both these 'forms' to assess more rigorously the taxonomic status of these taxa. The closely related *P. deceptor* (Calv.) and *P. jucunda* (Ramb.) were included in the data set to provide a baseline for comparisons. Specimens from all 4 taxa were from pools of the flood plain of the Sabie R., Kruger National Park, South Africa, and were potentially able to interbreed. Both phylogenetic analyses and comparisons of sequence divergence levels strongly support the hypothesis that the 2 'forms' of *P. lucia* are reproductively isolated and should be accorded full species status as *P. lucia* (Drury, 1773) and *P. portia* (Drury, 1773).

INTRODUCTION

Intense debate over the taxonomic status of *Palpopleura lucia* (Drury) has continued since the description of *P. lucia* and *P. portia* as separate species in 1773 (PINHEY, 1962). PINHEY (1951, 1985) treated "*lucia*," "*portia*," and the West African "*graffei*" as "forms" of *P. lucia*. Current taxonomy posits two subspecies, *P. l. lucia* and *P. l. portia*. While females of the two subspecies are difficult to distin-

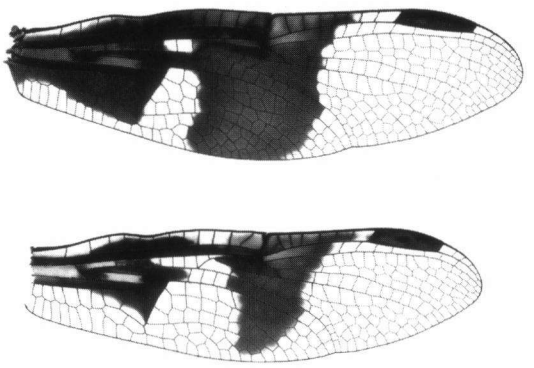

Fig. 1. Wing patterning of *Palpopleura l. lucia* (above) and *P. l. portia* (below).

guish, the males show distinctive patterning on the wings (Fig. 1). The subspecies are sympatric over much of their geographical ranges, from the Eastern Cape to East and West Africa, suggesting that they are not biological subspecies. Although at some localities both occur as adults at the same pool, although *P. l. lucia* overall seems to prefer warmer conditions (PINHEY, 1985). The question of their taxonomic status is also ecologically significant, because if they were indeed conspecific, this would possibly be balanced polymorphism, unknown to date in the Odonata, raising the question of what selection pressures might be responsible for maintaining such polymorphism. We investigate here the taxonomic status of these taxa using DNA sequences of the second internal transcribed spacer region (ITS2) of the nuclear ribosomal DNA repeat region, and the mitochondrial cytochrome oxidase I (COI) gene.

MATERIAL AND METHODS

We sequenced DNA from 21 male specimens, including ten specimens of *P. l. lucia*, five specimens of *P. l. portia*, three specimens of *P. jucunda* (Ramb.) and three specimens of *P. deceptor* (Calv.). Important is that all specimens were collected on the same day (14 March 2002 within a km of each other, and therefore highly sympatric, along the flood zone of the Sabie River, Kruger National Park, South Africa (24° 59'S, 31° 28'E., 320 m a.s.l.), and preserved directly into 100% ethanol.

Total genomic DNA was extracted from approximately 20 mg of flight muscles by means of the Qiagen DNeasy™ Tissue Kit.

For the ITS2 gene, initial PCR amplifications used the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and 28S-25R (5'-TATGCTTAAAYTCAGCGGGT-3'), yielding PCR fragments of approximately 900 bp in length. Each PCR fragment was fractionated on a 1% agarose gel, 2-5 µl of gel containing the PCR fragment was removed with a sterile micropipette tip and used in a reamplification reaction using an internal upstream primer, ITS2-1F (5'-CATGAACATCGACATYTTGAACGC-3'), and the original downstream primer (28S-25R) yielding a PCR fragment of approximately 520 bp. The primers used for the COI gene were Jerry/C1-J-2183 (5'-CAACATTTATTTTGATTTTTTGG-3') and Georgina/C1-N-2786 (5'-GGATAATCTGAATAWCGWCG-3'), yielding PCR fragments 647 bp in length.

PCR was performed on a Perkin Elmer GeneAmp PCR System 2400 under the following conditions: 94°C for 3 minutes, 32 cycles (or 24 cycles for reamplifications) of (94°C for 30 sec., 55°C for 30 sec, 72°C for 60-90 sec), 72°C for 7 min, 4°C hold. Reaction volumes were 30-50µl, and the reaction mixture contained: 1X PCR buffer, 2mM $MgCl_2$, 200µM of each dNTP, 10 pmol of each PCR primer, 0.7 units of Roche *Taq* DNA polymerase, and approximately 250ng of genomic DNA/RNA. PCR reactions were cleaned using Qiagen PCR purification columns and directly sequenced in both directions using the ABI BigDye™ Terminator v3.0 Cycle Sequencing Kit, following the manufacturer's recommended

conditions. Sequences were visualized on an ABI 3100 Genetic Analyser.

DNA sequence chromatograms were edited and contigs assembled using the Staden package (STADEN, 1996). Consensus sequences were automatically aligned using ClustalX (THOMPSON et al., 1997) and then manually corrected using Se-Al v2.0a7 (RAMBAULT, 2001). Phylogenetic analyses were performed under both the maximum parsimony (MP) and maximum likelihood (ML) criteria using PAUP – 4.0b10 (SWOFFORD, 1998). A 10,000 iteration partition homogeneity test was first performed. Each data set was analysed separately, and finally, the combined data set was analysed. Branch and bound searches were performed under MP, while 200-replicate random addition sequence heuristic searches were performed under ML. The extent of support for internal nodes was estimated by means of both parsimony-based and distance-based (ML-model) bootstrap analyses, each employing 1,000 iterations. Following FRATI et al. (1997), 16 different models of sequence evolution were compared for their fit to each data set (ITS2, COI and combined data) by means of likelihood ratio tests. The model chosen for ML analysis was the one with the least number of free parameters, and which was not significantly different from the most complex model, the general time-reversible model (GTR + I +G). For all three data sets, the best model proved to be the Hasegawa, Kishino and Yano model (HASEGAWA et al., 1985), with gamma-distributed rates (HKY85 + G). Model parameters were estimated from the data for each data set separately. Alternative phylogenetic hypotheses were assessed by performing ML constraint searches and comparing the resulting trees with the ML tree, using Kishino-Hasegawa (K-H) and Shimodaira-Hasegawa (S-H) tests, as implemented in PAUP*4.

RESULTS AND DISCUSSION

All DNA sequences reported here are deposited in GenBank (accession numbers ITS2: AY582759-AY582777; COI: AY582778-AY582796). The data set consisted of 474 nucleotides of ITS2 sequence and 595 nucleotides of COI sequence, for a total of 1069 sites. For ITS2 there were 59 variable sites (12.4% of the total) while for COI there were 143 variable sites (24%). Of the 21 specimens sampled, two lacked ITS2 sequence and another two lacked COI sequence, due to sequencing difficulties. Analyses of the combined data set therefore used only the 17 complete

Table I
Uncorrected pairwise divergence values (%)

Comparison	ITS 2					COI				
	mean	s	min	max	n[1]	mean	s	min	max	n
Within *deceptor*	-	-	-	-	-	0.78	0.09	0.67	0.84	3
Within *jucunda*	-	-	-	-	-	3.40	2.43	0.68	5.34	3
Within *portia*	0.30	0.13	0.28	0.46	3	2.72	1.29	0.67	4.37	10
Within *lucia*	0.86	0.46	0.22	1.59	15	0.74	0.36	0.17	1.51	28
lucia vs *portia*	3.66	0.18	3.28	3.91	18	10.82	0.32	10.25	11.61	40
jucunda vs *lucia*	4.35	0.16	4.14	4.60	6	10.17	0.47	9.16	11.24	24
jucunda vs *portia*	3.75	0.00	3.75	3.75	3	10.37	0.45	9.67	11.11	15
deceptor vs *jucunda*	9.76	0	9.76	9.76	1	10.71	0.43	10.09	11.27	9
deceptor vs *lucia*	9.17	0.36	8.83	9.72	6	13.46	0.26	12.94	14.02	24
deceptor vs *portia*	9.48	0.17	9.40	9.61	3	12.07	0.50	11.09	12.77	15

[1] For ITS2 a number of specimens showed identical sequences (e.g. within *P. deceptor* and *P. jucunda*) and all duplicate sequences were eliminated before comparison of divergence levels.

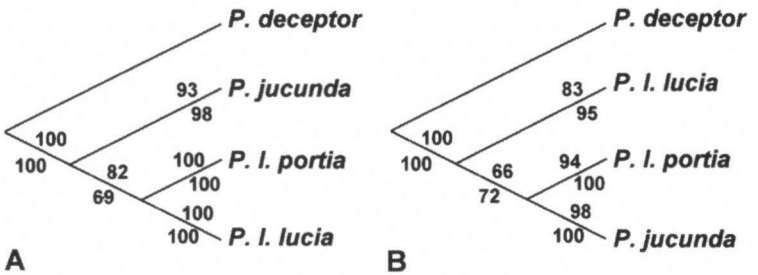

Fig. 2. Summary of relationships among the four sympatric *Palpopleura* taxa recovered under both ML and MP criteria. Bootstrap support levels under ML and MP criteria are shown above and below branches, respectively: (A) COI data only; – (B) ITS2 data only.

sequences. The partition homogeneity test was non-significant (p = 0.095), therefore the data were analysed both separately and in combination.

Table I summarizes the uncorrected divergence values observed in pairwise comparisons among all sequences. For ITS2 sequences, the average pairwise divergence levels between *P. l. lucia* and *P. l. portia* are not statistically different to those between these subspecies and *P. jucunda*. In fact, the maximum divergence observed between specimens of *P. l. lucia* and *P. l. portia* (3.91%) is greater than the maximum divergence observed between *P. jucunda* and *P. l. portia* (3.75%). The more distantly related *P. deceptor* shows 8.8 - 9.7% divergence from all other taxa. A similar pattern is seen for COI sequences, with the mean and maximum divergences between *P. l. lucia* and *P. l. portia* (10.8% and 11.6% respectively) being greater than the corresponding values for comparisons between either subspecies and *P. jucunda* (10.3% and 11.1% respectively). Again, comparisons involving *P. deceptor* give larger values (up to 14.2%).

For COI data alone, parsimony analysis of the 17 taxa data set produced 30 shortest trees of length = 202 steps, CI (excluding uninformative characters) = 0.77, and RI = 0.91. For ITS2 data alone, parsimony analysis of the 17 taxa data set produced 78 shortest trees of length = 72 steps, CI (excluding uninformative characters) = 0.96, and RI = 0.98. Both analyses recovered the four nominate taxa with >90% bootstrap support in every case. The branches that collapsed in the strict consensus trees were those indicating relationships within the four taxa. Analyses of the complete 19 taxa data sets for each gene were in agreement but gave no additional information, therefore they are not considered further here. Maximum likelihood analyses gave almost identical results to the MP analyses, the only differences being in the weakly supported relationships within the four taxa.

Figure 2A summarizes the relationships recovered among the four taxa for the COI data alone, under both MP and ML criteria, while Figure 2B shows the same for the ITS2 data alone. These trees differ in their placement of *P. l. portia*. The COI data places *P. l. lucia* and *P. l. portia* as sister groups, with bootstrap support of 82% under ML and 69% under MP, while the ITS2 data places *P. l. portia* and

P. jucunda as sister groups, with bootstrap support of 66% under ML and 72% under MP.

The combined data set recovered the same relationships among taxa as the COI analysis, with Figure 3 clearly showing the unambiguous and broad separation of the subspecies of *P. lucia*. Visual inspection of internal branch lengths confirms the initial findings suggested by examination of pairwise divergence levels, that the distance between *P. l. lucia* and *P. l. portia* is at least as great as that between either taxon and *P. jucunda*. The monophyly of each subspecies also is strongly supported.

To test the significance of differences in ML score between the competing hypotheses, heuristic searches were conducted on the COI (and combined) data sets while constraining the topology to that shown in Figure 2B (obtained with the ITS2 data), and vice versa. Resulting trees were compared to the ML tree for each data set by means of K-H and S--H tests (Tab. II). All of the test statistics were non-significant. Thus, despite seemingly reasonable bootstrap support values for both of the competing topologies, none of the data sets has the resolving power to distinguish among them. Furthermore, reverse constraint searches in which either *P. l. lucia* or *P. l. portia* were constrained to be not monophyletic yielded trees which were significantly different from the ML trees (K-H tests, p = 0.000-0.003; S-H tests, p = 0.001-0.018).

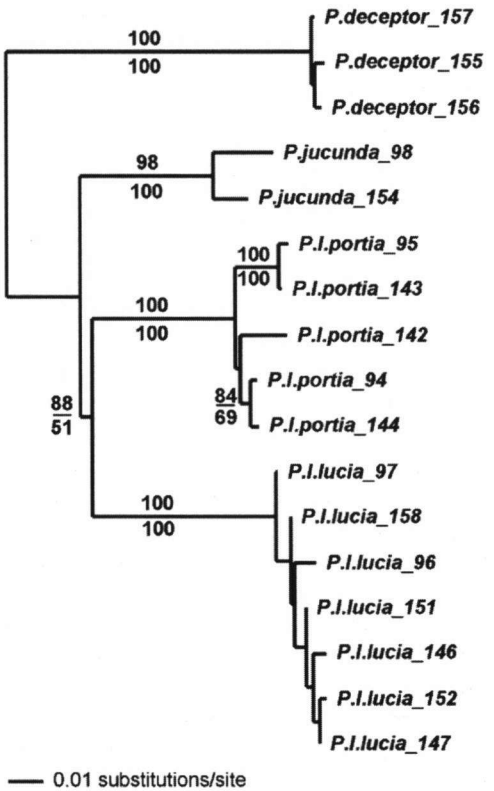

Fig. 3. ML tree for the combined data set, - ln L = 2,949.27958. HKY85 model parameters were as follows: A = 0.25575, C = 0.22220, G = 0.22755, T = 0.29450; Ts/Tv ratio = 3.54019 (κ = 7.1506344); shape parameter (α) = 0.1591.

These results suggest that the two subspecies of *P. lucia* each should be accorded full species status. Specimens of these taxa were collected from sympatric populations, apparently with full opportunity for interbreeding. Indeed, some specimens of *P. l. lucia* and *P. l. portia* were even collected within a few metres of each other from the same pool. Sampling of such specimens provides a very conservative test of the taxonomic status of these taxa. That is, while limited gene flow between the taxa

Table II
Summary of tests for the significance of likelihood differences between trees with the topologies shown in Figures 2A and 2B

	K-H test	S-H test
Combined data	p = 0.770	p = 0.803
ITS2 only	p = 0.624	p = 0.778
COI only	p = 0.849	p = 0.824

at this locality would not necessarily indicate that they were conspecific, the apparent lack of gene flow between the taxa provides very strong evidence that they are reproductively isolated, and therefore full species.

Instead of sampling specimens from across the geographical range of the species, we chose to concentrate on specimens that had been collected in sympatry in order to avoid the confounding effects of geographical variation. In essence, we have examined the "worst case scenario" of sympatric populations and yet we still obtained very clear results that *Palpopleura l. lucia* and *P. l. portia* are reproductively isolated from one another, and should be regarded as separate species, *Palpopleura lucia* (Drury, 1773) and *P. portia* (Drury, 1773).

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