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Molecules, Wing Pattern and Distribution: an Approach to Species Delimitation in the "*loxurina* group" (Lepidoptera: Lycaenidae: *Penaincisalia*)

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Abstract

The wide range of morphological variations in the "*loxurina* group" makes taxa identification difficult, and despite several reviews, serious taxonomical confusion remains. We make use of DNA data in conjunction with morphological appearance and available information on species distribution to delimit the boundaries of the "*loxurina*" group species previously established based on morphology. A fragment of 635 base pairs within the mtDNA gene cytochrome oxidase I (COI) was analysed for seven species of the "*loxurina* group". Phylogenetic relationships among the included taxa were inferred using maximum parsimony and maximum likelihood methods. *Penaincisalia sigsiga* (Bálint *et al*), *P. cillutincarae* (Draudt), *P. atymna* (Hewitson) and *P. loxurina* (C. Felder & R. Felder) were easily delimited as the morphological, geographic and molecular data were congruent. *Penaincisalia ludovica* (Bálint & Wojtusiak) and *P. loxurina astillero* (Johnson) represent the same entity and constitute a sub-species of *P. loxurina*. However, incongruence among morphological, genetic, and geographic data is shown in *P. chachapoya* (Bálint & Wojtusiak) and *P. tegulina* (Bálint *et al*). Our results highlight that an integrative approach is needed to clarify the taxonomy of these neotropical taxa, but more genetic and geographical studies are still required.

Introduction

Interspecific and intraspecific variation in DNA sequences has been used for assessing morphological variability between closely related species in several studies of invertebrate taxa (e.g. Falniowski & Wilke 2001, Mengual *et al* 2006, Iguchi *et al* 2007). The utility of short Cytochrome c oxidase subunit I (COI) regions has been offered as a tool for the discovery of cryptic butterfly and Diptera species (Hebert *et al* 2004a, Smith *et al* 2006, van Velzen *et al* 2007), in the understanding of the species boundaries of taxa (e.g. Micó *et al* 2003, Ståhls & Savolainen 2008) and to accurately classify species in a number of studies (e. g. Hebert *et al* 2004b, Kerr *et al* 2007).

The genus *Penaincisalia* (Eumaeine) was established by Johnson (1990) for a small group of high Andean butterflies related with Austral biomes. More recently, Robbins (2004) synonymized other four related genera (*Thecloxurina*, *Pons*, *Abloxurina*, *Candora*) with *Penaincisalia* forming a genus with highly variable wing shapes. Although the latter taxonomy of the *Penaincisalia* genus is relatively well accepted, additional morphological and molecular characters need to be explored to improve our knowledge on the relationships among the species of various Eumaeine genera and species groups.

Prieto (2008) considered six preliminary species groups within *Penaincisalia*, including the "*loxurina* group" (*Thecloxurina*, *sensu* Johnson 1992), this group is characterized for a hind wing vein CuA2 terminus extended as a rigid tail in both sexes. The "*loxurina* group" is restricted to the tropical Andean habitats where some species are abundant, particularly in the northernmost Andes. Only minor differences in wing pattern characters differ between taxa within the "*loxurina* group", and it is difficult to determine species boundaries, especially considering that the proposed diagnostic characters in the dorsal and ventral surfaces are frequently variable.

Several studies have been conducted on the species related with *Penaincisalia loxurina* (Felder & Felder) and despite several proposed classifications, checklists and nomenclatural descriptions of new species (Table 1), serious taxonomical confusion remains. Although Prieto (2008) recognized eight species in the "*loxurina* group" including several recently described taxa (Table 1), this is the first taxonomic study of this species group.

In some cases wing pattern of the species of the "*loxurina group*" is so variable that parapatric or allopatric populations have often been considered different species. This creates an undesirable over-abundance of redundant species names. On the other hand, synonymy may also occur by "lumping" together species into a single entity even though several species do exist. Most questions in evolutionary biology, ecology, conservation priorities or biogeography depend on our knowledge of species (Dayrat 2005, Bickford *et al* 2007), so there is a need for rigorously delimit species boundaries for producing accurate species inventories.

In spite of the morphological variability of the group, no genetic studies on *Penaincisalia* species have been reported as yet. In this study we use mtDNA COI sequences to clarify the taxonomy of *Penaincisalia*, particularly for the taxa where morphological and taxonomical confusion has been most apparent, under the concept of "integrative taxonomy", the use of DNA data in conjunction with morphological characters and available distribution information to define biological species for comparison with previously established species boundaries based on morphology (Dayrat 2005, Mengual *et al* 2006).Therefore, we aim to delineate the species boundaries of the "*loxurina* group" based on three criteria: a) sympatry/allopatry as an indication of interbreeding, b) wing pattern differentiation and intermediate forms as possible indicator of interbreeding, and c) genetic distance.

Material and Methods

Specimens and molecular techniques

We analysed partial nucleotide sequences of mtDNA COI of 17 specimens belonging to seven species and one subspecies of the "*loxurina* group" from several populations occurring along the tropical Andes (Fig 1). Two additional *Penaincisalia* species, *Penaincisalia browni* (Johnson) and *Penaincisalia magnifica* (Johnson), belonging to sister species group (Prieto 2008) were sequenced as outgroups.

Thorax and legs were used for DNA extraction from single individuals of either dry, pinned or ethanol preserved specimens. DNA was extracted from these

Table 1 Summary of the taxonomic history of the "*loxurina* group".

Draudt 1919	Johnson 1992	Bálint & Wojtusiak 2003	Robbins 2004	Prieto 2008
Thecla loxurina	*Thecloxurina loxurina*	*Thecloxurina loxurina*	*Penaincisalia loxurina*	*Penaincisalia loxurina*
- T. l. quindiensis	*- Th. l. lustra*	*- Th. l. astillero*	*- P. l. astillero*	*- P. l. astillero*
- T. l. atymnides	*- Th. l. astillero*	*Thecloxurina atymna*	*Penaincisalia atymna*	*Penaincisalia atymna*
- T. l. cillutincarae	*Thecloxurina quindiensis*	*Thecloxurina atymnides*	*Penaincisalia cillutincarae*	*Penaincisalia cillutincarae*
- T. l. fassli	*Thecloxurina atymnides*	*Thecloxurina cillutincarae*	*Penaincisalia atymnides*	*Penaincisalia alcacera*
Thecla atymna	*Thecloxurina cillutincarae*	*Thecloxurina amazona*		*Penaincisalia felizitas*
	Thecloxurina fassli	*Thecloxurina fassli*		*Penaincisalia sigsiga*
	Thecloxurina atymna	*Thecloxurina contracolora*		*Penaincisalia santamarta*
	Thecloxurina browni	*Thecloxurina chachapoya*		*Penaincisalia tegulina*
	Thecloxurina truncta	*Thecloxurina ludovica*		
	Thecloxurina costarica			
	Thecloxurina eiselorum			
	Thecloxurina bolivatymna			
	Thecloxurina feminina			

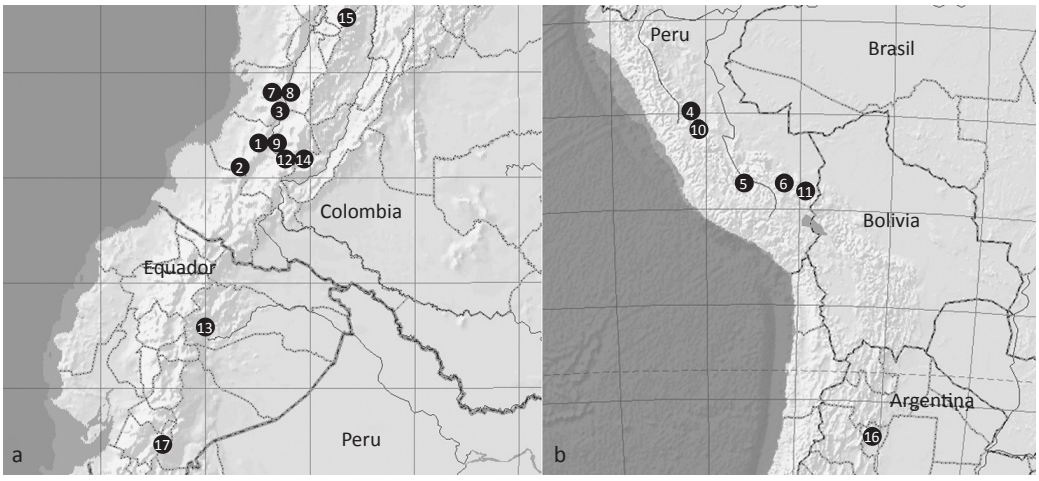

Fig 1 a) Map of the sampling sites in Colombia and Ecuador; b) Map of the sampling sites in Peru and Argentina. Numbers belong to the specimen number in Table 2.

parts using the QIAGEN DNeasy Tissue extraction kit. We used the forward primer C1-J-1751 (5'-GGATCACCTGATATAGCATTCCC-3') and the reverse primer TL2-N-3014 (5'-TCCAATGCACTAATCTGCCATATTA-3') (Simon *et al* 1994) in PCR amplifications in 25 µl reactions containing 3 µl DNA extract, 1 µl of each primer (primers at 10 pmol/ µl), 0.25 µl of Ampli*Taq* DNA polymerase (250 units, 5 U/µl), 3 µl 25 mM $MgCl_2$, 2.5 µl Buffer (Perquin-Elmer®), 4 µl 10 mM dNTP (Perquin-Elmer®) and ultra-pure water. Thermocycler conditions were 96ºC for 1 min (1x), followed by 29 cycles at 96ºC for 30s, 50ºC for 15s, and 60ºC for 4 min. PCR products were purified using the QIAquick® PCR Purification kit QIAGEN. Amplified PCR samples were sequenced including a second forward primer, C1-J-21835 (5'-CAACATTTATTTTGATTTTTTGG -3') (Simon *et al* 1994) with an ABI PRISM 310 (Applied Biosystems) sequencer.

Data analysis

The sequences were inspected, edited for base-calling errors and submitted to GenBank (accession numbers are presented in Table 2).

We used the program PAUP* version 4.0b10 (Swofford 2002) for a parsimony analysis (MP) using the heuristic search procedure. Gaps were treated as missing data. We also inferred the phylogenetic relationships among

Table 2 Taxa and specimens examined.

Species	Collection sites	Location	GenBank Accession Nº.
1. *Penaincisalia browni*	Cauca, Colombia	2º 40' N 76º 55' W	EU682666
2. *Penaincisalia browni*	Cauca, Colombia	2º 12' N 77º 21' W	EU682680
3. *Penaincisalia magnifica*	Valle, Colombia	3º 19 N 76º 36' W	EU682681
4. *Penaincisalia chachapoya*	Huanacuare, Perú	9º 48' S 75º 52' W	EU682667
5. *Penaicisalia tegulina*	Karkatera, Perú	13º 34' S 72º 58' W	EU682674
6. *Penaincisalia ludovica*	Cuzco, Perú	13º 30' S 70º 53' W	EU682682
7. *Penaincisalia loxurina*	Cali, Colombia	3º 36' N 76º 39' W	EU682669
8. *Penaincisalia loxurina*	Cali, Colombia	3º 36' N 76º 39' W	EU682670
9. *Penaincisalia loxurina*	Cauca, Colombia	2º 40' N 76º 55' W	EU682671
10. *Penaincisalia loxurina*	Oxapampa, Perú	10º 36' S 75º 26' W	EU682673
11. *Penaincisalia loxurina astillero*	Puno, Perú	14º 00' S 69º 38' W	EU682668
12. *Penaincisalia atymna*	Cauca, Colombia	2º 21' N 76º 23' W	EU682676
13. *Penaincisalia atymna*	Tulcán, Ecuador	0º 51' N 78º 03' W	EU682679
14. *Penaincisalia atymna*	Cauca, Colombia	2º 21' N 76º 23' W	EU682678
15. *Penaincisalia atymna*	Quindío, Colom.	5º 03' N 75º 20' W	EU682677
16. *Penaincisalia cillutincarae*	Tucumán, Argen.	26º 46' S 65º 25' W	EU682672
17. *Penaincisalia sigsiga*	Sigsig, Ecuador	3º 03' S 78º 47' W	EU682675

all species using maximum-likelihood (ML). Bootstrap support values (Felsenstein 1985) were calculated using 200 replicates for the MP and ML analyses, using HKY85 model (ln = -1691.639).

Results and Discussion

The final aligned sequences yielded 635 nucleotides that also include 60% of the "Folmer fragment" which is the fragment proposed by the DNA Barcoding Council. Of the obtained 635 nucleotides, 98 were variable and 72 sites were parsimony-informative. Parsimony analysis produced seven equally parsimonious trees, with a consistency index (CI) of 0.74, a retention index (RI) of 0.79. Results of the ML analysis are shown in Fig 2, one of the seven MP trees is shown in Fig 3. Monophyly of the "*loxurina* group" was not rejected and was supported with very high bootstrap values for maximum-likelihood and parsimony analyses (Figs 2, 3).

Uncorrected pairwise divergences between ingroup taxa ranged from 2.52% to 6.15%, and among individuals within each clade from 0.0 % to 0.78% (Table 3, Fig 2). Divergences between outgroup and ingroup taxa ranged from 6.29% to 7.57% (Table 3, Fig 2). A total of ten different haplotypes were detected in the "*loxurina*" species-group. Most clades were associated with distinct morphologies, especially in wing upper surface appearance. In addition, within the clade I, the dorsal surface for each individual is very distinct (Fig 3).

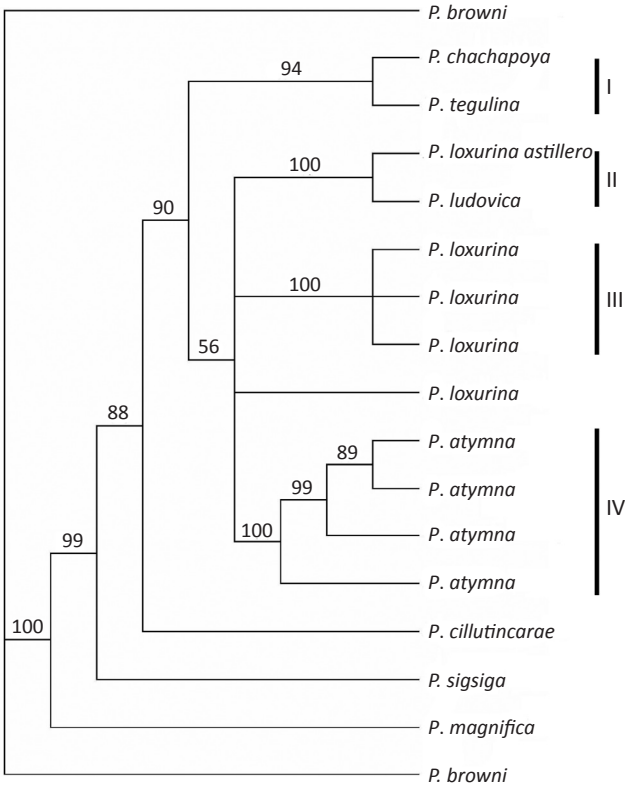

Fig 2 Maximun-likelihood tree (-Ln Likelihood = 1691.639, HKY85 model) showing the genetic relationships among haplotypes of seven "*loxurina* group" species from analysis of mitochondrial COI sequences. *Penaincisalia browni* and *Penaincisalia magnifica* were used as outgroup. Clades used for comparisons are numbered I-IV.

Congruence among data

Several species were easily defined, as the morphological, geographic and molecular data were congruent. *Penaincisalia sigsiga* (Bálint *et al*) was considered as a reproductively isolated taxon due to its relatively high genetic divergence when compared with all species of the "*loxurina* group" (4.7% to 6.15%) (Table 3), external morphology and confirmed sympatry with the most similar species *P. atymna* (Hewitson). Although sympatry was not confirmed for *P. cillutincare* (Draudt) with any other "*loxurina* group" species, its relatively high genetic divergence when compared with other "*loxurina* group" species (3.5% to 4.7%) (Table 3) and morphological differentiation suggest that this is a reproductively isolated taxon following current usage (Fig 3).

Clade IV (*P. atymna*) exhibits the highest intraspecific divergence (0.0% to 0.78%) with the specimen from the Quindío population as the most distinctive (Fig 3). Although clade III [*P. loxurina* (C. Felder & R. Felder) from Colombia] and *P. loxurina* from Peru presented higher genetic divergences than the average within clades, these entities are geographically isolated and differ morphologically by the lighter dorsal blue surface and the larger size of *P. loxurina* from Peru. Both morphological characters are very variable in the group, allowing us to consider that both are slight geographical forms of the same species.

The species *P. ludovica* (Bálint & Wojtusiak) and the subspecies *P. loxurina astillero* (Johnson) had identical COI sequences (clade II) and could not be separated based on the sequences analysed (Figs 2, 3; Table 3). Moreover, it was not possible to distinguish these taxa based on morphological characters. The genetic distances among the ingroup clades were larger than those within clades with the exception of clades II and III. The average genetic distance among clades II and III was not significantly larger than within clades ($P > 0.09$). Moreover, in our samplings, sympatry was not confirmed between clades II (*P. loxurina astillero* + *P. ludovica*), III (*P. loxurina*) and *P. loxurina* from Peru (Figs 2, 3). Thus, clade II most likely constitutes a subspecies of *T. loxurina* restricted to the eastern slope of the Ucayali river on the eastern mountain range in "Madre de Dios" (Peru) (Fig 1).

Incongruence among data

Two strongly divergent phenotypes were grouped in clade I (Fig 3) [*P. chachapoya* (Bálint & Wojtusiak) + *P. tegulina*]

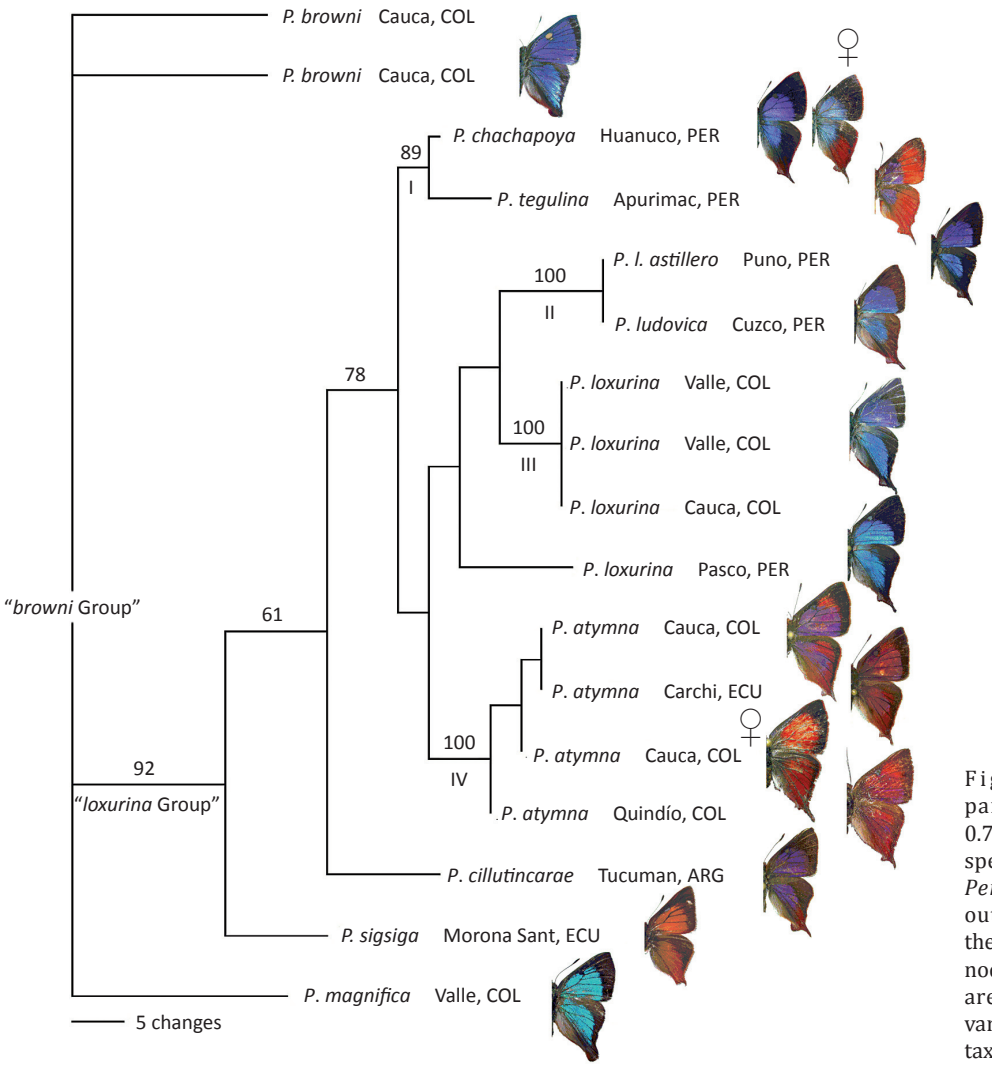

Fig 3 One of seven most parsimonious cladograms (CI = 0.74, RI = 0.79) for "*loxurina* group" species. *Penaincisalia browni* and *Penaincisalia magnifica* are the outgroups. Bootstrap support of the strict consensus is placed above nodes. Clades used for comparisons are numbered below nodes. The variability of dorsal wing pattern of taxa is presented.

and had very low genetic divergence ("P" = 0.011). The phenotype of *P. tegulina* would be considered as a wing pattern variant of *P. cillutincarae*. However, our results show that this phenotype (from Peru) is more closely related to *P. chachapoya* (from Peru).

The genetic divergence between *P. chachapoya* and *P. tegulina* was lower than the genetic divergence between clades. Although, this could indicate that these two phenotypes constitute the same species, it could be a case of "false negative", where little or no sequence variation in the COI fragment is found between different biospecies (Meyer & Paulay 2005, Wiemers & Fiedler 2007) easily distinguishable by their strong phenotypical differentiation. To decide whether these lineages belong to distinct species or to the same polymorphic species requires further study.

Penaincisalia chachapoya has been considered as a geographic form of *P. loxurina* (Robbins 2004). However, although *P. chachapoya* and *P. loxurina* has not been found exactly at the same locality, sympatry is not rejected due to the inexistence of geographic barriers between the very close localities where they have been collected and to the similarity of their ecosystems (Fig 1). This fact, together with their observed genetic distinctness, suggests *P. chachapoya* and *P. loxurina* are most likely reproductively isolated.

Results from morphological and molecular analyses suggest the following conclusions: *P. ludovica* constitutes the same entity as *P. loxurina astillero* and constitutes a subspecies of *P. loxurina*. Although we cannot decide whether *P. chachapoya* and *P. tegulina* are distinct species based on our results, we can confirm that the *P. tegulina* phenotype is not related to *P. cillutincarae* from Argentina.

Limits of non-integrative analysis of the taxonomy of Penaincisalia

In agreement with Dayrat (2005), our results show that traditional morphology-based taxonomy has limits. Cases such as *P. chachapoya*, *P. loxurina* and *P. ludovica*

Table 3 Genetic distance (%) between taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *P. browni* (1)	-																
2. *P. chachapoya* (4)	7.244	-															
3. *P. l. astillero* (11)	7.559	2.835	-														
4. *P. loxurina* (7)	7.244	2.677	2.520	-													
5. *P. loxurina* (8)	7.258	2.681	2.524	0.000	-												
6. *P. loxurina* (9)	7.244	2.677	2.520	0.000	0.000	-											
7. *P. cillutincarae* (16)	6.772	3.465	3.937	3.780	3.784	3.780	-										
8. *P. loxurina* (10)	7.717	3.150	3.780	3.150	3.154	3.150	5.197	-									
9. *P. tegulina* (5)	7.717	1.102	3.307	3.622	3.627	3.622	3.622	3.780	-								
10. *P. sisiga* (17)	6.780	4.891	5.682	5.207	5.213	5.207	4.735	6.152	5.364	-							
11. *P. atymna* (12)	7.402	2.677	3.465	3.150	3.153	3.150	4.567	3.937	3.465	5.678	-						
12. *P. atymna* (15)	6.929	2.047	2.677	2.835	2.839	2.835	4.094	3.150	2.835	5.048	0.787	-					
13. *P. atymna* (14)	7.402	2.520	3.150	3.307	3.312	3.307	4.567	3.622	3.307	5.521	0.315	0.472	-				
14. *P. atymna* (13)	7.402	2.677	3.465	3.150	3.153	3.150	4.567	3.937	3.465	5.678	0.000	0.787	0.315	-			
15. *P. browni* (2)	0.000	7.244	7.559	7.244	7.258	7.244	6.772	7.717	7.717	6.780	7.402	6.929	7.402	7.402	-		
16. *P. magnifica* (3)	6.142	6.299	7.244	7.087	7.099	7.087	6.299	7.087	6.457	6.939	7.402	7.244	7.402	7.402	6.142	-	
17. *P. lucovica* (6)	7.571	2.837	0.000	2.522	2.527	2.522	3.939	3.782	3.309	5.686	3.466	2.679	3.151	3.466	7.571	7.254	-

The number in brackets is the locality of Fig 1 and taxon number of Table 2.

show the need to test observed "morphodiversity" via different approaches and with different kinds of data in order to delimit species boundaries. On the other hand, the genetic methods alone may present some problems, such as the fact that gene trees may differ from species trees (Pamilo & Nei 1988, Maddison 1997). Based on genetic data alone, *P. chachapoya* and *P. tegulina* could have been considered as the same species, but their strong wing pattern differentiation suggests otherwise. Therefore, as Valdecasas *et al* (2008) suggested, part of the problem of species delineation is the fact that molecular biology, cytogenetics, enzymology, ecology, among others approaches for species delimitation also have certain limits.

While the success rate of barcoding undoubtedly varies among groups, problems in insects and other invertebrates have been frequently observed, including mitochondrial introgression between taxa and interbreeding that obscures species identification and limits (e.g. Croucher *et al* 2004, Kaila & Ståhls 2006). Moreover, some groups in which recent speciation rates are high and effective population sizes large and stable, as in many tropical insects, are particularly likely to be subject to difficulties (Elias *et al* 2007). As the "*loxurina* group" seems to be a result of recent speciation processes (Prieto 2008), the use of molecular methods alone to understand species boundaries could be unreliable. Thus, a multidisciplinary approach to taxonomy of this group is necessary.

In the studied group, as well as many tropical insects, measuring intra- and interspecific sequence divergence is hampered by the fragmentary knowledge of most taxa. More information is necessary on the amount of intraspecific genetic and morphological variation among closely related species. Our study group needs to be rigorously tested with sequence data from samples that cover the geographic range more comprehensively. Further studies of COI profiles, correlated with sequence data from additional nuclear genes and informative morphological, geographical and ecological characters are necessary for the understanding of species boundaries of this group of butterflies.

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