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Hybridization in Phlebotominae (Diptera: Psychodidae): a mini-review

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Abstract

Taxonomy based only on morphology, although extremely important for the classification of sandflies, has been shown to be insufficient for the delimitation of some taxa. Thus, integrative taxonomy could play a fundamental role in clarifying these and other taxonomic issues, since data from different areas are used to aggregate greater reliability in species classification. Experimental crosses are important taxonomic tools, since the presence of reproductive barriers when associated with divergence between two evolutionary lineages, confirms the specific status of taxa based on the biological species concept. In the subfamily Phlebotominae, experimental crosses were mostly focused on the study of the *Lutzomyia longipalpis* complex, which helped to identify different evolutionary lineages for that group of vectors. Considering the difficulty of classifying some Phlebotominae species and the importance of hybridization studies for taxonomy, we grouped all the information associated with experimental crosses in sandflies in a mini-review. In view of the results grouped in this review, it is evident that i) experimental crossings are important tools to aggregate studies of integrative taxonomy in the Phlebotominae subfamily; ii) these analyses should be applied in the taxonomic studies of cryptic species; iii) *Lu. longipalpis* populations have pre and/or post-zygotic reproductive barriers; iv) *Lu. longipalpis* represents more than one species and efforts must be applied to differentiate the taxa of the *Lu. longipalpis* complex; v) *Phlebotomus* populations do not present intraspecific reproductive barriers; vi) the absence of reproductive barriers between *Ph. sergenti* from Israel and Turkey (representing populations of the same evolutionary lineage) does not rule out the possible existence of cryptic species, it being necessary to perform experimental crosses between the different strains indicated by the molecular markers; and finally, vii) different species of *Phlebotomus*

have post-zygotic barriers, confirming the specific status of *Ph. duboscqi*, *Ph. papatasi*, and *Ph. bergeroti*.

Keywords: Sand fly; Leishmaniasis; Reproductive isolation; Integrative taxonomy

1. Review

There are three main forms of leishmaniasis, visceral (also known as kala-azar, which is the most serious form of the disease), cutaneous (the most common), and mucocutaneous, which together cause around 700 thousand to one million new cases annually (WHO, 2020). These diseases are caused by protozoa of the genus *Leishmania* Ross, 1903 (Kinetoplastida, Trypanosomatidae), which are transmitted to humans through the bite of infected female phlebotomine sandflies (Diptera, Psychodidae, Phlebotominae), since males are not hematophagous (Forattini, 1973; WHO, 2020). Currently, there are more than 1000 species of sandflies (including fossils), about half of which are present in the Americas (Galati, 2018).

Taxonomy based only on morphology, although extremely important for the classification of sandflies (Akhoundi et al., 2016; Galati, 2018), has been shown to be insufficient for the delimitation of some taxa. This is the case of *Lutzomyia longipalpis* (Lutz and Neiva, 1912) which through morphological analysis is classified as just one species, while in studies using other approaches [such as phenotypic, ecological, cytogenetic, isoenzymatic, morphometric differences, as well as divergences in sexual pheromones and characterization of reproductive barriers (Mangabeira-Filho, 1969; Ward et al., 1983; Lenzaro et al., 1993; Dujardin et al., 1997; Yin et al., 1999; Souza et al., 2008; Spiegel et al., 2016; Palframan et al., 2018)], is shown to be a complex of cryptic species (Souza et al., 2017; Reis et al., 2020). Thus, integrative taxonomy can play a fundamental role in clarifying these and other taxonomic issues, since data from different areas of biology, such as ecology, behavior, and evolution, are used to aggregate greater reliability in species classification (Dayrat, 2005).

Experimental crosses are important taxonomic tools, since the presence of reproductive barriers (pre and/or post-zygotic) when associated with divergence

between two evolutionary lineages, confirms the specific status of taxa based on the biological species concept (Mayr, 1963, 2001; Dobzhansky, 1970). Hybridization studies based on the characterization of reproductive barriers have already contributed substantially to the elucidation of the taxonomy of cryptic species of different groups of arthropods, such as Acari (Tixier et al., 2006; Sourassou et al., 2012), Copepoda (Monchenko, 2000), Hymenoptera (Heimpel et al., 1997; Desneux et al., 2009), Hemiptera (Horton et al., 2005; Xu et al., 2010), Coleoptera (Takano et al., 2011), and Diptera (Lanzaro et al., 1988; Rull et al., 2013). In the subfamily Phlebotominae, experimental crosses were mostly focused on the study of the *Lu. longipalpis* complex, which helped to identify different evolutionary lineages for that group of vectors (Ward et al., 1983; Lanzaro et al., 1993; Souza et al., 2008).

Considering the difficulty of classifying Phlebotominae species and the importance of hybridization studies for taxonomy, we grouped all the information associated with experimental crosses in sandflies in a mini-review.

1.1. Experimental crosses performed in Phlebotominae

1.1.1. Interspecific crosses between New World Phlebotomines

The only work involving interspecific hybridization for sandflies in the New World was carried out by Andrade-Filho et al. (1999) who crossed *Evandromyia carmelinoi* (Ryan, Fraiha, Lainson & Shaw, 1986) and *Ev. lenti* (Mangabeira, 1938). These species are quite similar (Andrade-Filho et al., 2001; Galati, 2018) and only produced hybrids in one direction of the crossing, *Ev. lenti* (male) x *Ev. carmelinoi* (female). When performing the intercrossing of hybrids from this crossing, the authors verified that although the individuals had fertility, all second generation (F2) larvae

died before reaching the pupal stage, suggesting, thus, hybrid collapse (Andrade-Filho et al., 1999).

Although these species are not considered vectors of leishmaniasis (Andrade-Filho et al., 2001; Queiroz et al., 2012), *Ev. lenti* has already been found naturally infected with *Le. infantum* and *Le. braziliensis* (Lana, 2015). Taking into account that these species have already been collected in sympatry (Andrade-Filho et al., 2001), if natural hybridization occurs, it is possible that the resulting offspring may also be able to become infected with *Leishmania* species.

1.1.2. Interpopulation crosses in *Lu. Longipalpis*

The first experimental crossings were carried out by Ward et al. (1983) between some populations of *Lu. longipalpis* from Brazil, from the states of Minas Gerais (Lapinha Cave), Ceará [one from Mocim Nova and two populations from Sobral that occur in sympatry, and that present one (S1) or two pairs (S2) of tergal spots] and one from Pará (Marajó Island) (Table 1). The authors observed lower egg hatching rates in crossings between populations from the different localities in relation to the control groups (Table 1) (WARD et al., 1983). Among the morphotypes from Sobral, the hatching rate was only 1.9% in one of the directions (female S1 x male S2), while in the reciprocal crossing there was no hatching of eggs (Table 1) (Ward et al., 1983). Although the eggs from this cross showed 1.9% hatching, the authors did not rule out the possibility of contamination.

Lanzaro et al. (1993) observed that the crossing between populations of *Lu. longipalpis* from Brazil, Colombia, and Costa Rica resulted in the production of hybrids in the first generation in all crosses (Table 1). Despite this, all male hybrids were infertile (Table 1), showing that these populations probably represent different

species of *Lutzomyia* França, 1924, since there are reproductive barriers between them. Interestingly, the number of hybrids that reached adulthood was greater in interspecific crossing than in the control, a fact attributed by the authors to heterosis (Lanzaro et al., 1993).

More recently, Souza et al. (2008) performed crossings between populations in Bahia (Jacobina), Rio Grande do Norte (Natal), and Minas Gerais (Lapinha Cave) and observed reproductive barriers in all crossings (Table 1). When assessing copulation rates, the authors noted that they were significantly lower in crossings between populations of the different localities compared to the control. Crossings were also performed between the two sympatric populations (S1 and S2) of Sobral (Ceará), corroborating the results of Ward et al. (1983) (Table 1). Surprisingly, among these populations, the pre-zygotic barriers are even more evident, since there were no copulations registered for any of the 119 crossings performed.

Since the first crossings performed by Ward et al. (1983), the role of some characteristics related to the strong reproductive isolation, observed among the populations of Brazil, has been elucidated over the years. The sounds emitted by the males during the courtship, for example, proved to be quite divergent between the populations of *Lu. longipalpis* that present at least seven distinct patterns, being five variations of the Pulse-type, one Burst-type, and one intermediary, called the Mix-type (Souza et al., 2004; Araki et al., 2009; Vigoder et al., 2015). In addition, there are also differences in the pheromones produced between the populations of these insects that can present cembrene-1, cembrene-2, 9-methylgermacrene-B, or himachalene (Hamilton et al., 1999a; 1999b; 2004; Araki et al., 2009), which, associated with the courtship songs, can play an important role in reproductive isolation (Araki et al., 2009).

These differences in the patterns of courtship songs and in the pheromones produced are reflected in the results obtained by the crossings performed by Ward et al. (1983) and Souza et al. (2008). For example, the reproductive isolation among the sympatric populations of Sobral (S1 and S2) (Table 1) may be related to these differences, since the S1 population has a Pulse-type courtship song (pattern 3) and 9-methylgermacrene-B pheromone, while the S2 population has a Burst-type courtship song and Cembrene-1 pheromone (Araki et al., 2009). The same can be observed in the crossing between the populations of Lapinha Cave and Jacolina (Table 1) [the first presents a Pulse-type courtship song (pattern 2) and 9-methylgermacrene-B pheromone, and the second, a Pulse-type courtship song (pattern 1) and himachalene pheromone (Araki et al., 2009)], as well as between the populations of Lapinha Cave and Marajó Island (Table 1) [the first presents a Pulse-type courtship song (pattern 2) and 9-methylgermacrene-B pheromone, and the second, a Burst-type courtship song and cembrene-1 pheromone (Araki et al., 2009)]. However, some differences can be noted in the crossings performed, such as the absence of copulations among the sympatric populations of Sobral, but presence among allopatric populations (Souza et al., 2008), although both have different combinations of courtship songs / pheromones (Araki et al., 2009). One possible explanation for this is that pre-mating isolation generally settles more quickly among sympatric populations, where reinforcement can take place (Coyne and Orr, 1989). This can be confirmed, as there are indications of the occurrence of introgression among these populations, signaling that natural hybridization events may have occurred throughout the evolutionary process (Araki et al., 2013).

In addition to taxonomic contributions, these crosses between different populations of *Lu. longipalpis* are also very important from an epidemiological point

of view, since this species is considered the main vector of *Le. infantum* Nicolle, 1908 in Latin America (WHO, 2020), and in the case of a species complex, it is possible that they have different vector capacities (Reis et al., 2020). In the state of São Paulo (Brazil), for example, there are populations of *Lu. longipalpis* that present the 9-methylgermacrene-B pheromone, associated with sites of transmission of visceral leishmaniasis, and cembrene-1, associated with sites without transmission (Casanova et al., 2015). Characterizing the specific status of these taxa is of great importance for public health, since it allows vector control programs to direct activities to species that really have vectorial importance.

1.1.3. Natural hybrids of New World sandflies

In addition to experimental crosses there are two reports of possible natural hybrids: Galati et al. (2010) describe a hybrid male and female between *Nyssomyia intermedia* (Lutz & Neiva, 1912) and *Ny. neivai* (Pinto, 1926) and reinforce the importance of experimental crosses between these species to elucidate the possible reproductive barriers, as well as the fitness of the hybrid offspring; after six years, Oliveira et al. (2016) present a female, probably hybrid, resulting from the crossing of species of *Psychodopygus* Mangabeira, 1941, which has characteristics similar to *Ps. arthuri* (Fonseca, 1936) and *Ps. ayrozai* (Barretto & Coutinho, 1940), and could lead to the mistaken identification of this possible hybrid (Oliveira et al., 2016).

1.1.4. Crosses carried out between species of *Phlebotomus*

In addition to the hybridization studies associated with the *Lu. longipalpis* complex, experimental crosses were also performed between species of *Phlebotomus* Rondani & Berté 1840 (Table 1). The first study evaluated the reproductive

compatibility between *Ph. papatasi* Scopoli, 1786 and *Ph. bergeroti* Parrot, 1934 (Fryauff and Hanafi, 1991). These species are morphologically similar, with the females differentiated by only a single character (relative length of the ascoids on antennal segment IV) (Lane and Fritz, 1986). However, while *Ph. papatasi* is demonstrably a vector of *Le. major*, which causes cutaneous leishmaniasis, the vectorial capacity of *Ph. bergeroti* for this parasite has not yet been fully elucidated (Killick-Kendrick, 1990, 1999; Akhoundi et al., 2016). The authors found that only the crossing between *Ph. bergeroti* (female) x *Ph. papatasi* (male) produced hybrids (F1) (Table 1) (Fryauff and Hanafi, 1991). These hybrids were intercrossed and backcrossed with both parents and only the backcross between *Ph. bergeroti* (female) x F1 hybrid (male) produced offspring (Fryauff and Hanafi, 1991), suggesting that only male hybrids are fertile, which discards the application of Haldane's rule (Haldane, 1922). It was also observed that when compared to the females of *Ph. bergeroti*, the hybrid females initiated hematophagy more quickly (Fryauff and Hanafi, 1991), which could represent greater capacity and vectorial competence of these hybrids.

Subsequently, Ghosh et al. (1999) performed interspecific crosses between *Ph. papatasi* from two locations (Israel and India) and *Ph. duboscqi* Neveu-Lemaire, 1906 (which is also a vector of *Le. major*). This species is morphologically similar to *Ph. papatasi* and *Ph. bergeroti* and can occur in overlap with them (Ghosh et al., 1999). The authors observed the production of hybrids in the first generation (F1) only when the female used was *Ph. duboscqi* (Table 1). Subsequently, interbreeding and backcrossing of the F1 hybrids with the parents were performed, but in all cases there was no hatching of eggs (Ghosh et al., 1999) suggesting hybrid sterility and corroborating the specific status of these species.

Several molecular studies have shown that *Ph. papatasi* is closer to *Ph. bergeroti* than *Ph. dubosqi* (Esseghir et al., 1997; Depaquit et al., 2008; Khalid et al., 2010). This evolutionary relationship corroborates the results of the crossings performed by Fryauff and Hanafi (1991) and Ghosh et al. (1999) (Table 1), since reproductive incompatibility tends to be lower among the closest species, because pre-zygotic barriers have not yet been installed (the action of post-zygotic barriers being necessary to break the hybrid) (Neves et al., 2020). This can be seen for both crosses that showed post-zygotic barriers related to hybrid sterility (total or partial) (Table 1).

Taking into account the difficulty of identifying some groups of sandflies, characterization of possible natural hybrids becomes even more problematic, being restricted to two studies (Galati et al., 2010; Oliveira et al., 2016). Khalid et al. (2010), using specific molecular markers, were able to differentiate some species of *Phlebotomus* and characterize hybrids by species-specific PCR bands, highlighting the potential of this analysis for the detection of natural hybrids.

1.1.5. Crosses carried out between populations of *Phlebotomus*

Some intraspecific crosses between allopatric populations have also been carried out with *Phlebotomus* species, but have not shown reproductive isolation (Table 1). The crossing of two populations of *Ph. papatasi* from Israel and India, separated by almost 3500 km, showed genetic compatibility, producing viable offspring until the second generation (F2) (Table 1) (Ghosh et al., 1999). These results are in agreement with those observed later by Depaquit et al. (2008) that verified molecular homogeneity (with divergence less than 1%) among 22 populations of *Ph. papatasi*, distributed in 16 countries (including Israel and India).

Other experimental crosses were also performed between populations of *Ph. sergenti* Parrot, 1917 (Dvorak et al., 2006), vector species of *Le. tropica*, which although normally causes cutaneous leishmaniasis, has recently been reported to cause cases of visceral leishmaniasis (Akhoundi et al., 2016; Sarkari et al., 2016). It has already been verified through phylogenetic analyses based on the ITS-2 marker that the populations of *Ph. sergenti* form two distinct clades: one grouping populations to the northeast of the Mediterranean, and the other populations from the south and west (Depaquit et al., 2002). Because of this, these populations could present different vector capacities, and may even represent a species complex (Depaquit et al., 2002), which led Dvorak et al. (2006) to perform experimental crossings between populations of these two clades (one from Turkey and one from Israel), in order to assess the specific status of these populations. The authors verified that these populations do not present reproductive barriers, with the offspring being fertile and viable to F2 (Table 1). Later, in molecular analyses with the mitochondrial cytochrome B (cyt-B) gene, it was seen that the populations of Israel are, in fact, grouped with the populations of Turkey (including the one used in the crossing) (Dvorak et al., 2011), which justifies the absence of reproductive barriers between them.

Although the crossing performed by Dvorak et al. (2006) does not corroborate the existence of cryptic species in *Ph. sergenti*, there are other mitochondrial strains that have already been identified and the potential reproductive barriers not yet evaluated. In the work by Dvorak et al. (2011), for example, the authors identified three strains among the populations of Israel, Syria, Turkey, and Uzbekistan. Barón et al. (2008) identified four strains between the populations of Spain and Morocco, which even have ecological differences (Merino-Espinosa et al., 2016). Thus, taking into account that the crosses between *Ph. sergenti* were carried out between specimens

of the same strain (Dvorak et al., 2011), crosses involving populations of these other strains may help to clarify the possibility of this species being a complex of cryptic species.

Interpopulation crossings between *Ph. orientalis* Parrot, 1936 have also been carried out, one of the main vector species of *Le. donovani*, which causes visceral leishmaniasis in Africa and India (Killick-Kendrick, 1990, 1999; Akhoundi et al., 2016). Despite the great vectorial importance, there are populations of *Ph. orientalis* in endemic and non-endemic areas in Ethiopia (Seblova et al., 2013). Based on this, Seblova et al. (2013) carried out experimental crossings between two geographically distant populations: one from Addis Zemen (endemic area) and one from Melka Werer (non-endemic area). The absence of reproductive barriers between these populations was observed, with viable offspring being produced up to the second generation (F2) (Seblova et al., 2013). In addition, when evaluating the susceptibility to infection by *Le. donovani* in both populations, the authors did not verify differences between them, showing that other factors may be related to the absence of transmission in Melka Werer (Seblova et al., 2013).

1.2. Conclusion

In view of the results grouped in this review, it is evident that i) experimental crossings are important tools to aggregate studies of integrative taxonomy in the Phlebotominae subfamily; ii) these analyses should be applied in the taxonomic studies of cryptic species; iii) *Lu. longipalpis* populations have pre and/or post-zygotic reproductive barriers; iv) *Lu. longipalpis* represents more than one species and efforts must be applied to differentiate the taxa of the *Lu. longipalpis* complex; v) *Phlebotomus* populations do not present intraspecific reproductive barriers; vi) the

absence of reproductive barriers between *Ph. sergenti* from Israel and Turkey (representing populations of the same evolutionary lineage) does not rule out the possible existence of cryptic species, it being necessary to perform experimental crosses between the different strains indicated by the molecular markers; and finally, vii) different species of *Phlebotomus* have post-zygotic barriers, confirming the specific status of *Ph. duboscqi*, *Ph. papatasi*, and *Ph. bergeroti*.

Ethical Standards

The experiments comply with the current laws of the country in which they were performed.

Author Contributions

Yago Visinho dos Reis: Conceptualization, Methodology, Investigation, Writing-Original Draft Preparation.

Kaio Cesar Chaboli Alevi: Conceptualization, Validation, Formal analysis, Writing - Original Draft Preparation, Writing - Review & Editing, Visualization, Supervision, Project administration and Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Legends to tables

Table 1. Experimental crosses carried between several populations and species of sand flies.

| crosses | Reproductive barrier | References |
|---|--|---|
| Local crosses | | |
| <i>Ph. papatasi</i> (Brazil) x Morada Nova (Brazil) | Post-zygotic: probably hybrid inviability (F1 viable but with low hatch rates in all crosses) | Ward et al. (2004) |
| <i>Ph. papatasi</i> (Brazil) x Marajó Island (Brazil) | Post-zygotic: hybrid inviability (F1 produced in only one reciprocal cross but in low hatch rates) | Ward et al. (2004) |
| <i>Ph. papatasi</i> (Brazil) x Jacobina (Brazil) | Pre-zygotic: post-mating (Copulations occurred but the eggs didn't hatch) | Souza et al. (2004) |
| <i>Ph. papatasi</i> x Jacobina (Brazil) | Pre-zygotic: post-mating (Copulations occurred but the eggs didn't hatch) | Souza et al. (2004) |
| <i>Ph. papatasi</i> (Brazil) x Natal (Brazil) | Pre-zygotic: post-mating (Copulations occurred but the eggs didn't hatch) | Souza et al. (2004) |
| <i>Ph. papatasi</i> (Brazil) x Sobral-S2 (Brazil) | Pre-zygotic: behavioral isolation (Copulation didn't occur) | Ward et al. (2004) Souza et al. (2004) |
| <i>Ph. papatasi</i> (Brazil) x Bahia | Post-zygotic: Hybrid sterility in F1 (Maybe partial - females weren't evaluated) | Lanzaro et al. (2004) |
| <i>Ph. papatasi</i> (Costa Rica) | Post-zygotic: Hybrid sterility in F1 (Maybe partial - females weren't evaluated) | Lanzaro et al. (2004) |
| <i>Ph. papatasi</i> (Costa Rica) | Post-zygotic: Hybrid sterility in F1 (Maybe partial - females weren't evaluated) | Lanzaro et al. (2004) |
| | Reproductive compatibility until F2 | Ghosh et al. (2004) |
| | Reproductive compatibility until F2 | Dvorak et al. (2004) |
| <i>Ph. papatasi</i> (Ethiopia) x Melka Werer | Reproductive compatibility until F2 | Seblova et al. (2004) |
| Other crosses | | |
| <i>Ph. papatasi</i> x <i>Ph. carmelinoi</i> | Post-zygotic: Hybrid breakdown | Andrade-Filho et al. (1999) |
| <i>Ph. papatasi</i> (Israel) | Post-zygotic: Hybrid sterility in F1 | Ghosh et al. (2004) |
| <i>Ph. papatasi</i> (India) | Post-zygotic: Hybrid sterility in F1 | Ghosh et al. (2004) |
| <i>Ph. papatasi</i> x <i>Ph. papatasi</i> | Post-zygotic: Partial hybrid sterility (F1 produced in only one reciprocal cross; backcrosses produced offspring - Males hybrid are fertile) | Fryauff and (2004) |

Highlights

- ✓ We group all information related to hybridization in Phlebotominae;
- ✓ Experimental crossings are important tools to integrative taxonomy of the Phlebotominae;
- ✓ These analyzes must be applied in the taxonomic studies of cryptic species;
- ✓ Efforts must be applied to differentiate the taxa of the *Lu. longipalpis* complex.