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Phlebotomus (Paraphlebotomus) chabaudi and Phlebotomus riouxi: closely related species or synonyms?

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Abstract -- Phlebotomus riouxi Depaquit, Killick-Kendrick & Léger 1998 was described as a species closely related to Phlebotomus chabaudi Croset, Abonnenc & Rioux 1970, differing mainly by the size and number of setae of the coxite basal lobe. Molecular studies carried out on several populations from Algeria and Tunisia and based on mitochondrial genes cytochrome b (Cytb) and cytochrome oxidase I (COI) supported the typological validity of these two species. Recently, specimens from a single population in southern Tunisia were morphologically identified as Ph. riouxi, Ph. chabaudi and intermediates, but were clustered in the same clade according to their Cytb and nuclear gene elongation factor-1 alpha (EF-1α) sequences. These species were thus synonymized. To further explore this synonymy, we carried out a molecular study on specimens from Algeria and Tunisia using the same molecular markers and a part of 28S rDNA. We did not find any morphologically intermediate specimens in our sampling. We highlighted differences between the genetic divergence rates within and between the two species for the three markers and we identified new haplotypes. The sequence analysis did not reveal any signature of introgression in allopatric nor in sympatric populations such as in the Ghomrassen population. Phylogenetic analyses based on our specimens revealed that the two main clades are Ph. chabaudi and Ph. riouxi, in agreement with the morphological identification. These results support the validity of Ph. riouxi and Ph. chabaudi as typological species.

Keywords: Phlebotomus chabaudi, Phlebotomus riouxi, mitochondrial, nuclear and ribosomal markers, phylogenetic, North Africa

Résumé -- Phlebotomus (Paraphlebotomus) chabaudi et Phlebotomus riouxi: espèces proches ou synonymes ? Phlebotomus riouxi Depaquit, Killick-Kendrick & Léger 1998 a été décrit comme une espèce proche de Phlebotomus chabaudi Croset, Abonnenc & Rioux 1970, se distinguant principalement par la taille et le nombre de soies sur le lobe basal du coxite. Des études moléculaires, menées sur plusieurs populations d’Algérie et de Tunisie, ont montré que ces deux espèces, Ph. riouxi et Ph. chabaudi, étaient morphologiquement identifiées dans le même clade. Cette étude, menée avec des marqueurs moléculaires, a permis de révéler des nouveaux haplotypes, et d’exploiter les différences génétiques pour la validité des deux espèces. Ces résultats, réalisés avec des échantillons d’Algérie et de Tunisie, confirment la validité de Ph. riouxi et Ph. chabaudi comme espèces typologiques.

Keywords: Phlebotomus chabaudi, Phlebotomus riouxi, mitochondrial, nuclear and ribosomal markers, phylogenetic, North Africa

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Introduction

Within the Phlebotomus genus (Diptera, Psychodidae), the subgenus Paraphlebotomus Theodor 1948 includes some proven and suspected vectors of leishmaniasis, e.g. Phlebotomus sergenti, the main vector of Leishmania tropica [2,20]. Our study focuses on two species of Paraphlebotomus from North Africa: Phlebotomus chabaudi Croset, Abonnenc & Rioux 1970 and Phlebotomus riouxi Depaquit, Killick-Kendrick & Léger, 1998 [9,10,35]. The presence of Ph. chabaudi has also been reported in southern Spain [34].

Although their vectorial role has never been demonstrated, these two species are recorded in several leishmaniasis foci [3,28,40] and are related to Ph. sergenti. In fact, Ph. chabaudi and Leishmania killicki have been described for the first time in the same locality (Tataouine) in Tunisia [36], and L. killicki was also found in Algeria [24,26], especially in Ghardaïa, where some Ph. riouxi were reported, even though Ph. sergenti was the main proven vector [4].

In previous studies, Ph. chabaudi and Ph. riouxi males collected in Algeria and Tunisia were clearly identified morphologically. Molecular processing used two mitochondrial genes: a partial sequence of cytochrome b (Cytb-CB3) [6], as proposed by Esseghir et al. [17], and cytochrome oxidase 1 (COI) [5]. In both studies, phylogenetic analyses emphasized the validity of the two species, supporting their typological status, meaning that the deposited type-specimens are fully justified.

Recently, several specimens from Southern Tunisia showed ambiguous morphological characters [40,41]. According to these authors, several morphological criteria described as specific characters were found together in some specimens that they described as intermediate specimens. They used the same mitochondrial marker as that of Bounamous et al. [6], called Cytb-CB3, in order to compare their sequences with those available in GenBank. They also sequenced a longer fragment of Cytb (called Cytb-CB) and the nuclear elongation factor-1alpha gene (EF-1α) [30,41]. Their molecular results did not match with the morphological identification, not only for the intermediate specimens, but also for the differentiation between Ph. chabaudi and Ph. riouxi: all specimens were clustered in the same clade. According to these results, based on specimens from the single locality of Ghomrassen, they proposed to consider Ph. riouxi as a junior synonym of Ph. chabaudi.

In order to better understand the situation, we decided to broaden the approach by performing a comparative and combined sequence analysis of three loci on larger samples from different geographical populations we previously investigated. We included the two markers used by Tabbabi’s team [40,41], Cytb-CB and nuclear EF-1α, and the D1-D2 domain of ribosomal 28S DNA which is known as a good marker for studying the interspecific genetic divergence between species [19,22,38]. This domain has specifically been used to perform analysis at the taxonomic level in Phlebotominae [11,13,31].

Material and methods
Sample collection

Samples analyzed in the present study were those used by Tabbabi et al., Bounamous et al. and Boudabous et al. [5,6,40,41]. For the Tabbabi samples included in this analysis, we only had access to published data. All the other specimens came from our laboratory, including samples used by Bounamous et al. and Boudabous et al. [5,6], for which we kept the same sample codes marked in bold in Tables 1 and 3. Our specimens were collected by CDC miniature light traps and sticky paper traps from two regions of Algeria (Ghardaïa and Aurès) and from three regions of Tunisia (Mahdia, Monastir and Ghomrassen) (see Table 1 and Figure 1).

Specimens were stored in 95% ethanol at −20°C, until dissection. After thawing, each specimen was dissected individually in 95% ethanol with sterile needles. The head and the genitalia were cleared in boilded Marc André solution and mounted in chloral gum between the slide and cover slide for microscopic observation. The rest of the body was dried and preserved at −20°C in a sterile microtube until DNA extraction.

Taking into consideration the difficulty in identifying females, we restricted the number of females in our sampling. The 9 females and 12 males of Tabbabi’s study [41] are represented in Figure 1 as black symbols. Unfortunately, we did not have access to these specimens. However, all the specimens studied by Bounamous et al. and Boudabous et al. [5,6], as well as the new samples, were morphologically examined or re-examined in the present study, according to the criteria previously described [6,9,10].

The morphological analyses were focused on the basal lobe of the coxite, known to be the differential character between the two species under study [6,10]. For each processed specimen, the number of coxite lobe setae was counted and morphometrics analysis, using Stream Motion 1.9.1 software (Olympus, Japan), was also

aucune signature d’introgression que ce soit dans les populations allopatrices ou sympatiques telle que la population de Ghomrassen. Les analyses phylogénetiques basées sur nos échantillons ont révélé que les deux principaux clades correspondent à Ph. chabaudi et Ph. riouxi, résultat en accord avec l’identification morphologique. Ces résultats soutiennent la validité de Ph. riouxi et Ph. chabaudi comme espèces typologiques.
**Table 1.** Analyzed samples. On a grey background, the samples processed by Tabbabi’s team. Samples in bold were used by Bouamoun et al. and Boulabous et al. [5,6]. Samples not sequenced with all markers are underlined and their accession number replaced by ND.

| Country | Area         | Species      | Number of specimens | Sex | Codes | Cytb-CB Accession Numbers | EF-1α Accession Numbers | D1/D2 Accession Numbers |
|---------|--------------|--------------|---------------------|-----|-------|----------------------------|------------------------|-------------------------|
| Tunisia | Ghomrassen   | *Ph. chabaudi* | 1                   | f   | 1377  | KC478308 \*                 | KC478288/KC478289 \*   | /                       |
|         |              |              | 3                   | m   | 573 1331, 1335 | KC478290, KC478288/KC478289, | KC478289/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288 | \*                       |
|         |              |              |                     |     |       | KC478305, KC478288/KC478288, | /                       | \*                      |
|         |              |              |                     |     |       | KC478306, KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288 | /                       |
|         |              |              |                     |     |       | KC478293, KC478300, ND       | /                       | /                       |
|         |              | *Ph. riouxi*  | Unclear             | 3   | 571 912, 1495 | KC478293, KC478288/KC478288, | /                       | /                       |
|         |              |              |                     |     |       | KC478292, KC478289/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288 | /                       |
|         |              |              |                     | 8   | 558 1311, 547, 556, 658, 892, 980, 1339 | KC478292, KC478289/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288/KC478288 | /                       |
|         |              |              |                     | 6   | 1520, 624, 906, 916, 954, 966 | /                       | /                       |
|         |              |              |                     |     |       | /                       | /                       | /                       |
| Tunisia | Ghomrassen   | *Ph. riouxi*  | 2                   | m   | TAT63, TAT74 | KY764775, KY764776           | KY764779, KY764780       | KY764613, KY764614     |
|         |              |              | 3                   | f   | TAT186, TAT23, TAT24 | KY764777, Ky764779 | KY764721-KY764723 | KY764615-KY764617     |
| Mahdia  | *Ph. chabaudi* | 8             | SMA149, SMA160, SMA161, SMA162, SMA163, SMA46, SMA62 | 8   | 558 1311, 547, 556, 658, 892, 980, 1339 | /                       | /                       |
| Monastir| *Ph. chabaudi* | 35            | SMO326, SMO332, SMO335, SMO336, SMO337, SMO340, SMO343 | 35  | SMO345, SMO359, SMO460, SMO463, SMO465, SMO469 | /                       | /                       |
|         |              |              | SMO475, SMO478, SMO485 | 35  | SMO490, SMO493, SMO497, SMO498, SMO499, SMO500 | /                       | /                       |
|         |              |              | SMO505, SMO508, SMO515, SMO516 | 35  | SMO517, SMO518, SMO520, SMO521, SMO522, SMO523 | /                       | /                       |
|         |              |              |                     | 3   | SMO524, SMO525, SMO526 | KY764740-KY764742 | KY764677-KY764679 | KY764635-KY764637     |
| Algeria | Ghadamis     | *Ph. riouxi*  | 8                   | m   | RX1, RX2, RX3, RX5, RX6, RX7, RX8, RX9 | KY764764-KY764787       | KY764724-KY764731       | KY764625-KY764628     |
|         |              |              | 3                   | m   | CBI, CB2, CB3 | KY764771-KY764777 | KY764715-KY764717       | KY764666-KY764668     |
|         |              |              | 1                   | f   | CBZAT5583 | KY764774-KY764777 | KY764666-KY764668       | KY764669               |

applied. In 1998, it was suggested that the number of setae, and the width and length of the coxite lobe were informative to differentiate the species [10]. The width measure did not cause any difficulty: a transversal line was perpendicularly traced in the larger part of the lobe. Considering the difficulties in measuring the length of the basal lobe of the coxite, we substituted it by its perimeter and area, as indicated in Figure 2.

**DNA extraction, PCR and sequence analysis**

Some DNA extracts (sample codes in bold in Tables 1 and 3) from previous studies [5,6] were simply thawed for direct PCR amplification. For the other specimens, we used the same procedure as for the older extracts: DNA extractions were individually carried out using a QIAamp DNA Mini Kit (Qiagen), according to the manufacturer’s protocol. Samples were crushed in ATL buffer with a piston pellet and DNA extracts were eluted in 180 μL to 200 μL of AE buffer and stored at −20 °C. Cytb-CB was amplified using CB1-SE: 5′-TATGTACTACCCTGAG-GACAATATC-3′ and CB-R06: 5′-TATCTATGTGTTCAAAACATGTC-3′ primers, as previously described [41]. For EF-1α amplification, primers EF-F05: 5′-CCT-GGACATCGTGTATTCGAT-3′ and EF-R08: 5′-CCCAATCTCTTGGTAGCAGCATCTCG-3′ were used [41]. The ribosomal domain D1-D2 was amplified using CI: 5′-ACCCTGCAATTTAGCCAGCAT-3′ and D2: 5′-TCCCTGTGTTTCAGACGGG-3′ primers [32]. The PCR conditions for Cytb-CB and EF-1α were exactly the same as those used by Tabbabi et al. [41]. All PCRs were performed in a 50 μL volume using 5 μL of extracted DNA solution of each specimen individually, and 12.5 pmol of the primer sets in a thermocycler. The PCR mix contained (final concentrations) 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.01% Triton X-100, 200 mM dNTP each base, and 1.25 units of Taq polymerase (Eppendorf, Germany). For each PCR run, a negative control using 5 μL of ultrapure sterile water and a positive control using a DNA extract with a known sequence, were included. PCR
programs were carried out with an initial denaturation step at 94°C for 3 min and finished by an extension step at 68°C for 10 min. Between these two steps, cycling program parameters depended on the markers: for Cytb-CB, 40 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec, and extension at 68°C for 1 min; for D1-D2, 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 68°C for 1 min; for EF-1α, 5 cycles of denaturation at 94°C for 30 sec, annealing at 44°C for 30 sec, and extension at 68°C for 1 min, followed by 30 similar cycles with an annealing temperature at 48°C [29]. All PCR products were first verified using the molecular weight marker 100 bp DNA Ladder (Promega) in GelGreen (Biotium) stained 1.5% agarose gel electrophoresis. The PCR products were then sequenced in both directions on a Beckman Coulter Genomics sequencer, with the same primers used for PCR except that CB1-SE was replaced by CB1: 5'-TATGTACTACCATGAGGACAATATC-3', as mentioned by Tabbabi et al. [41]. Sequences were then aligned with the alignment editor implemented in BioEdit 7.0.8.0 [23] and checked by eye. Sequence alignments were performed respecting the following criteria: (1) to minimize the number of inferred mutations (number of steps); (2) to prefer substitution to insertion-deletion, and (3) to prefer transitions to transversions. Genetic divergences between the sequences were measured using the Tamura-Nei model and the presence of open reading frames (ORFs) was checked using MEGA6 [42]. Tabbabi’s sequences were used as the reference to perform alignment. The primer sequences were removed. Phylogenetic inferences derived from maximum likelihood (ML) for each gene separately were performed using the PhyML 3.0 software program [21]. For these analyses, the best fitting nucleotide substitution model was determined through the automatic model selection tool available on the PhyML server. Then, we used RAxML software [39] for a partitioned ML analysis with a GTR (general time reversible) model for the combined analysis of the three genes. Each gene was considered as a different partition and a specific separate substitution model was assigned. For each analysis, bootstrapping was used to test the branch strength of the phylogenetic trees. For all phylogenies, we used one sequence of Ph. sergenti as the outgroup. The trees were then visualized using TreeDyn, version 198.3 [7]. Whenever possible, sequences of Ph. chabaudi published by Tabbabi et al. [41] were added to the analyses.

Figure 1. Sampling locations. Numbers indicate the number of specimens studied with round and square symbols corresponding to Ph. chabaudi and Ph. riouxi, respectively. Samples with a black fill come from Tabbabi’s sampling while those with a white fill come from our sampling. The three uncertain specimens of Tabbabi are indicated by a rounded square.

Figure 2. Illustration of the procedure of morphometrical measures of area and perimeter of the basal lobe of the coxite. Left, unmarked; right, marked with perimeter and area.
identiﬁed 17 haplotypes of Tabbabi from the Ghomrassen area, clade was composed of a mix of our own Cytb-CB between Ph. chabaudi < geographical subdivision between Algeria (bootstrap value = 97.4%). The second clade (98.6%) and a lower one with 17% and 11%, respectively. Concerning our sampling, haplotypes in our 43 Ph. chabaudi samples had an intraspeciﬁc divergence of 0.60% (SD = 0.13%), and interestingly, a higher intraspeciﬁc divergence in the 13 Ph. riouxi with 12 haplotypes: 4.38% (SD = 0.59%).

Results

Morphological identiﬁcation

All of our specimens were correctly identiﬁed as Ph. chabaudi and Ph. riouxi, according to morphological criteria of initial descriptions for males [9,10] and to the pharyngeal armature for females [6]. All females were morphologically identiﬁed based on the presence or absence of anterolateral teeth in the pharynx. All measures related to the coxite lobe are listed in Table 2. Considering these data, we found signiﬁcant interspeciﬁc differences between all measurements of the basal lobe of the coxite, without signiﬁcant geographical variability (data not shown). We did not ﬁnd any morphological intermediate specimen. All Ph. riouxi were found in the south of Tunisia and in the south of Algeria.

Sequence analyses

A total of 63 specimens were analyzed and compared to the sequences of the 21 specimens from Tabbabi et al. [41] (Table 1).

Cytochrome b analyses

For the 56 specimens successfully ampliﬁed, the length of the cytochrome b fragment Cytb-CB used for analysis was 628 bp. The phylogenetic tree obtained by adding 17 haplotypes of Tabbabi’s data [41] highlighted two distinct clades (Figure 3). The ﬁrst one was only composed of Ph. chabaudi as supported by a bootstrap value of 88.6%, with a geographical subdivision between Algeria (bootstrap value < 50%) and Tunisia (bootstrap value = 97.4%). The second clade was composed of a mix of our own Ph. riouxi and the 17 haplotypes of Tabbabi from the Ghomrassen area, identiﬁed as Ph. chabaudi, Ph. riouxi and intermediates. For this clade, the bootstrap value was lower (59.6%). Within this clade, the specimens were also subdivided into two clusters according to the geographic area, i.e. southern Tunisia versus southern Algeria but with low bootstrap values < 50% and = 62.2%, respectively.

We obtained congruent results between morphology and molecular observations in our samples, with an interspeciﬁc genetic divergence of 12.2% (SD = 1.38%) for Cytb-CB between Ph. chabaudi and Ph. riouxi. Eleven haplotypes in our 43 Ph. chabaudi samples had an intraspeciﬁc divergence of 0.60% (SD = 0.13%), and interestingly, a higher intraspeciﬁc divergence in the 13 Ph. riouxi with 12 haplotypes: 4.38% (SD = 0.59%).

EF-1α analyses

EF-1α ampliﬁcation gave a fragment of 454 bp in length. All variable sites of 83 sequences alignment are shown in Table 3. One genotype R01 (01/01) was found in all 13 specimens we identiﬁed as Ph. riouxi. They corresponded to genotype 01/01 of Tabbabi’s samples (=genotype R01 according to our label). Interestingly, Ph. chabaudi showed very different genotypes (C01 to C16) compared to R01, with ﬁve different bases between the two clades, as seen in the phylogenetic tree based on genotypes (Figure 4). These data led to a high bootstrap value for the Ph. riouxi clade (98.6%) and a lower one (51.4%) for Ph. chabaudi.

Analyses considering genotypes were performed as we obtained several heterozygous positions (double peaks), represented by ambiguous bases in the sequences, for three specimens (Table 3). Indeed, several ambiguous positions were detected in some specimens in the same sequence, precluding the haplotype determination. This is the case for genotypes C12 and C14, corresponding to three Ph. chabaudi. All double peaks were located in the third position of the codon without changing the amino acid translation. Overall, on the 46 samples for which the haplotypes could be determined, the frequency of the major haplotype was 30% (haplotype 05), followed by haplotype 10 and haplotype 06 with 17% and 11%, respectively. Concerning our sampling, interspeciﬁc divergence of EF-1α between Ph. riouxi and Ph. chabaudi was 1.15% (SD = 0.53%), and intraspeciﬁc divergences were 0 and 0.009%, respectively, corresponding to normal values for this marker [43].

Conversely to Tabbabi’s studies [40,41], the sequences obtained in our sampling were congruent with the morphological identiﬁcation.

D1-D2 analyses and concatenate analyses

D1-D2 ampliﬁcation of our 55 samples gave fragments from 712 to 714 bp in length. For this marker, the

| Table 2. Morphological data of male samples: for each species, mean and standard deviation (sd) are calculated and minimal (min) and maximal (max) values are indicated. |
|---|---|---|---|---|
| Species | Coxite lobe area (µm²) | Coxite lobe perimeter (µm) | Coxite lobe width (µm) | Number of setae per coxite lobe |
| **Ph. riouxi** | | | | |
| mean | 1154 | 160 | | |
| sd | 118 | 7 | | |
| min | 926 | 148 | 23 | 28 |
| max | 1331 | 167 | 32 | 43 |
| **Ph. chabaudi** | | | | |
| mean | 415 | 95 | 14 | 11 |
| sd | 94 | 11 | 2 | 2 |
| min | 209 | 65 | 10 | 7 |
| max | 609 | 116 | 18 | 17 |
Figure 3. Phylogenetic tree inferred from cytochrome B data of *Phlebotomus chabaudi* and *Ph. riouxi* specimens. We added to the analysis the sequences of *Ph. chabaudi* published by Tabbabi et al. (2014). The phylogram results from bootstrapped data sets obtained using the PhyML 3.0 program [21] using GTR (general time reversible) + $\Gamma$ distribution (gamma distribution of rates with four rate categories). The tree was visualized using the TreeDyn program, version 198.3 [7]. The percentages above the branches are the frequencies with which a given branch appeared in 500 bootstrap replications. Only bootstrap values higher than 50% on the early branches are shown. A sequence of *Ph. sergenti* (AF161216) was used as the outgroup. The sequences marked by * were published by Tabbabi et al. (2014); R = sequences found in specimens morphologically characterized as *Ph. riouxi*. C = sequences found in specimens morphologically characterized as *Ph. chabaudi*. RC = sequences found in specimens morphologically characterized as *Ph. chabaudi* or *Ph. riouxi*. Int = sequences found in specimens morphologically characterized as intermediate between *Ph. riouxi* and *Ph. chabaudi*. 
interspecific genetic divergence between Ph. chabaudi and Ph. riouxi was 0.50% (SD = 0.26%) and the intraspecific divergence was 0 and 0.1%, respectively. This value (0.50%) was close to the genetic divergence observed between the two well-separated species Ph. chabaudi and Ph. sergenti (0.74%, SD = 0.31%). The lack of or the very low intraspecific divergence can be explained by the low mutation rate of this conserved marker [19,22,38].

Phylogenetic analysis based on the sequences of the D1-D2 domain of 28S rDNA allowed us to differentiate the two species by their clustering into two main clades (Figure 5). In spite of a low genetic divergence, the bootstrap value was strong for the Ph. riouxi clade (89.6%) and 68.6% for Ph. chabaudi.

Concatenated analyses of the three loci using a partitioned ML model also showed clear clustering in two clades corresponding to the morphological identifications of Ph. chabaudi and Ph. riouxi (Figure 6). The bootstrap values were 70.6% for the Ph. chabaudi clade and 92.8% for the Ph. riouxi clade. As indicated by the comparison of the trees, we did not find any signs of introgression.

**Discussion**

*Phlebotomus chabaudi* was described for the first time in Tunisia [9] as *Paraphlebotomus* with a sharply pointed aedeagus (Figure 7, A and D). The same year, this species was also recorded in Algeria [33] and was described with a larger and more tufted basal lobe (Figure 7, E and F). The authors linked this observation to variability due to the geographically segregated populations. These two morphs have been found in sympatry without intermediate specimens [10], justifying the description of a new species: Ph. riouxi (Figure 7, B, C, E, F).

The morphological identification of the female remains very difficult. It is clearly not possible to differentiate the spermathecae of the two species (Figure 8, C and D).

### Table 3. Base variability in EF-1α genotypes. On a grey background: four alleles found by Tabbabi et al. (2014) (01 to 04 = KC478288 to KC478291). On a white background: 16 new genotypes found in our sample (n = 62), composed of 12 new haplotypes (05 to 16); ND = Non Determinate. Samples in bold were used by Bounamous et al. and Boudabous et al. [5,6].

| Species | Number of specimen | Genotype | Codes | Positions of polymorphic sites |
|---------|-------------------|----------|-------|--------------------------------|
|          |                   |          |       | 1 1 1 1 2 2 2 3 3 4 4 4 4 4 4 |
| Ph. riouxi / Ph. chabaudi | 17 | 01/01 | 558, 1311, 571, 1520, 547, 556, 573, 624, 658, 906, 916, 954, 966, 1331, 1335, 1339, 1495 | G G A T A G G G A C C T C C A |
|          | 2 | 01/02 | 892, 1377 | S S S S S S S S S S S S S S S S |
|          | 1 | 02/03 | 980 | S S S S S S S S S S S S S S S S |
|          | 1 | 01/04 | 912 | . . . . . . . . . . . . . . . . |
| Ph. riouxi | 13 | R01 (01/01) | RX1, RX2, RX3, RX5, RX6, RX7, RX8, RX9, TAT186, TAT23, TAT24, TAT63, TAT74 |
| Ph. chabaudi | 8 | C01 (05/05) | SMA60, SM01022, SM0194, SMO430, SMO554, SMO873, SMO877, SMO911 |
|          | 2 | C02 (06/06) | CB1, SMA149 |
|          | 1 | C03 (07/07) | CB2 |
|          | 2 | C05 (09/09) | SMO919, SMO64 |
|          | 4 | C06 (10/10) | CBZAT583, SNA69, SMO310, SMO565 |
|          | 3 | C07 (11/11) | SMO892, SMO929, SMO929 |
|          | 6 | C08 (08/11) | CB3, SMA161, SM01024, SMO575, SMO983, SMO931 |
|          | 2 | C09 (12/12) | SMO1021, SMO443 |
|          | 1 | C10 (13/13) | SMA163 |
|          | 6 | C11 (13/14) | SMA160, SM0112, SMO569, SMO578, SMO895, SMO898 |
|          | 1 | C12 (ND) | SM0891 |
|          | 1 | C13 (15/15) | SM0562 |
|          | 2 | C14 (ND) | SMO560, SM0899 |
|          | 1 | C15 (05/16) | SMO921 |
|          | 8 | C16 (05/10) | SMA162, SM0419, SMO559, SMO886, SMO888, SMO918, SMO926, SMO941 |
Figure 4. Phylogenetic tree inferred from *Phlebotomus chabaudi* and *P. riouxi* specimens using the data of elongation factor 1-α gene. Sequences of *P. chabaudi* published by Tabbabi et al. (2014) were added to the analyses. The phylogram results from bootstrapped data sets obtained using the PhyML 3.0 program [21] using the HKY85 [25] + I (proportion of invariant sites) model. The tree was visualized using the TreeDyn program, version 198.3 [7]. Percentages shown above the branches are the frequencies at which a given branch appeared in 500 bootstrap replications. Only bootstrap values higher than 50% on the early branches are shown. A sequence of *P. sergenti* (EF416841) was used as the outgroup. The sequences marked by * were published by Tabbabi et al. (2014); R = sequences found in specimens morphologically characterized as *P. riouxi*. RC = sequences found in specimens morphologically characterized as *P. chabaudi* or *P. riouxi*. Int = sequences found in specimens morphologically characterized as intermediate between *P. riouxi* and *P. chabaudi*. RCint = sequences found in specimens morphologically characterized as *P. riouxi*, *P. chabaudi* and intermediate specimens between the two species.
Figure 5. Phylogenetic tree inferred from *Phlebotomus chabaudi* and *Ph. riouxi* specimens using the data of D1-D2 domain of 28S rDNA. Sequences of *Ph. chabaudi* published by Tabbabi *et al.* (2014) were added to the analyses. The phylogram results from bootstrapped data sets obtained using the PhyML 3.0 program [21] using the HKY85 model [25]. The tree was visualized using the TreeDyn program, version 198.3 [7]. The percentages above the branches are the frequencies with which a given branch appeared in 500 bootstrap replications. Only bootstrap values higher than 50% on the early branches are shown. A sequence of *Ph. sergenti* (KY764627) was used as the outgroup.
Although Depaquit et al. [10] suggested examining the appearance of the armature in the genital atrium, this criterion remains uncertain. Regarding the pharynx, Bounamous et al. [6] noted the presence of anterolateral teeth in *Ph. chabaudi*, a character not found in *Ph. riouxi* (Figure 8, A and B). They suggested the use of this character to identify these two species, pending a larger sampling. Nevertheless, it seems that the individual variability of the pharyngeal armature of *Ph. chabaudi* makes this distinction hazardous for a non-trained entomologist.

Consequently, we selected a majority of males in the present study in order to reduce the risk of misidentification. Only a few females for which the morphological identifications were congruent with molecular analyses were included (those previously processed by Bounamous et al. and Boudabous et al. [5,6]) (Table 1). In the study by Tabbabi et al. [41], out of 21 specimens, two-thirds (n = 14) were morphologically identified as *Ph. riouxi* out of which 6 were males and 8 were females. Interestingly, despite the difficulty in identifying the females, all ambiguous specimens were males. Unfortunately, we could not include the specimens processed by Tabbabi in the present study and we did not find any intermediate specimens in our collection. Consequently, we performed a detailed phylogenetic analysis of *Ph. chabaudi* and *Ph. riouxi* specimens selected from our collection, and we added the sequences published by Tabbabi et al. [41]. Our molecular study included three independent markers. The long fragment of Cytb-CB codes for a partial protein sequence of this gene, whereas the shorter fragment (Cytb-CB3) is more frequently used for taxonomic studies [17]. Tabbabi et al. [40,41] analyzed the Cytb-CB fragment that they considered more informative than the Cytb-CB3 fragment. The interspecific (between closely related and/or vicariant species) and intraspecific divergence values observed for the mitochondrial cytochrome b (Cytb-CB and Cytb-CB3) in *Phlebotomus* spp. are 2.7–11% and 0.1–2.5%, respectively [12,17,27]. The 12.2% interspecific value between *Ph. chabaudi* and *Ph. riouxi* supports the genetic differentiation of these two taxa. Regarding the intraspecific values, the value for *Ph. chabaudi* (0.6%) was in the commonly accepted range. For *Ph. riouxi*, the value (4.38%) is higher than the accepted range but close to the value observed in *Sergentomyia clydei* Sinton 1928, displaying a value of 5.5% due to a divergent population from the Seychelles [32].

The nuclear EF-1α is known to be a good phylogenetic marker in Metazoa [37] and was previously used in several molecular studies in Phlebotomine sandflies [1,16,18,29,43]. Its utility in other groups has also been demonstrated in heliothine moths [8] and in Triatominae [14]. We selected this marker to compare our data with those of Tabbabi et al. [41]. Several studies successfully compared haplotypes of EF-1α [41,44], although EF-1α also showed considerable diversity of haplotypes for a same specimen, thus complicating the analyses [29,43]. In the present study, we also noted significant haplotype diversity. Ribosomal marker D1-D2 does not have this disadvantage. Indeed, this marker is present in many homogeneous copies in the genome, thus providing a good signal that is easy to use as a genetic marker [38]. However, the nuclear ribosomal DNA may provide only a short-term
marker for introgression, because of the homogenization of the multi-copy genes at this locus [15]. This marker is independent of the two previous ones and is more conserved [13]. We used this marker in previous studies and showed its usefulness for phylogenetic analysis [11,31,32].

Several ambiguous bases were observed in EF1-α sequences. Nevertheless, these ambiguous positions do not correspond to intermediate profiles between the two species. All our Ph. riouxi specimens from Algeria and Tunisia revealed the genotype R01 (homozygotes) corresponding to the EF_chab01 haplotype defined by

Figure 7. Differentiation criteria of males (A to F) 100X. A and D: aedeagus and basal lobe of coxite of Ph. chabaudi (SMO562); B and E: aedeagus and basal lobe of coxite of Ph. riouxi from Algeria (RX2); C and F: aedeagus and basal lobe of coxite of Ph. riouxi from Tunisia (TAT63). All photographs are set on the same scale.
Tabbabi et al. [41] (Table 3), in agreement with the results of Boubidi et al. [4]. In contrast, we obtained many new sequences of EF-1α in Ph. chabaudi called C01 to C16 (Table 3), providing five synapomorphic nucleotide substitutions that distinguished the two species.

Cytb and EF-1α have already been combined to demonstrate mitochondrial introgression in New World Phlebotomine sandflies [43]. Our study did not find any introgression between the two species under examination, as confirmed by the ribosomal D1-D2 analyses.

The independent phylogenetic analyses of the three genes (Figures 3, 4 and 5) underlined the subdivision of Ph. chabaudi and Ph. riouxi specimens into two independent clades. Nevertheless, our data support low genetic divergence between the two species, suggesting recent differentiation between these two species. This low divergence is confirmed by the low bootstrap values observed in Cytb phylogeny for the Ph. riouxi branch (Figure 3), in EF-1α phylogeny for the Ph. chabaudi branch (Figure 4), and in D1D2 phylogeny for the Ph. chabaudi branch (Figure 5). The phylogeny of the concatenated genes revealed bootstrap values above 70% for the two branches, suggesting that added data can only increase the differentiation between the two species.

When we consider the two phylogenies including the sequences published by Tabbabi et al. [41], i.e. Cytb and EF-1α phylogenies (Figures 3 and 4), all Tabbabi’s sequences are included in the Ph. riouxi branch, without clear distinction between our specimens and Tabbabi’s specimens. From these data, it is difficult to explain the disagreement between morphological characters and molecular data observed by Tabbabi et al. It would thus appear essential to further investigate these samples on genetic and morphological grounds to make a comparison with Ph. chabaudi and Ph. riouxi specimens from Algeria and North Tunisia.

It is worth noting that the specimens in the Ph. riouxi branch were only collected in Southern Algeria and Southern Tunisia, and that the specimens in the Ph. chabaudi branch were only collected in Northern Algeria and Northern Tunisia. This suggests that related evolution of these two taxa between the South of these two countries for Ph. riouxi and between the North of these two

![Ph. chabaudi](image1.png)

![Ph. riouxi](image2.png)

**Figure 8.** Differentiation criteria of females (A to D), 100X. A and C: pharynx and spermathecae of Ph. chabaudi (CBZAT583); B and D: pharynx and spermathecae of Ph. riouxi (TAT186 and TAT24). All photographs are set on the same scale.
countries for *Ph. chabaudi*. The molecular clock of Cytb has been calculated for *Ph. papatasii* (Scopoli, 1786) and *Ph. duboscqi* Neveu-Lemaire 1906, two vicariant species separated by the Sahara. Its estimated calibration ranged from 1 to 2.5% variability per million years [17] or from 1.34 to 2.64% per million years [18]. *Ph. chabaudi* and *Ph. riouxi* exhibit a Cytb mean interspecific genetic divergence of 12.2%. If we apply this calibration to the latter species, we hypothesized their speciation started between 12.2 and 4.62 Mya. This period corresponds to the aridification of the Sahara desert (10 to 6 Mya). The vicariance of *Ph. chabaudi* and *Ph. riouxi* could result from the same event as the vicariance of *Ph. papatasii* and *Ph. duboscqi*. The presence of intermediate specimens as described by Tabbabi et al. [41], as well as specimens with morphological criteria corresponding to *Ph. chabaudi* in the South of Tunisia suggests the sympathy of the two species in Ghomrassen, which could be explained by a mixing of the two species. Only the investigation of sympatric populations will answer the unresolved question of whether or not the two lineages usually behave as true biological species when they meet. Further morphological and molecular studies on a larger sample of *Ph. riouxi* (e.g. from Ghomrassen) and on more genes remain necessary to help in determining the evolutionary history of these two species.

Finally, these results still support the existence of two species, and their typological validity, thus refuting *Ph. riouxi* as a junior synonym. The close genetic relationships and the intermediate specimens detected by Tabbabi et al. [41], however, suggest a recent speciation phenomenon followed by several migration events. Further genetic and morphological studies of specimens from Algeria, Tunisia and Morocco will help to better understand the evolution of these two species in North Africa.

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