# DNA Taxonomy and the identification of immature insect stages: the true larva of *Tauriphila argo* (Hagen 1869) (Odonata: Anisoptera: Libellulidae)

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**Abstract.** For many insect taxa, larval morphology plays a decisive role in various fields like taxonomy, phylogeny or ecology. However, species identification is usually based on imaginal characters and the identification of larvae depends upon an established link to unequivocally identified imagines. This taxonomic correspondence of larvae and imagines is far from being established in many odonate species. We have employed a molecular approach to link larval and adult specimens in *Tauriphila argo* (Hagen, 1869). The sequenced mt SSU gene fragments of the reared female, supposedly a *T. argo* female, and a clearly identified male specimen of the species were identical. However, the larva of the reared female clearly differed from the described *T. argo* larva, previously matched to the species. From this observation, we conclude that the previously described larva of *T. argo* does not belong to this species because of too many phenotypic differences that far exceed the generally observed intraspecific variation.
It can be foreseen that the molecular approach will prove to be effective in identifying unknown larvae in many insect species. Additionally, the discrimination of sibling species or the linkage of allotypes and holotypes will become feasible with this approach.

**Résumé. Taxonomie ADN et identification des stades larvaires : la véritable larve de *Tauriphila argo* (Hagen 1869) (Odonata : Anisoptera : Libellulidae).** La connaissance des larves de certains groupes d'insectes s'avère importante dans des domaines aussi variés que la taxonomie, la phylogénie ou l'écologie. Or la correspondance entre la larve et l'adulte est loin d'être établie pour la majorité des espèces. Jusqu'à présent la seule méthode pour réaliser ce lien est d'élever la larve jusqu'à l'obtention de l'imago. Cette méthode est longue, fastidieuse et n'aboutit pas toujours. Nous avons utilisé l'outil moléculaire pour établir ce lien en comparant les séquences ADN de deux spécimens. Cet outil peut également s'avérer très efficace pour discriminer des espèces jumelles ou faire correspondre un type mâle avec une femelle supposée appartenir à la même espèce. C'est grâce à cet outil que nous avons pu déterminer de façon sûre la larve de *Tauriphila argo* (Hagen, 1869). La larve précédemment décrite attribuée à *T. argo* ne devrait pas appartenir à cette espèce étant donné le nombre important de différences qui dépasse de loin celui d'une simple variation intraspécifique.

**Keywords:** Molecular taxonomy, ribosomal genes, identification of larvae

Taxonomy is one of the foundations of ever biological discipline. Without taxonomy, evolutionary analyses, ecological questions or issues of conservation biology can not satisfyingly be addressed. Traditionally, taxonomy is based on phenotypical analyses. This approach has still its merits since phenotypical variability is readily accessible and inexpensively scored and it is obvious that morphological variability can be regarded as a straightforward approximation of biological species delimitation. However, taxonomy based on morphological characters has also its limitations. Limitations of morphological taxonomy are particularly striking in cases where it becomes necessary to discriminate between closely related species like sibling species which are often hardly recognizable on a phenotypic level. The advent of molecular methods offers an elegant solution to these obstacles. With molecular characters it becomes possible to increase resolution of taxonomic work by including genotypic information on species differentiation. The application of DNA taxonomy was recently advocated by several authors (Godfray 2002, Hebert *et al.* 2002, Pilgrim *et al.* 2002, Tautz *et al.* 2002, 2003). It is quite obvious that DNA taxonomy will display its major strength in groups of negligible morphological differentiation and economic importance. Among those are certainly many soil organisms or insects. In addition to species identification, DNA taxonomy offers also other fields of application. In many insects, it is impossible to identify species from immature stages. This problem

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Accepté le 12 mai 2005

is not restricted to holometabolous insects, since in many hemimetabolous insects immature stages are adapted to completely different ecological conditions compared to imagines, complicating the process of species identification. Molecular methods allow to establish a link between immature stages and safely identified adult organisms. This link can become important if immature stages are the most easily accessible organismal life stages. For example, the imagines of many tropical dragonfly species are indeed rarely caught, because their flight period often lasts only a few weeks and in this time their behaviour depends on meteorological conditions, additionally they often are swift or stay in the canopy (Fleck, Misof pers. obs., Legrand, Theischinger, pers comm.). On the contrary, larvae are frequently collected in rainforest water bodies (Fleck, Misof, pers. obs, Legrand, Theischinger, pers comm.).

We have used a molecular approach to establish the link between known adult dragonfly species and undescribed larvae. For many odonate species, larvae are completely unknown. Many larvae are described, but the link to already described adult species is only established by supposition. The classical way to establish a link between a larva and its adult organism is to rear larvae until they moult into imagines. However, this procedure is time consuming, tedious and often fails. Additionally, for many species the most reliable identification is provided by male specimens. Furthermore, for a few dragonfly species groups, imagines are readily identified but the corresponding larvae lack morphological differentiation between species, e.g. within the genus *Macromia* (Macromiidae). Due to this lack, the degree of biodiversity within a given water body might often become grossly underestimated. In large scale biodiversity assessments the correct identification of immature stages is a central task. DNA based identification is a clear solution to these problems. With a molecular approach it is possible to establish a link between undescribed larvae and described imagines, indistinguishable larvae and described imagines, or undescribed allotypes and described males or females.

DNA taxonomy has to rely on highly variable molecular markers in order to yield high resolution in taxonomic problems. If fairly conserved gene fragments will be used the informativeness of the approach is strongly diminished. In our study we used mitochondrial SSU rRNA gene fragments which appear appropriate for distinguishing between odonate species. Mitochondrial rRNA sequences contain portions of high sequence conservation disrupted by hypervariable sequence stretches. These features make them ideally suited for taxonomic purposes. The conserved portions of the gene allow to use generally applicable primers and hypervariable sections provide enough resolution to separate species.

*Tauriphila argo* (Hagen 1869) is a well known South American libellulid species. Its larva was only recently described by Costa & De Assis (1994) based

**Table 1.** List of Specimens used in Divergence Plots.

| Family | Genus | Species | Collection Localities |
|---|---|---|---|
| Libellulidae | *Sympetrum* | *vulgatum* (L.,1758) | Austria, 1997 |
| | | *striolatum* (Charpentier 1840) | France, 1998 |
| | | *meridionale* (Sélys 1841) | France, 1998 |
| | | *danae* (Sulzer 1776) | France, 1998 |
| | | *sanguineum* (Müller 1764) | France, 1998 |
| | | cf. *fonscolombii* (Sélys 1840) | Iran, 2002 |
| | | cf. *fonscolombii* (Sélys 1840) | Iran, 2002 |
| | *Orthetrum* | *albistylum* (Sélys 1848) | Greece, 1998 |
| | | *albistylum* (Sélys 1848) | France, 1998 |
| | | *brunneum* (De Fonscolombe 1837) | France, 1998 |
| | | *brunneum* (De Fonscolombe 1837) | Iran, 2002 |
| | | *brunneum* (De Fonscolombe 1837) | Iran, 2002 |
| | | *brunneum* (De Fonscolombe 1837) | Iran, 2002 |
| | | *coerulescens* (Fabricius 1798) | France, 1998 |
| Gomphidae | *Onychogomphus* | *uncatus* (De Charpentier 1840) | France, 1999 |
| | | *f. forcipatus* (L. 1758) | Greece, 1997 |
| | | *f. forcipatus* (L. 1758) | France, 1998 |
| | | *forcipatus unguiculatus* (van der Linden 1823) | France, 1999 |

on a reared female specimen. We reared a female *Tauriphila* specimen from a quite differently shaped larva compared to Costas & De Assis. This female *Tauriphila* specimen perfectly fits the *T. argo* description, however our larva and that one described by Costas & De Assis are clearly different (see description below). Within the genus *Tauriphila*, females are notoriously difficult to distinguish, therefore it remained uncertain, whether the described larva and female were indeed conspecific to *Tauriphila argo* (Hagen 1869). Since reared male specimens are not available, we used a molecular approach to tackle the problem.

## Material and methods

### Material studied

*Tauriphila argo* (Hagen 1869). male : Montabo, Cayenne, French Guyana, XII 1997, G. Fleck collector; exuvia (reared female) : small pond near Petit Saut dam, French Guyana, 28 XI 2001 (imago 20 XII 2001), G. Fleck collector.

We used sequence comparisons within the genera *Orthetrum* (Libellulidae), *Sympetrum* (Libellulidae) and *Onychogomphus* (Gomphidae) to plot absolute nucleotide differences within the amplified mt SSU fragment.

List of specimens for divergence plots see table 1.

### DNA extraction

DNA samples were prepared from individual insects by extraction of total DNA from ethanol preserved animals. We used thoracic musculature for our DNA extractions in order to avoid contamination with food particles from the gut system.

Total DNA was extracted with the DNA Extraction Tissue Kit (Qiagen) following the manufactures instructions. Extracted DNA was stored at 4 °C.

### PCR amplification

The SR-N-14233/SR-J-14756 standard primer sets (Simon *et al.* 1994) was used to amplify a roughly 550 bp long 3' fragment of the SSU gene. Amplification of SSU rRNA gene fragments where successfully carried out using the following set up (Misof *et al.* 2001). Amplifications were performed in 50 µl reaction volume on a Perkin Elmer 9600 (Perkin Elmer). PCR was successful using a touchdown protocol that contained 1–2.5 units of Taq Polymerase (Sigma), 10xPCR Buffer (Sigma), 2.5 mM $MgCl_2$ and 2 mM dNTP-Mix (Sigma). The 1–2.5 units of Taq Polymerase and 50% of the sterile water were added after 2 minutes denaturation at 94 °C. Following an initial denaturation step, each reaction underwent 30 cycles of: 94 °C for 30 seconds, 50–35 °C for 20 seconds, and 72 °C for 1 minute, followed by a final five minutes at 72 °C. Within the 30 cycling steps the annealing temperature was gradually decreased by 0.5 °C each cycle, starting at 50 °C ending at 35 °C.

### DNA sequencing

Prior to sequencing unincorporated nucleotides and primers were removed by passing PCR products over Sephadex columns (Qiagen) following manufacturer's protocol. Purified PCR products were sequenced directly using the ABI Big Dye Terminator Cycle sequencing kit (Perkin Elmer) and run on an Applied Biosystems 377 automated sequencer. Both strands were sequenced for every PCR product. PCR primers where used for directly sequencing the purified PCR products.

### Sequence Analyses

Sequences were analysed using the BioEdit (Hall 1999) and ClustalX (Higgins *et al.* 1996) software packages. In ClustalX, default parameters were used for aligning sequences. Differences between sequences were counted as the absolute number of nucleotide differences between pairs of aligned sequences.

## Results and Discussion

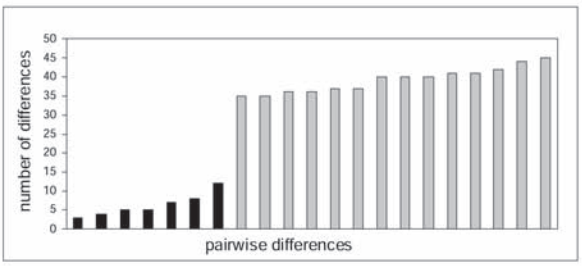
The amplified mt SSU fragments were between 673 (*Orthetrum*, *Sympetrum*) and 730 (*Onychogomphus*) aligned positions long. All positions were clearly alignable within genera. A pairwise comparison within the genera *Sympetrum*, *Orthetrum* and *Onychogomphus* shows that there are clear distinctions in nucleotide differences between morphologically well separated species ranging from 18 to 23 substitutions in *Onychogomphus*, from 35 to 45 in *Orthetrum* and from 23 to 50 in *Sympetrum*. Differences within species are much less pronounced (Fig. 1). There is a steep increase of absolute nucleotide differences for comparisons within species compared to between morphologically recognized species. This implies that sequence variability of the mt SSU fragment is a good predictor of morphological differentiation, and consequently a useful tool to link larvae and imagines.

We concluded that the sequenced mt SSU fragment will be suitable to reliably distinguish conspecific specimens from specimens of separate species. It is important to keep in mind that in all three reference genera, clear conspecific specimens do show sequence variability. This observed sequence variability among conspecifics constrains the accuracies of the molecular approach. Our plot of species differences within selected genera (Fig. 1) suggests that persistent gene flow keeps the level of genetic variance low, but an interruption of gene flow between populations quickly results in an increase of genetic differentiation.

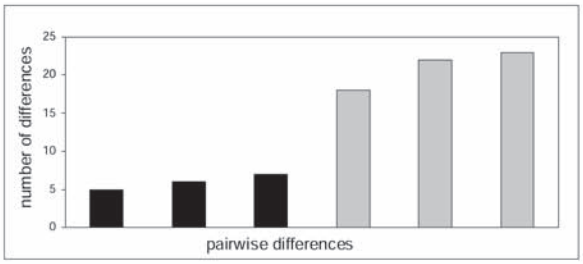
It is clear that sister species of very recent origin might not have accumulated enough nucleotide differences within this gene fragment and might therefore be indistinguishable. However, it is very likely that these two species will than be indistinguishable at the phenotypic level as well. In such cases only a population genetic approach will help to follow up the degree of gene flow and possible speciation between populations.

Is it possible to develop guidelines for identification? Theoretically, it would be necessary to describe sequence variability for each recognized species which

| | *Orthetrum* | *1* | *2* | *3* | *4* | *5* | *6* | *7* |
|---|---|---|---|---|---|---|---|---|
| *1* | *O. albistylum* | 0 | | | | | | |
| *2* | *O. coerulescens* | 35 | 0 | | | | | |
| *3* | *O. albistylum* | **5** | 35 | 0 | | | | |
| *4* | *O. brunneum* | 37 | 40 | 36 | 0 | | | |
| *5* | *O. brunneum* | 42 | 45 | 41 | **8** | 0 | | |
| *6* | *O. brunneum* | 41 | 44 | 40 | **4** | **12** | 0 | |
| *7* | *O. brunneum* | 37 | 40 | 36 | **3** | **5** | **7** | 0 |




| | *Onychogomphus* | *1* | *2* | *3* | *4* |
|---|---|---|---|---|---|
| *1* | *O. f. forcipatus* | 0 | | | |
| *2* | *O. f. unguiculatus* | **6** | 0 | | |
| *3* | *O. uncatus* | 18 | 22 | 0 | |
| *4* | *O. f. forcipatus* | **7** | **5** | 23 | 0 |




| | *Sympetrum* | *1* | *2* | *3* | *4* | *5* | *6* | *7* |
|---|---|---|---|---|---|---|---|---|
| *1* | *S. vulgatum* | 0 | | | | | | |
| *2* | *S. striolatum* | 23 | 0 | | | | | |
| *3* | *S. fonscolombii* | 52 | 48 | 0 | | | | |
| *4* | *S. meridionale* | 28 | 22 | 50 | 0 | | | |
| *5* | *S. danae* | 38 | 33 | 46 | 34 | 0 | | |
| *6* | *S. sanguineum* | 29 | 26 | 50 | 22 | 32 | 0 | |
| *7* | *S. fonscolombii* | 54 | 50 | **3** | 53 | 49 | 53 | 0 |

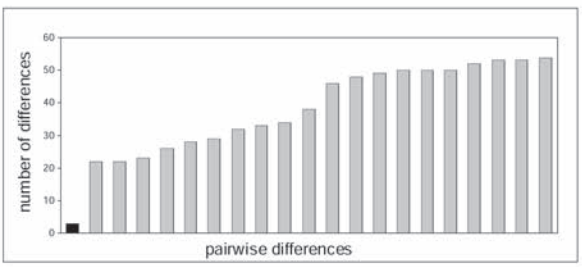



**Figure 1**
Absolute nucleotide differences between specimens.
The absolute pairwise nucleotide differences between conspecific specimens and specimens of different species are illustrated in distance tables and graphical visualisations. Differences in distance tables for conspecific specimens are given in bold letters. A fairly sharp distinction is visible between intra - and interspecific comparisons.

would than for example allow to tell apart conspecific larvae from larvae of different species. In many cases this is an unrealistic approach. For many "hard to come by" it will not be possible to characterize sequence variability. Consequently, species identification will rest on the assumption that sequence variability within species will not exceed certain threshold levels and molecular differentiation between species will exceed this intraspecific variability. In most cases, this will be a safe assumption, but a much better sampling for selected gene fragments is clearly warranted. A better sampling will help to characterize the phylogenetic variance of species differentiation within genera and therefore improve the accuracy of species assignments.

The analyses of the mt SSU variability within *Sympetrum*, *Orthetrum* and *Onychogomphus* implies that species differences a roughly comparable between taxa, even between genera of different families. Nevertheless, it is certainly premature to define a level of genetic difference at which conspecificity can be excluded within Odonates. Conspecificity seems obvious in cases, where sequences of larvae and imagines are identical but remains a preliminary assignment in cases of nucleotide differences without knowing the complete spectrum of sequence variability. Different to breading larvae, species assignment by means of sequence variability becomes an estimation process, similar to morphological analyses.

Pilgrim *et al*. (2002) used sequence variability of the nuclear ITS I sequence to demonstrate that *Cordulegaster bilineata* and *Cordulegaster diastatops* (Odonata: Cordulegastridae) are indeed two separate species. They found that ITS I sequences of these two species differ by a 51 bp long insertion. In such

instances, species assignment becomes a clear cut decision. This example shows that the exploration of different gene loci will be an important step towards increasing the power of the molecular approach.

**Molecular taxonomy**

We used mt SSU rRNA sequence variability to confirm that the reared female was indeed a *Tauriphila argo* female. For comparison, a *Tauriphila argo* male collected in 1997 in French Guyana was sequenced for a SSU rRNA fragment and its sequence compared to the SSU rRNA fragment of the reared female. The male was reliably identified due to its diagnostic shape of its anal appendages and copulatory organs. The SSU ribosomal DNA (SSU), comprising 544 bp, is 100 % identical between the male and the reared female specimen collected approximately 100 km apart. Thus, the male and bread female are most likely conspecific and identified as *Tauriphila argo*.

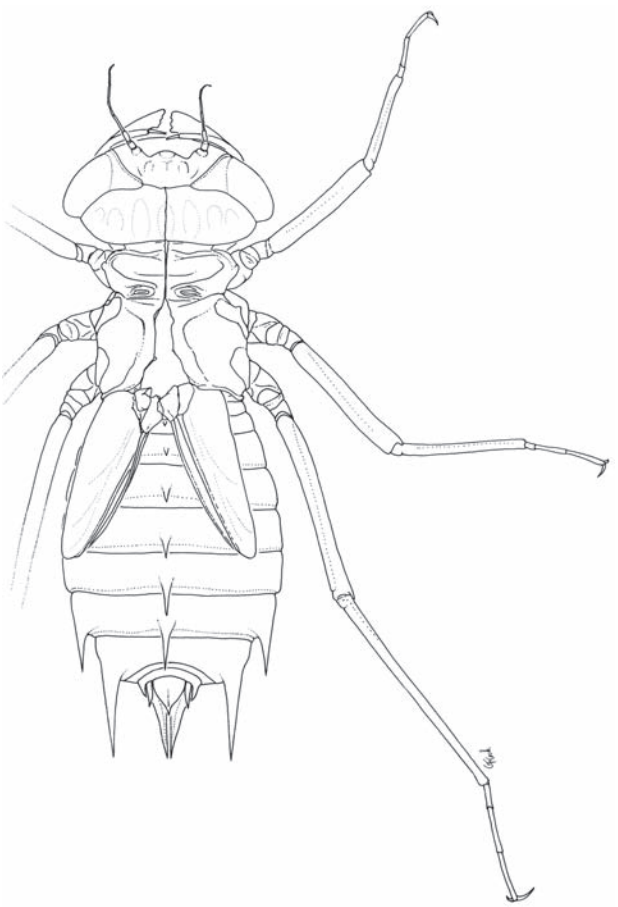


**Figure 2**
*Tauriphila argo*, general habitus of exuvia, dorsal view (setae omitted).

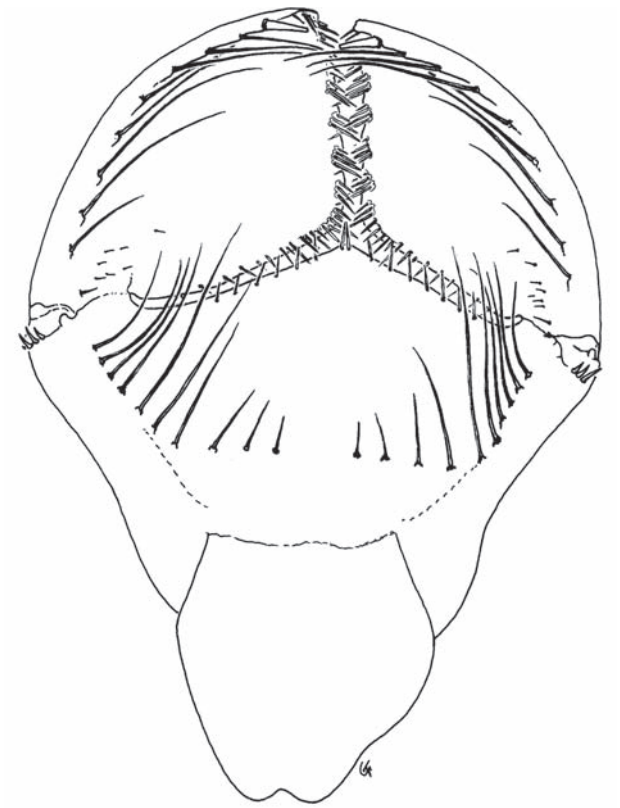


**Figure 3**
*Tauriphila argo*, labium, dorsal view.

## Morphological taxonomy

### Description of the last instar larva of *Tauriphila argo* (Hagen 1869) fig. 2-6

**Body.** robustly built, light brown, covered by minute spine like setae (fig. 2).

**Head.** massive, twice as wide as long, broader than thorax and nearly as broad as the abdomen; frontal margin rounded in dorsal view and aligned with the anterior margin of the eyes; antennae long and thin with seven segments; eyes very large and laterally protruding, their lateral apex well beyond the occipital lateral margin and their free posterior margin long and being at right angle to the sagittal axis; occiput well developed, narrowed posteriorly, its posterior margin nearly strait (fig. 2) and with strong spiniform setae; mask rather voluminous compared to the body dimension, the submentum-mentum articulation just reaching the level of metacoxae; dorsal side of mentum with two rows of ten mental setae, its distal margin ('ligula') well developed anteriorly, carrying a row of acute setae, increasing in size towards the apex, at the apex two very close setae; palpus with eight palpal setae, its external margin glabrous, carrying near the articulation with mentum eight small setae, the most proximal seta being the strongest one; distal margin of palpus with shallow crenulations, each tooth asymmetric and carrying three or four acute raptorial setae; distal part of movable hooks broken, but strong at the base (figs 4,5).

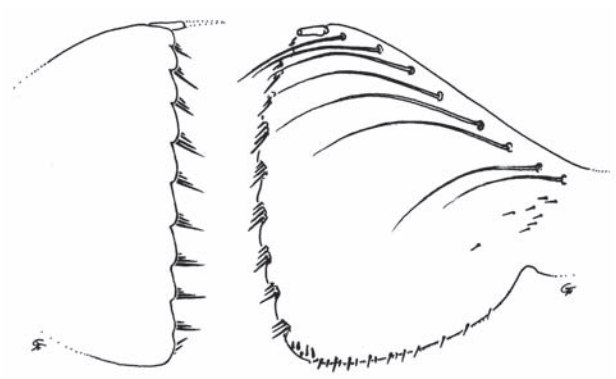


**Figure 4-5**
*Tauriphila argo*, right labial palp, 4, frontal view; 5, internal view.

**Thorax.** prothorax well developed, its pronotal shield weakly developed; proepisternum and proepimeron not clearly separated, proepisternum with long and strong setae, proepimeron small and rounded; spiracles well developed and exposed; on the living larva, all costal margins of wing pads parallel, the apex of the posterior wing pads slightly protruding beyond the posterior margin of segment 6; legs very long and rather stout, the mesothoracic legs reaching the anal pyramid when fully extended, the metathoracic legs as long as the entire body; claws long and thin.

**Abdomen.** Lateral margins not parallel; dorsal hooks on segments 3 to 8, the one of segment 3 small and erect, the remainders stronger and gradually directed backwards (fig. 6); lateral spines present on segment 8 and 9, those of segment 8 reaching the posterior margin of abdominal tergite 10 (at sagittal line), thus the apex of lateral spines and the apex of the dorsal hook of the same segment are at the same level, those of segment 9 very long, nearly as long as the three last abdominal segments together (at sagittal line); lateral margins of segments 7 to 10 and the external margin of lateral spines with spiniform setae larger than the average; few hair-like setae at the posterior margin of the last abdominal tergites; anal pyramid well developed, epiproct long and acutely pointed, much shorter than the paraprocts; paraprocts very long, their apex at the same level as the apices of the posterior lateral spines; cerci acute and slightly divergent (figs 2, 6).

**Dimensions (exuvia).** Total length, excluding antennae and mask, including anal pyramid: 17.9 mm; maximal width of head: 6.2 mm; length of antennae: 3.5 mm; maximal width of abdomen: 6.5 mm; length of posterior legs: 17.9 mm.

## Taxonomic considerations, comparison with the larva of Costa & De Assis (1994)

The herein described larva of *Tauriphila argo* differs in many aspects compared to the larva described by Costa & De Assis (1994), therefore we conclude that these authors did not describe the true larva of this species. The differences to the described larva of Costa & De Assis are manifold:

head shape very different (see Costa's fig. 1), (1) eyes protruding prominently with free posterior margin long and being at right angle to the longitudinal axis (not prominent and continuously "rounded laterally" as described for the larva by Costa & De Assis) and (2) occiput well developed (distinctly lesser developed in the larva described by Costas & De Assis); (3) only 10 mental setae (instead of 14 mental setae); (4) labial palp with only 9 not very shallow crenulations (instead of 11 "very shallow" crenulations) and (5) the most of the crenulations with 4 raptorial setae (instead of 3); (6) dorsal hook on abdominal segments 3 to 8, those of segments 4 and 5 well developed (only of segments 4 to 8, those of segments 4 and 5 small); (7) lateral spine on segment 8 very long and acute, reaching segment 10 (small and broad, reaching half of the mid-dorsal length of segment 9); (8) lateral spine on segment 9 not up curved, very long and acute, its apex at the same level of the apices of the paraprocts ("up curved", shorter and its apex well before the apices of the paraprocts); (9) anal pyramid long (distinctly shorter) and the cerci reaching the 3/4 of the length of epiprocts (reaching only 1/3 of the length of epiprocts); (10) body broader : length (mm) / width (mm) = 17.9 / 6.5 = 2.75 (instead of 19.65 / 6 = 3.27); (11) shape of paraprocts in dorsal view 'normally' build: close together and meeting at segment 10 (in larva of Costa & De Assis very unusual: well apart and not meeting); (12) no minute spine on the "dorsal border" of the epiproct (minute spine present in larva description of Costa & De Assis).

Intraspecific morphological variation is know from the genus *Leucorrhinia* (see for example Johansson & Wahlström 2002), but they concern only light variations in the length of dorsal and lateral abdominal spines. Furthermore, one of us (G.F.), working on the description of still undescribed larvae of more than thirty species or genera (see for example Fleck 2002a, 2002b, 2003a, 2003b, 2004a, 2004b) and rearing hundreds of larvae, observed that most of the differences between the two described larval specimens of *T. argo* far exceed the morphological variance found within one

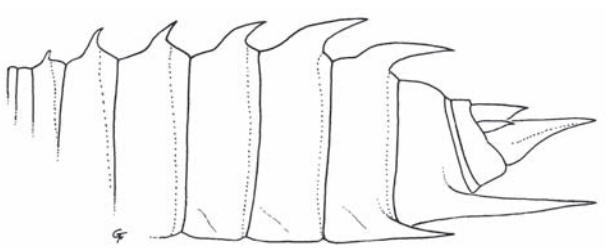


**Figure 6**
*Tauriphila argo*, abdomen, left lateral view.

species. Possibly, Costa & De Assis described a larva of a still undescribed additional *Tauriphila* species. Their description is based on a female specimen only, the identification of corresponding male specimens is necessary to complete a morphological and molecular description of a possible new species within this genus.

According to Westfall (1998), five valid species are included in the genus *Tauriphila* Kirby, 1889: *T. argo* (Hagen 1869) (South and Central America, and Cuba), *T. australis* (Hagen 1867) (South and Central America, and southern United States), *T. azteca* Calvert 1906 (Central America), *T. risi* Martin 1896 (South America) and *T. xiphea* Ris 1913 (South America).

The larvae of *T. australis* and *T. risi* have been described by Westfall (1998) and Rodrigues Capítulo (1996) respectively. In South America, the larva of *T. xiphea* remains unknown. Even if the females of *T. xiphea* and *T. argo* are rather similar (shape of anal and external sexual appendages), they can easily be separated by the general colour of the wings, the basal dark brown spot at the basis of the hind wing and the black pattern on the abdominal tergits.

**Remark.** Westfall (1998) reports that he had received (before 1994) a reared specimen of *T. argo* (male or female?) from Dr. A. Machado collected in Brazil, and that he had drawn the larva, but never published it. He mentions that Costa & De Assis (1994) finally described the larva (from a single specimen). As neither Westfall (loc. cit.) nor Needham et. al. (2000) refer to the description of Costa & De Assis (1994), it is likely that the specimen of Costa et De Assis (from Brazil) and the one given by Machado to Westfall (also from Brazil) might belong to the same still undescribed species.

## Conclusions

The application of molecular techniques proved to be a valuable tool in identifying unknown odonate larvae. It can be foreseen that the application of molecular techniques will speed up the identification of unknown larvae from various groups. The establishment of suitable molecular markers as standardized tools for the application of DNA taxonomy should have high priority. We showed the potential of mt SSU genes in this field but the incorporation of nuclear gene fragments within this tool kit should be a central task. We are currently exploring the suitability of ITS genes for these purposes (compare Pilgrim *et al.* 2002, Wheekers *et al.* 2001). Ribosomal genes as well as ITS genes are among the most promising canditates since generally applicable primers to amplify regions of interest are readily available.

The creation of a tool kit will be necessary but the maintance of a data base in which all known sequences are stored for easy access and scoring has to accompany the creation of this tool kit. Promising software solutions are under development for several taxonomic groups, for example the ARB project (Hille *et al.* 2001, Ludwig *et al.* 2004, *www.arb-home.de*) and should be explored for there suitability.

**Acknowledgements.** This study is part of the program 'Pluriformation Guyane'. We are indeed grateful to all people who permitted the realization of the project, therefore we particularly thank Professor Jean-Marie Betsch (Institut d'Ecologie et de Gestion de la Biodiversité, MNHN). We also thank all members of the team of the HYDRECO laboratory (Petit Saut dam, French Guyana) for their hospitality, and particularly Dr. Véronique Horeau for her help and kindness. Finally we thank Sébastien Lacau, myrmecologist, for his help in the field and Catherine Brunet, for reading and correcting the manuscript.

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