

# On the Relationship between *Zaprionus spinipilus* Chassagnard & McEvey and *Z. vittiger* Coquillett, the Type Species of the Genus *Zaprionus* (Diptera: Drosophilidae)

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**Abstract.** *Zaprionus vittiger* Coquillett is the type species of the genus *Zaprionus* Coquillett. However, the species is only known from five old museum specimens collected from South Africa and Malawi. It has often been confused with many other *Zaprionus* species, especially with *Z. spinipilus* Chassagnard & McEvey, a widespread species in Africa known from Madagascar, Malawi, Ethiopia and Cameroon. We have recently collected flies from the type localities of both species (South Africa and Madagascar, respectively). This has prompted us to test the taxonomic boundaries of these two nominal species using molecular (the mitochondrial *COII* and the nuclear *Amyrel* genes), chromosomal, morphological (internal and external genitalia), and reproductive isolation analyses. The results suggest *Z. spinipilus* to be a junior synonym to *Z. vittiger*.

**Résumé.** La relation entre *Zaprionus spinipilus* Chassagnard & McEvey et *Z. vittiger* Coquillett, l'espèce type du genre *Zaprionus* (Diptera : Drosophilidae). *Zaprionus vittiger* Coquillett est l'espèce type du genre *Zaprionus* Coquillett. Cependant, l'espèce n'est connue que par cinq anciens spécimens de musée, récoltés en Afrique du Sud et au Malawi. Elle a souvent été confondue avec plusieurs autres espèces de *Zaprionus*, en particulier avec *Z. spinipilus* Chassagnard & McEvey, une espèce répandue en Afrique et connue de Madagascar, du Malawi, de l'Ethiopie et du Cameroun. Nous avons récolté récemment des individus provenant des localités types des deux espèces (Afrique du Sud et Madagascar). Ceci nous a permis d'étudier les différences taxonomiques entre ces deux espèces nominales, en utilisant des caractères moléculaires (le gène mitochondrial *COII* et le gène nucléaire *Amyrel*) ; chromosomiques, morphologiques (genitalia externes et internes), ainsi que l'isolement reproducteur. Les résultats suggèrent de mettre *Z. spinipilus* en synonymie avec *Z. vittiger*.

**Keywords:** Africa, mitotic chromosomes, morphometry, molecular phylogenetics, speciation.

The genus *Zaprionus* was created by Coquillett (1902) for a few specimens from the Cape Province (South Africa), and the type species was named *Z. vittiger*. The generic name referred to the presence of a row of protruding warts each bearing a spine and a long bristle on the antero-ventral surface of the forefemur in both sexes, whereas the species name referred to the presence of four longitudinal silvery white stripes (vittae) on the frons and the mesonotum. With the eventual discovery of many new species of *Zaprionus* in Africa, the notal pattern turned out to be a constant character and thus a generic diagnostic, whereas forefemoral ornamentation was shown to

be very diverse among species. However, one very common species in Africa with a series of forefemoral armed warts has usually been assigned to *Z. vittiger*. Tsacas (1980) corrected by examining Coquillett's types in the US National Museum in Washington the misidentification of the common species and named it *Z. collarti*. Tsacas (1985) recognized later that his *Z. collarti* had already been described from India as *Z. indianus* Gupta (1970). Following the priority rule of nomenclature, *Z. indianus* thus became the valid name of this most common African species, which has also invaded the Americas during the last decade (Yassin *et al.* 2008b).

Tsacas (1980) also created the *vittiger* complex for five species having the forefemoral ornamentation of the type species. The complex was then raised to the rank of subgroup by Chassagnard & Tsacas (1993)

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to comprise 12 species. One of these species, *Z. spinipilus* Chassagnard & McEvey, was described from Madagascar and differed from *Z. vittiger* in having only less pubescent male terminalia (epandrium and hypandrium). *Zaprionus spinipilus* on the one hand was shown to be a widespread species in Africa as it was also collected from Cameroon, Ethiopia and Malawi (Chassagnard *et al.* 1997). On the other hand, *Z. vittiger* was still only known from the Coquillett's three Cape specimens in the US National Museum and two male specimens pinned in the Natal Museum, collected from Natal (South Africa) in 1967 and from Ntchisi Forest Reserve (Malawi) in 1980 (Tsacas 1990; Chassagnard *et al.* 1998).

We have recently conducted a molecular phylogenetic revision of *Zaprionus* (Yassin *et al.* 2008a), where a strain from Kibalé (Uganda) was assigned, on the basis of dark spots on the posterior margin of abdominal tergites, to *Z. vittiger*. The phylogenetic positioning of this species was shown to be distant from cryopreserved specimens of the type strain of *Z. spinipilus*. This work was submitted for publication in April 2007. At that time, one of us (M. D.-T.) had made several collections of *Zaprionus* in Stellenbosch (South Africa). Among several easily identified species, one female was noticeable by its big size, dark pigmentation especially on the scutellum. The female was used to found a laboratory strain in our laboratory (LEGS). The comparison of the dissected genitalia of the male offspring to Tsacas' (1980) illustrations of *Z. vittiger* suggested the strain to belong to the true *vittiger*, although different from the Ugandan strain. The latter turned out to be a new species (A. Y. & J. R. D., in lit.). In the meanwhile, two of us (A. Y. & J. R. D.) made a recent collection in Mandraka (Madagascar), the type locality of *Z. spinipilus*, and were able to bring back several isofemale lines of the latter species. The recent availability of these living strains in our laboratory has prompted us to conduct the present study, in order to investigate the boundary between the two nominal species, *Z. vittiger* and *Z. spinipilus*, by using molecular, karyological, morphological and hybridization analyses.

## Materials and Methods

### Molecular analyses

Yassin *et al.* (2008a) presented a molecular phylogenetic hypothesis for 21 Afrotropical species of *Zaprionus sensu stricto*, using a mitochondrial gene (*COII*) and a nuclear gene (*Amyrel*). Since their study, four additional species of *Zaprionus s.s.* became available for molecular analyses, besides the type species *Z. vittiger*. These were *Z. campestris* Chassagnard, *Z. gabonicus* Yassin & David, *Z. litos* Chassagnard & McEvey,

and *Z. simplex* Chassagnard & Tsacas. *Zaprionus gabonicus* is endemic to Gabon and belongs to the *vittiger* subgroup of the *armatus* group (Yassin *et al.* 2008b), whereas *Z. litos* and *Z. simplex* are endemic to Madagascar and belong to the *inermis* group (Chassagnard & McEvey 1992). *Zaprionus campestris* is a widespread species throughout the Afrotropical region and belongs to the *armatus* subgroup of the *armatus* group (Chassagnard & Tsacas 1993). For comparative purposes the same two genes (*COII* and *Amyrel*) used by Yassin *et al.* (2008a) were sequenced for the five species. DNA extraction, PCR amplification and sequencing were as in Yassin *et al.* (2008b) for *COII* and Da Lage *et al.* (2007) for *Amyrel*. Sequence alignment and phylogenetic analyses were performed using Molecular Evolutionary Genetic Analysis (MEGA) version 4 (Tamura *et al.* 2007) and MrBayes version 3.2 (Ronquist & Huelsenbeck 2003) software packages. Bayesian inference of phylogeny was conducted using the same parameters as in Yassin *et al.* (2008a). Analyses other than molecular ones have only been applied to the two species *Z. vittiger* and *Z. spinipilus*.

### Chromosomal analyses

Third instar larvae were used for this study. Larvae grown at 17 °C were used because of their bigger size. Dissections were made in the *Drosophila* Ringer solution. The cerebral ganglia were isolated, then put in an orcein-acetic acid fixative and squashed to find cells in a proper state. Digital photomicrographs were taken using a magnification of 100X.

### Morphological analyses

Species were kept as laboratory strains at 17 °C on standard *Drosophila* culture medium following the special rearing precautions for *Zaprionus* species described in David *et al.* (2006). For the study of internal reproductive system, mature, about 10 days old adults, were dissected in a *Drosophila* Ringer solution. For the male reproductive system (see drawing in Araripe *et al.* 2004), testes were uncoiled before a linear measurement could be done. This operation was facilitated by allowing the Ringer solution to evaporate a little so that the testis loses its rigidity. For each species, two or three individuals were dissected.

### Reproductive isolation analyses

From each strain, groups of 15 virgin females and 15 males were established and kept for at least a week in culture vials. This is necessary for the adults to reach sexual maturity. Then crosses were made, *i.e.* females of one strain were introduced in the same vial with males of the other strain. Then the adults were transferred to a fresh vial twice a week and the culture examined for progeny. Serial transfers were done for at least four weeks. When F<sub>1</sub> progeny was produced, the hybrids were transferred to fresh vials and the culture examined for F<sub>2</sub> production. The transfers of F<sub>1</sub> were done during a month. The two reciprocal crosses were performed and in each case four repeats were done.

## Results

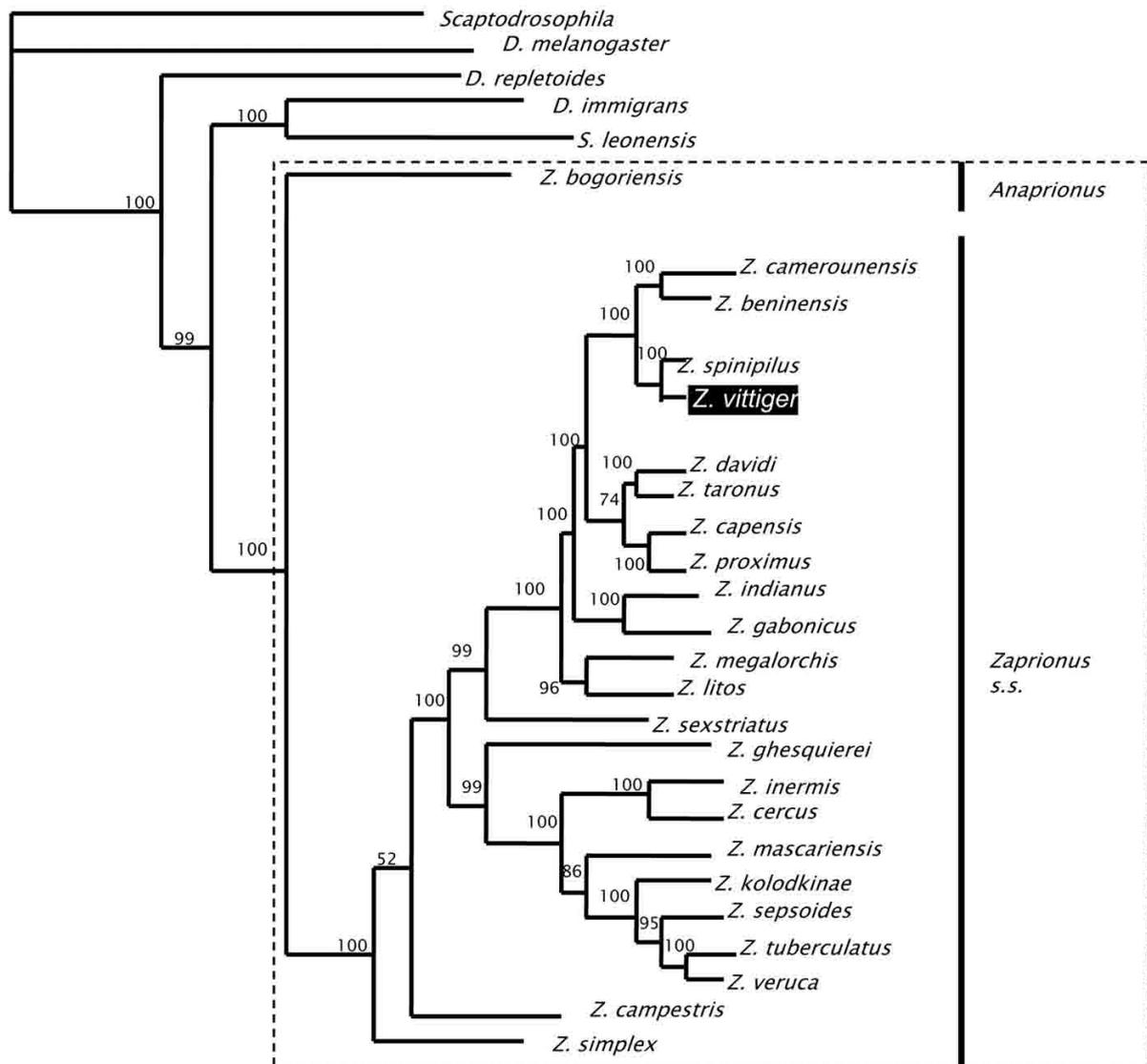
### Molecular analyses

Figure 1 shows the phylogenetic positioning of *Z. vittiger* in the Bayesian tree inferred from concatenated *COII* and *Amyrel* sequences. This species forms a very

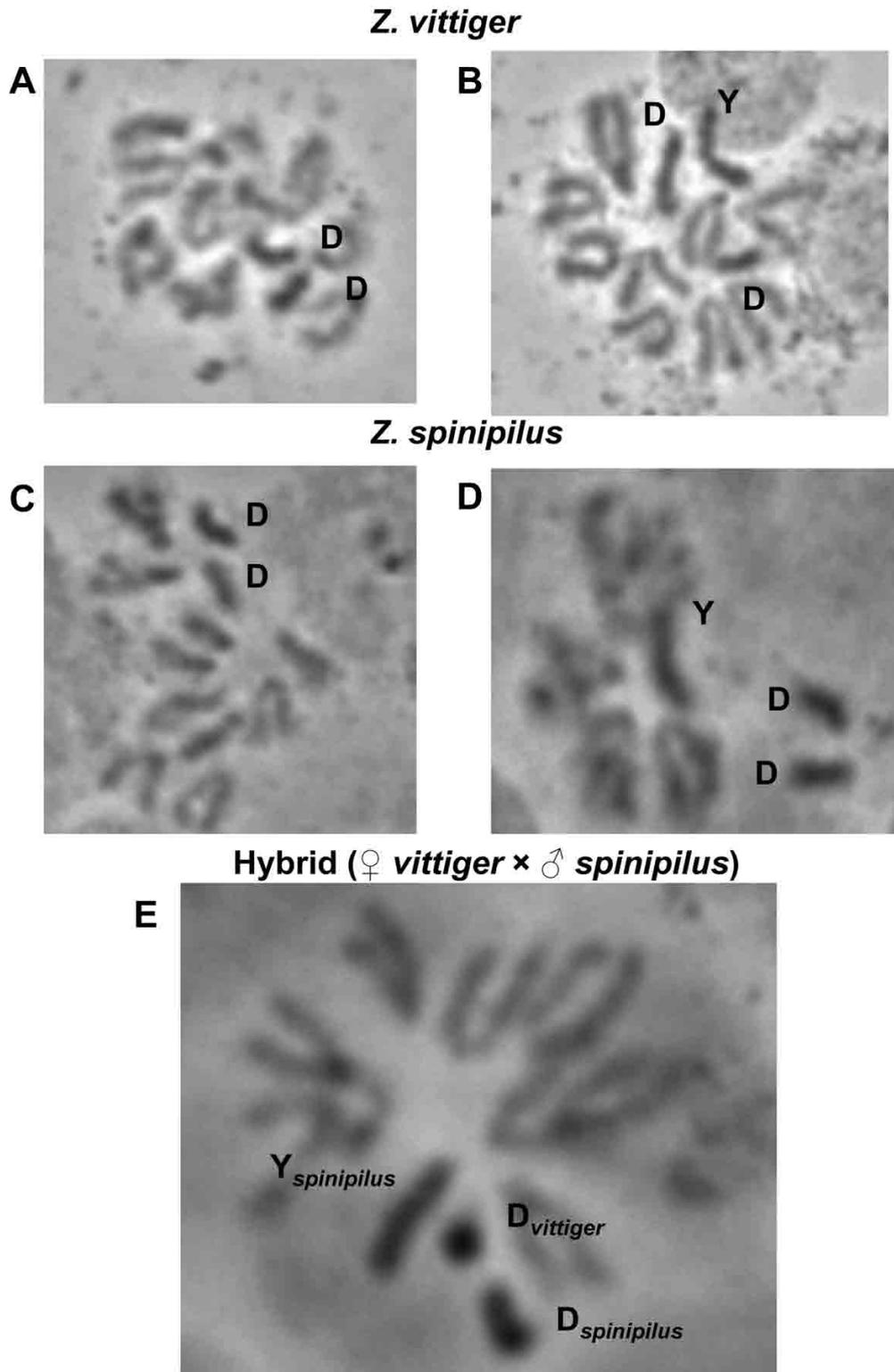
highly supported clade with *Z. spinipilus* (*a posteriori* probability of 100%). This confirms the close relation between the two species, previously suggested from their high morphological similarity. The divergence of mitochondrial sequences (*COII*) between the two species was found to be 1.6%, a value below the 1.9% maximal within-species sequence divergence previously observed in *Z. indianus* (Yassin *et al.* 2008b).

Chassagnard & Tsacas (1993) suggested the *armatus* group to contain three subgroups: *armatus*, *tuberculatus* and *vittiger* based on morphological data. Yassin *et al.* (2008a) transferred the *tuberculatus*

subgroup to the *inermis* group. Yassin *et al.*'s (2008a) molecular phylogenetic revision of *Zaprionus s.s.* did not include any representative of the *armatus* subgroup. However, on morphological basis, they suggested this subgroup to be polyphyletic, forming three distinct monophyletic species complexes that they called *montanus*, *armatus* and *spinosus*. *Zaprionus campestris* used here belongs to the *montanus* complex. It does not form a monophyletic clade with the remaining *vittiger* species (Fig. 1) in disagreement with Yassin *et al.*'s (2008a) hypothesis. Instead, it appears as one of the earliest branches of the subgenus. Its relationships with



**Figure 1** Bayesian phylogenetic positioning of *Zaprionus vittiger* (black background) using concatenated *COII* and *Amyrel* sequences. Support values (posterior probability) are given above internal branches.



**Figure 2**  
Metaphase chromosomes of: A) *Z. vittiger* (female); B) *Z. vittiger* (male); C) *Z. spinipilus* (female); D) *Z. spinipilus* (male); and E) hybrid male (female *vittiger* × male *spinipilus*). Symbols: D = dot chromosome, and Y = Y chromosome.

other species previously classified under the *armatus* subgroup by Chassagnard & Tsacas (1993) need to be revised when other specimens available for molecular analyses can be captured.

Yassin *et al.* (2008a) also suggested that *Z. litos* did not belong to the *inermis* group. Indeed, it forms here a monophyletic clade with *Z. megalorchis* Chassagnard & McEvey, a member of the *vittiger* subgroup. Yassin *et al.* (2008a) erected an *ornatus* species complex containing *Z. ornatus* Séguéy and *Z. megalorchis*. Although lacking the characteristic forefemoral ornamentation of the *vittiger* subgroup, *Z. litos* is included within the *ornatus* complex of this subgroup.

*Zaprionus simplex*, which traditionally belongs to the *inermis* group, appears as the earliest branching lineage of *Zaprionus s.s.* Yassin *et al.* (2008a) suggested on the basis of morphological characters this species to belong to the *armatus* species complex. Until additional *armatus* species becomes available for molecular analyses, such relationship needs to be confirmed.

#### Comparison of mitotic chromosomes between *Zaprionus vittiger* and *Z. spinipilus*

Figure 2 shows the metaphase chromosomes of the two species and their hybrids. Both species have five pairs of rod and one pair of dot chromosomes, which is the typical karyotype of the genus. Like other species of *Zaprionus s.s.*, the Y chromosome is highly heteropycnotic. However, the two species differ mainly in the size of their dot chromosomes, being larger in *Z. spinipilus* than in *Z. vittiger*, as can be shown in the karyotype of their hybrid male (Fig. 2E). A single male of *Z. vittiger* was polymorphic for the dot chromosomes (Fig. 2B).

#### Anatomical comparison between *Zaprionus vittiger* and *Z. spinipilus*

In most *Zaprionus* species the intensity of testis pigmentation increases with age, but the colour remains the same, yellow or orange. The *Z. vittiger* strain exhibited a colour polymorphism which, so far, seems unique in the genus. About half of the males had yellow testes while the others exhibited a darker pigmentation, and a characteristic brownish-purple colour. In *Z. spinipilus*, 10 isofemale lines were investigated and males with a light purple colour were observed in only two of them, with a low frequency.

#### Reproductive isolation experiments between *Zaprionus vittiger* and *Z. spinipilus*

As stated in Methods, interspecific groups of 15 females and 15 males were put together in single vials, allowing them to mate and produce progeny.

Examination of the culture vials evidenced mating pairs in most vials. Thus behavioural isolation, if any, is not important.

Concerning the production of F<sub>1</sub> progeny, this occurred in all cases, but the offspring production was often delayed. Indeed, in one case, progeny was obtained only one month after the beginning of the cross. From the overall examination of the data, a conclusion is that progeny production was easier between *Z. spinipilus* female and *Z. vittiger* male, than in the reciprocal cross.

Viable F<sub>2</sub> progeny was obtained in many, but not all cases. It was often noticed that a group of many F<sub>1</sub> flies, transferred regularly to fresh food, never produced any offspring. Moreover, the progeny of the same parental group, obtained a few days later, did produce an F<sub>2</sub>.

These results are difficult to interpret. For the moment, a provisional conclusion is that a reproductive isolation exists between the two species and that this is manifested by reduction of the F<sub>1</sub> number and quite erratic production of an F<sub>2</sub>. In other words, many F<sub>1</sub> males seem to be sterile (egg production was always observed) but the sterility is not complete.

#### Discussion

Since its original description, *Z. vittiger*, the type species of the genus, has been collected twice. The few specimens available could not give major systematic insights. Indeed, in their revision of the *vittiger* species subgroup, Chassagnard & Tsacas (1993) pointed out that six out of the then 12 species of the subgroup had very similar male genitalia and that they only differ on the basis of somatic characters prone to intraspecific variation. These were *Z. beninensis* Chassagnard & Tsacas, *Z. camerounensis* Chassagnard & Tsacas, *Z. koroleu* Burla, *Z. proximus* Collart, *Z. spinipilus* and *Z. vittiger*. As noted by Chassagnard & McEvey (1992), the species most to be confused with *Z. vittiger* was *Z. spinipilus*. The recent recollection of *Z. vittiger* in South Africa and of *Z. spinipilus* in Madagascar has allowed the conduction of the present comparative study using their topotype strains.

The molecular phylogenetic analysis showed the two species to be very close, with their mitochondrial DNA sequence divergence falling within the intraspecific range previously observed in *Z. indianus* (Yassin *et al.* 2008b). Chromosomal analyses showed only slight differences in the size of the dot chromosomes between the two species. In the *Z. tuberculatus* species subgroup, differences in the heteropycnosis level of the dot chromosomes are diagnostic between sibling species (Tsacas *et al.* 1977). However, *Z. vittiger* appears

polymorphic for the size of the dot chromosomes and thus the difference in the heteropycnosis level between the two species is not diagnostic. Finally, the examination of the internal reproductive system of the males revealed a unique testis colour polymorphism in these two species not found in other *Zaprionus* species. The diagnostic differences in epandrial pilosity mentioned by Chassagnard & McEvey (1992) were also variable among the few individuals dissected from each species.

There are numerous examples of interspecific hybridization in the genus *Drosophila* (Patterson & Stone 1952), but only two cases have recently been recognized in the genus *Zaprionus*. Both are in the *tuberculatus* species subgroup (Yassin 2008). Here we present the third case of incomplete reproductive isolation that differs from that in the *tuberculatus* subgroup by producing a small fraction of fertile  $F_1$  hybrid males. Sterility of hybrid heterogametic sex remains the most reliable operational criterion in delineating a species (Funk & Omland 2003).

From the molecular to the reproductive levels, the differences do not guarantee *bona fide* status for the two species. Instead, they may be better considered two isolated, recently diverged populations of the same species. Indeed, using the molecular clock estimates presented by Yassin *et al.* (2008a) their divergence appears to have occurred only during the last 1 million years. *Zaprionus spinipilus* Chassagnard & McEvey is thus considered a junior synonym to *Z. vittiger* Coquillett, whose geographical distribution includes now Cameroon, Ethiopia and Madagascar besides South Africa and Malawi. The type species of the genus *Zaprionus* has no longer to be considered rare or of limited distribution in Tropical Africa.

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