



## The mystery of the lesser stag beetle *Dorcus parallelipedus* (L.) (Coleoptera: Lucanidae) mycangium yeasts

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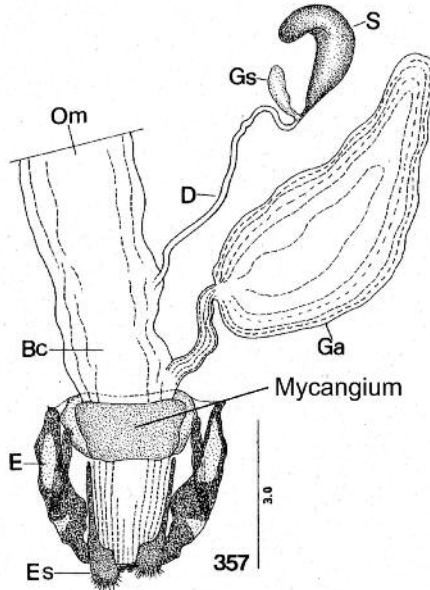
### Introduction

The mycangium is a microbe-storage organ present in several wood-feeding invertebrates; for example, it has been known for some time that bark and ambrosia beetles, and even leaf-roller weevils carry symbiotic fungi in special structures on their bodies (Beaver, 1989; Kobayashi *et al.*, 2008). However, this organ has only been discovered recently in stag beetles (Tanahashi *et al.*, 2010) and in a certain lizard beetle (Toki *et al.*, 2012), probably because it is not an external structure. In their case, the mycangium is an ovipositor-associated organ, that is, an exoskeletal organ normally folded and hidden under the last tergal plate of the female's abdomen.

Remarkably, this organ is clearly represented, albeit without a description, in Franciscolo's drawings of the dorsal view of the female reproductive system for a couple of Lucanidae species: *Lucanus cervus* and *L. tetraodon* (1997). However, he opted for ventral views of all the other species, including *Dorcus parallelipedus*, in which case the mycangium is obscured by the reproductive organs. Since the author is no longer alive and the book is currently out of print, we reproduce below one of his diagrams with the mycangium marked by us (Figure 1).

When laying, stag beetle females swab the everted mycangium close to their eggs, thus providing the future larvae with micro-organisms that will facilitate their development (MT, unpubl.; pers. observ.). Sometimes this organ everts when a female *L. cervus* is accidentally trodden on by humans, a not uncommon occurrence in the urban areas favoured by this species because the females tend to walk around in search of suitable places to lay their eggs even during the day (Fremlin, 2012). Possibly the mycangium is everted by pressure of body fluid like the endophallus (inner sac) of the male genitalia.

As MT is currently studying the species-specific associations between the microbial community in the mycangium and the Lucanidae, and the range of *D. parallelipedus* is confined to Europe, Middle East, Russia and a bit of North Africa (Klausnitzer & Sprecher-Uebersax, 2008), MF sent one Colchester female to Japan for further studies.



**Figure 1.** Female *Lucanus cervus* reproductive apparatus: Bc = bursa copulatrix, D = spermathecal duct, E = hemitergite, Es = hemisternite (or, gonocoxite), Ga = accessory gland, Gs = spermathecal gland, Om = median oviduct, S = spermatheca; dorsal view (scale bar, 3 mm). The hindgut (not shown) is normally attached between the mycangium and the bursa copulatrix. (After Franciscolo, 1997).

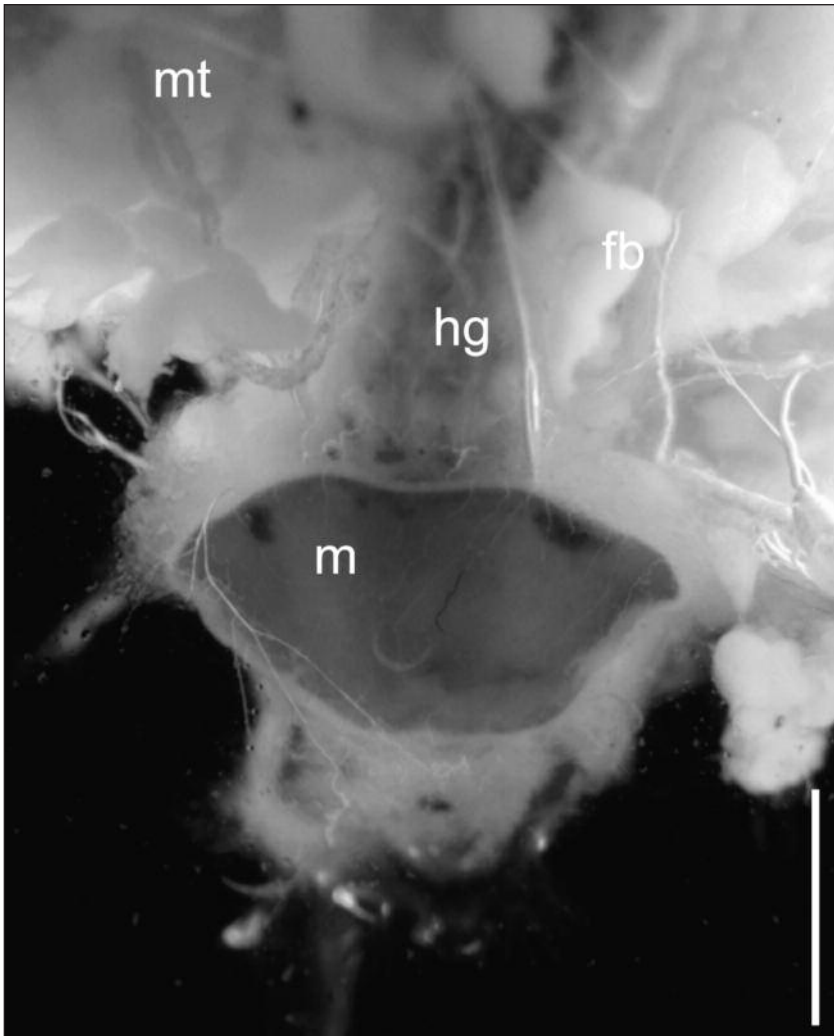
## Methods

A female Lesser stag beetle collected from an old *Acer negundo* which supports a large *D. parallelepipedus* colony (Fremlin & Hendriks, 2013) was sent to Japan.

The female insect was anaesthetised on ice and dissected under the microscope. The mycangium was cut away from the body, rinsed with sterilised phosphate-buffered saline (PBS) and then homogenised in PBS. The homogenate was spread onto culture medium plates. The plates were incubated at 25°C until yeast colonies appeared. For the identification of the yeasts, a total of eight yeast colonies were subjected to DNA analysis as described in Tanahashi *et al.* (2010).

## Results

The Lesser stag beetle female survived the journey and was successfully dissected. The mycangium is clearly seen in Figure 2; it is the dark trapezoidal organ above the hindgut, close to the ovipositor. DNA



**Figure 2.** Female *Dorcus parallelipedus* mycangium and other organs: m = mycangium, hg = hindgut, mp = Malpighian tubules, fb = fat bodies; dorsal view (scale bar, 1mm).

sequences of the ITS-D1/D2 region of ribosomal RNA gene (1,202 base pairs in total) of the yeasts isolated from the mycangium were all identical to those of *Pichia stipitis* CBS 5773, the type strain of this species (Pignat, 1968). Note that *P. stipitis* is now assigned to the new genus *Scheffersomyces* (Kurtzman & Suzuki, 2010).



This result agrees with a previous study on the *Dorcus* species in Japan (Tanahashi *et al.*, 2010). Conspecific yeast strains generally have fewer than 1% sequence difference in the D1/D2 region (Kurtzman & Robnett, 1998). Interestingly, there was no sequence difference between the type strain of *P. stipitis* and the yeasts isolated from our *D. parallelipipedus*, although *Pichia* yeasts obtained from different Lucanidae species usually have different sequences in that region (Tanahashi *et al.*, 2010). In addition, we preliminarily tested newly developed, hyper-variable DNA markers (MT, unpubl.) and found that the yeast from *D. parallelipipedus* is possibly the same as CBS 6054, which is another authentic strain of *P. stipitis*. This strain is of industrial interest because it ferments D-xylose to ethanol, with application to bio-fuels (Agbogbo & Coward-Kelly, 2008). More important, it has had its genome sequenced (Jeffries *et al.*, 2007) and consequently it is being used in biogenetics all over the world. This coincidence came as a big surprise. However, to complicate things, nobody knows its origin; all that is known is that it was deposited in the CBS\* in the early 70's by J. Santa María, an eminent Spanish mycologist.

### **The mystery of the origin of the genome-sequenced yeast**

It would be very important to find out if the host of the original strain was also *D. parallelipipedus*; this would prove that there really is a strong species-specificity in the Lucanidae. So we set out to unravel this mystery and, after many false leads, finally, we managed to gather relevant information about its author.

Professor Juan Santa María Ledochowski (his mother was Polish), died in 2006, aged 90, and his collaborators have either died or retired, some at least 20 years ago. He published a lot and even wrote a book about yeasts (1978).

According to one of his PhD students, Manuel Nuñez, Santa María in the 70's was based in the Instituto Nacional de Investigaciones Agronómicas (INIA), Madrid, Spain; when he left in the 80's he took with him the yeast collection (and all the information about it) to the Escuela Técnica Superior de Ingenieros Agronomos, Madrid, where he spent his last years of activity in research.

When he retired, the yeast collection was transferred to the Colección Española de Cultivos Tipo (CECT, [www.cect.org](http://www.cect.org), the Spanish equivalent to CBS), at Valencia, together with the information about the strains. But the genome-sequenced *Pichia stipitis* strain is not deposited in that collection.

\* CBS stands for Centraalbureau voor Schimmelcultures - Fungal Biodiversity Centre - an institute of the Royal Netherlands Academy of Arts and Sciences which maintains a world-renowned collection of living filamentous fungi, yeasts and bacteria. As a rule, when a particular yeast strain is registered there, it is assigned a specific CBS number in chronological order. However there is no field for the deposit date. Their strain database can be searched here <http://www.cbs.knaw.nl/collections/BioloMICS.aspx?Link=T...All>



However, we discovered that, in the CBS collection, the two preceding yeasts were also deposited by him, one of which has valuable information attached to its deposit. *P. castillae*, CBS 6053, was isolated from frass of insects collected in an exotic tree, *Gymnocladus canadensis* (sic), Botanical Gardens, Madrid; no details about the insects (Santa María & García Aser, 1970), but the tree was probably *G. dioicus*, the Kentucky coffee tree. Interestingly, only this strain is known for *P. castillae*, now known as *Priceomyces castillae* (Kurtzman *et al.*, 2011). It may not be a specific symbiont of Lucanidae or other saproxylic (wood-feeding) insects. However, the related yeasts are sometimes found in the guts of adult Lucanidae and their larvae, and we guess that it is a facultative symbiont of wood-boring insects.

Later, Santa María deposited a third *Pichia* yeast, *segobiensis*, now known as *Scheffersomyces segobiensis*, CBS 6857; it is one of several strains of *P. segobiensis*. Two were isolated from wood-feeding beetle larvae: the jewel beetle *Chalcophora mariana massiliensis* and the longhorn beetle *Rhagium inquisitor*; and a third from insect frass, all found under the bark of *Pinus sylvestris* in Segobia (Santa María & García Aser, 1977).

Unfortunately the co-author, Concépcion García Aser, retired 30 years ago and even if she were still alive she would probably not remember the origin of the now famous genome-sequenced yeast, CBS 6054, because she did only the chemical side of the work. One possibility is that it could well have been the same substrate of isolation as in the preceding yeast, CBS 6053, and the field was overlooked when filling in its deposit form which is rather incomplete by comparison; for instance, the country of origin is not stated.

Thus this secret could be either in his lab books or in his yeast collection book.

### History of Pignal's yeast

Regarding the oldest xylose-fermenting yeast, *P. stipitis* CBS 5773, its history is well known. It was deposited earlier by Marie-Claire Pignal. She isolated it from over 20 wood-feeding larvae of two beetles: the Lesser stag beetle *D. parallelipipedus* and the rose chafer *Cetonia aurata*; and one robber-fly (*Laphria* sp.), all found in an old fruit tree stump in France (Pignal, 1968). It is unfortunate that she did not specify the exact origin of the deposited strain, since in the 70's there might not have been methods to detect strain-level variations between these isolates. It is not uncommon that more than two different *P. stipitis* strains are identified from different insects living in the same decaying wood (MT, pers. observ.).



However, the presence of the Lesser stag beetle larvae, together with the rose chafer larvae, was an interesting coincidence; MF often finds the latter together with the Stag beetle *L. cervus*. It has been observed that when Cetoniinae larvae coexist with the Rainbow stag beetle *Phalacrognathus muelleri*, they may be feeding on the latter's faeces (Wood *et al.*, 1996). At the same time, it is known that Cetoniinae larvae produce large quantities of faecal pellets and that they do not reingest them (Jönsson *et al.*, 2004; Micó *et al.*, 2011; Li & Brune, 2005; MF, pers. observ.). MT examined *C. aurata* and another Cetoniinae species, *Paratrichius doenitzi*, which in Japan usually shares decaying wood with stag beetle larvae. Although they do not have a mycangium, *P. stipitis* is identified from the larval gut of *P. doenitzi* with high frequency (Tanahashi *et al.*, 2010; MT, pers. observ.). Possibly, yeasts that were inoculated in the first place by the lesser stag beetle female/s during oviposition had become part of the shared pabulum at the time of collection by Pignal.

And if this hypothesis were true then the same could well have happened within the substrate of that tree in Madrid's Botanical Gardens, the presence of *P. castillae* in the guts of Lucanidae points to that, but this is just speculation.

### Conclusion

The mystery of the true origin of genome-sequenced yeast remains unsolved for the time being.

A lot more research needs to be done to establish whether female stag beetles do inoculate the substrate with their mycangium yeasts. More importantly, to prove that the *P. stipitis* isolated from the Colchester lesser stag beetle is exactly the same as the famous CBS 6054 strain, we should make a whole-genome comparison between these two strains, as well as a wide range sampling of the beetle in Europe, particularly in Spain where this remarkable Lesser stag beetle is undeservingly known as l'olvidado, the forgotten one.

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## References

- Agbogbo, F. K. and Coward-Kelly G. (2008). Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. *Biotechnology Letters*, 30, 1515-1524.
- Beaver, R. A. (1989). Insect-fungus relationship in the bark and ambrosia beetles. In: *Insect-fungus interactions*. Wilding N., Collins N. M., Hammond P. M. and Webber J. F. (Eds). London: Academic Press
- Franciscolo, M. E. (1997). *Coleoptera: Lucanidae, Fauna d'Italia*: Volume 35. Bologna: Calderini
- Fremlin, M., (2012). Protruded organs of a crushed female. URL [http://maria.fremlin.de/stagbeetles/flc\\_trodden.html](http://maria.fremlin.de/stagbeetles/flc_trodden.html)
- Fremlin, M. and Hendriks, P. (2013). Lesser Stag Beetles *Dorcus parallelipipedus* (L.) (Coleoptera: Lucanidae) longevity – at least three years. *Bulletin of the Amateur Entomologists' Society*, 72, 48-56.
- Jeffries, T. W., Grigoriev, I. V., Grimwood, J., Laplaza, J. M., Aerts, A., Salamov, A., Schmutz, J., Lindquist, E., Dehal, P., Shapiro, H., Jin, Y. S., Passoth, V. and Richardson, P. M. (2007). Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nature Biotechnology*, 25, 319-326.
- Jönsson, N., Méndez, M. and Ranius, T. (2004). Nutrient richness of wood mould in tree hollows with the Scarabaeid beetle *Osmoderma eremita*. *Animal Biodiversity and Conservation*, 27, 79-82.
- Klausnitzer, B. and Sprecher-Uebersax, E. (2008). Die Hirschkäfer oder Schröter (Lucanidae). Die Neue Brehm-Bücherei. Band 551. Magdeburg: Westarp Wissenschaften.
- Kobayashi, C., Fukasawa, Y., Hirose, D. and Kato, M. (2008). Contribution of symbiotic mycangial fungi to larval nutrition of a leaf-rolling weevil. *Evolutionary Ecology*, 22, 711-722.
- Kurtzman, C. P. and Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek*, 73, 331-371.
- Kurtzman, C. P. and Suzuki, M. (2010). Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*. *Mycoscience*, 51, 2-14.
- Kurtzman, C. P., Fell, J. W. and Boekhout, T. (2011). *The Yeasts, a taxonomic study*: Volume 2. (5th edition). London: Elsevier.
- Li, X., and Brune, A. (2005). Digestion of microbial biomass, structural polysaccharides, and protein by the humivorous larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Soil Biology and Biochemistry*, 37, 107-116.
- Micó, E., Juárez, M., Sánchez, A. and Galante, E. (2011). Action of the saproxylic scarab larva *Cetonia aurataeformis* (Coleoptera: Scarabaeoidea: Cetoniidae) on woody substrates. *Journal of Natural History*, 45, 41-42 and 2527-2542.
- Pignal, M.-C. (1968). Une nouvelle espèce de levure isolée de larves d'insectes: *Pichia stipitis*. *Bulletin Mensuel de la Société linnéenne de Lyon*, 36, 163-168.
- Santa María, J. and García, Aser, C. (1970). *Pichia castillae*, nov. spec., aislada de insectos. *Boletín del Instituto Nacional de Investigaciones Agronomicas*, 62, 51-55.
- Santa María, J. and García, Aser, C. (1977). *Pichia segobiensis*, sp. nov. *Anales del Instituto Nacional de Investigaciones Agrarias, Serie general*, 5, 45-50.
- Santa María, J. (1978). Biotaxonomic studies on yeast. *Comunicaciones del Instituto Nacional de Investigaciones Agrarias. Serie general*, 3, 5-62.
- Tanahashi, M., Kubota, K., Matsushita, N. and Togashi, K. (2010). Discovery of mycangia and associated xylose-fermenting yeasts in stag beetles (Coleoptera: Lucanidae). *Naturwissenschaften*, 97, 311-317.
- Toki, W., Tanahashi, M., Togashi, K. and Fukatsu, T. (2012). Fungal farming in a non-social beetle. *PLoS One*, 7, e41893.
- Wood, G. A., Hasenpusch, J. and Storey, R. I. (1996). The life history of *Phalacrognathus muelleri* (Macleay) (Coleoptera: Lucanidae). *Australian Entomologist*, 23, 37-48.