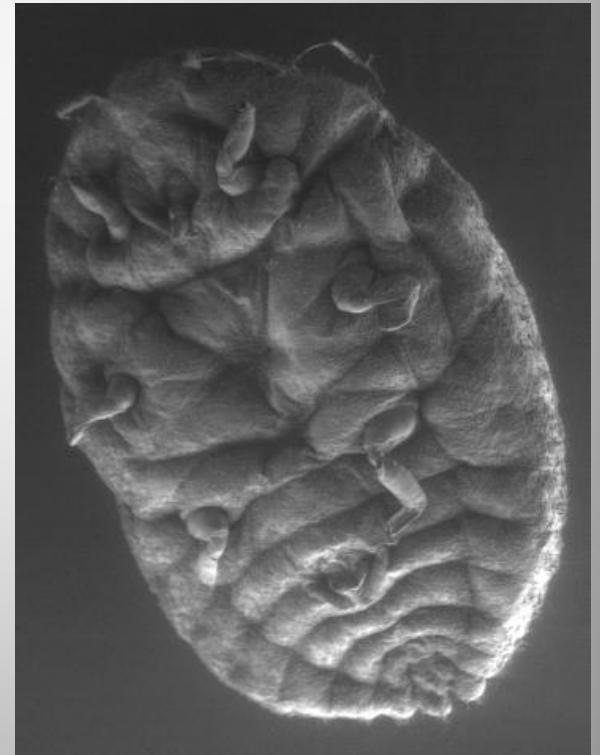


A PCR-BASED APPROACH FOR THE IDENTIFICATION OF CACAO *SWOLLEN SHOOT VIRUS* MEALYBUG VECTORS

Andy Wetten, Colin Campbell & Joël Allainguillaume



Summary

- Tissue culture for the interruption of CSSV
- Mealybugs as pests of cacao
- They all look very similar
- How COI-based DNA barcoding can help

Somatic Embryogenesis (SE)

A process by which haploid or diploid cells develop into differentiated plants through embryological stages without fusion of gametes



'Hamlin' sweet orange somatic embryos

Advantages of somatic embryogenesis

- Allow rapid clonal multiplication
- Fungal and bacteria-free
- Interruption of virus movement – lack of vascular connection.

Cocoa somatic embryogenesis



Flower bud



Floral staminode



4 weeks

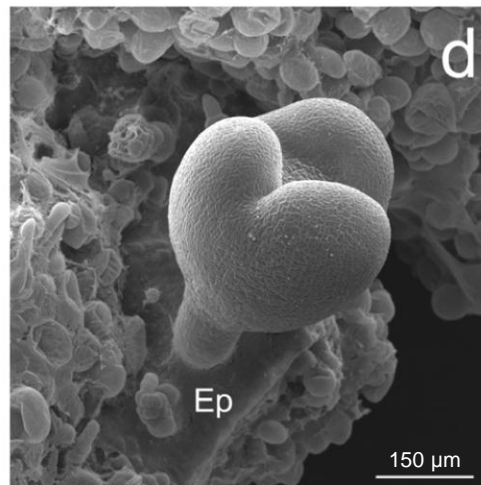
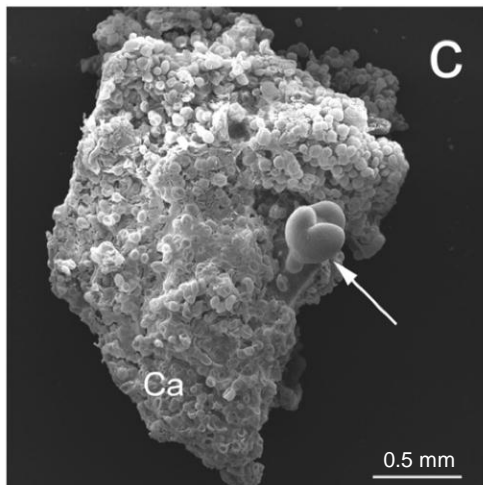
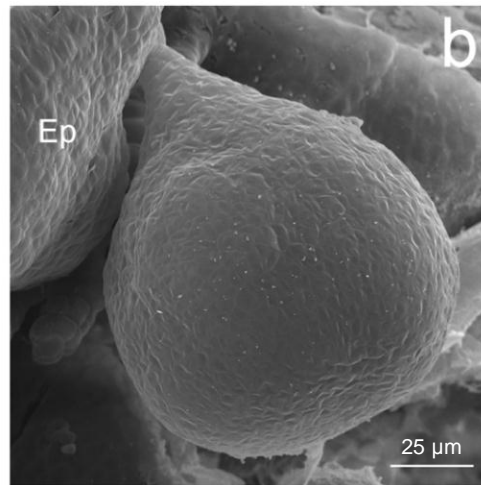
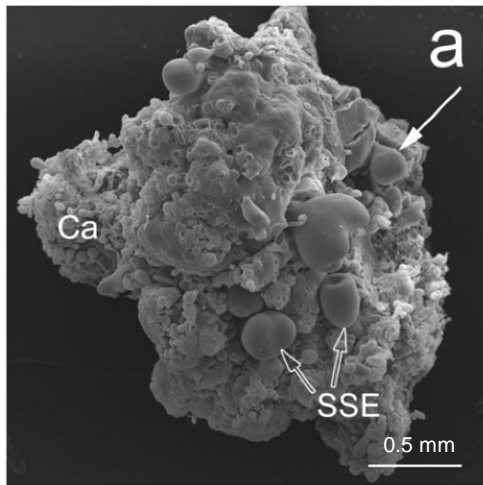


Somatic embryos

30 d – 1 year



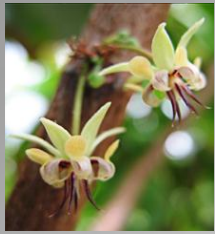
Regenerated plant



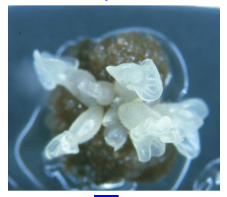
Secondary somatic embryos developing on cotyledonary explants from primaries (a & c). Arrows show SSEs (magnified in b & d) arising from epidermis. Ca = callus, Ep = epidermis.

Germplasm conservation via cryopreservation

Staminodes harvested from closed flowers



1° somatic embryos initiate after 6 weeks



2° embryos initiate on cotyledonary explants from 1° embryos

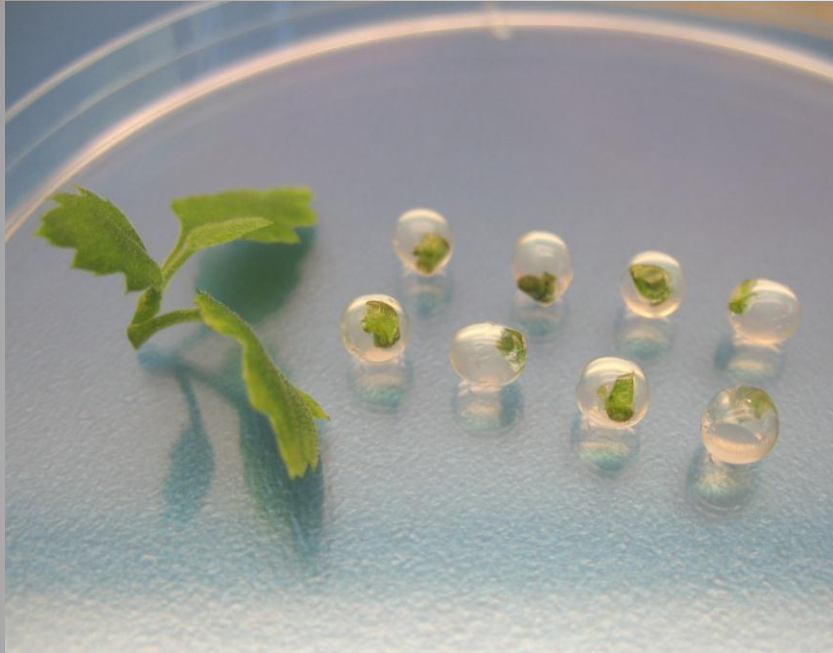


Dehydrated alginate encapsulated embryos cryopreserved

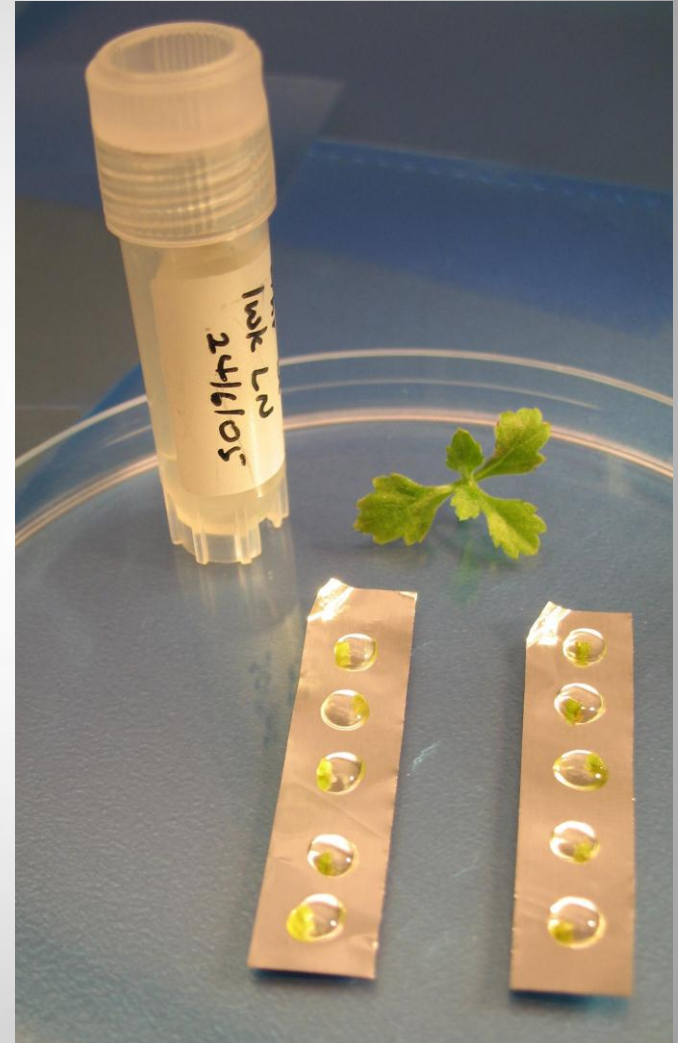


Embryos weaned after appearance of first true leaves

Cryopreservation methodologies



Encapsulation/dehydration



PVS2-based vitrification

PVS2 protocol:

- 2° SEs (2-5mm) precultured on 0.5 M sucrose – 5 days



- Loading solution (2M glycerol / 0.4M sucrose) – 20 min



- 10 SEs in 2 ml cryovials + 1 ml PVS2 on ice – 1 h



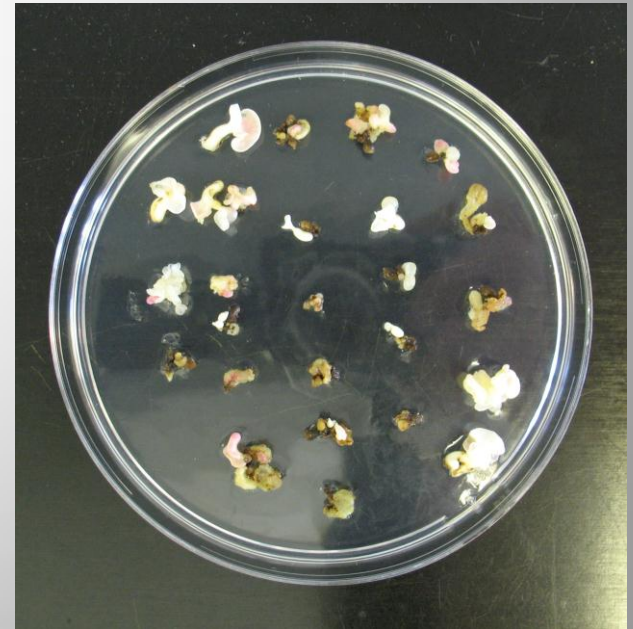
- LN - at least 1 h



- 42°C water bath



- remove PVS2 and wash twice with liquid ED



Mealybug classification

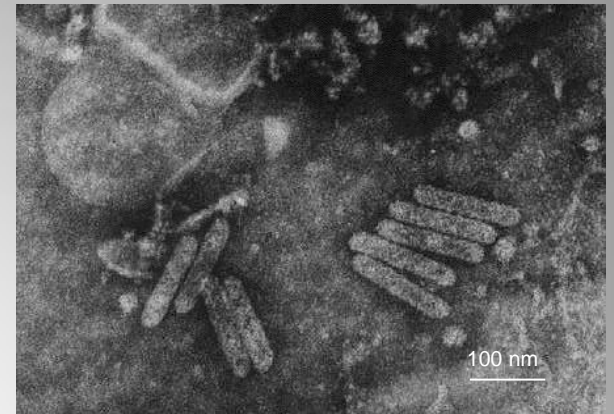
- Order – Hemiptera (so, incomplete morphogenesis – nymphs resemble adults)
- Family - Pseudococcidae



- Sooty moulds can be problematic on cacao arising from their sap feeding habit
- Major pest status as virus vectors

Cacao Swollen Shoot Virus (CSSV)

- **Bacilliform DNA virus**



- red vein banding



- swellings on chupons



CSSV transmission

- ≥ 15 mealybug spp. thought to transmit



- Dominance of particular species in part due to associations with specific attendant ants





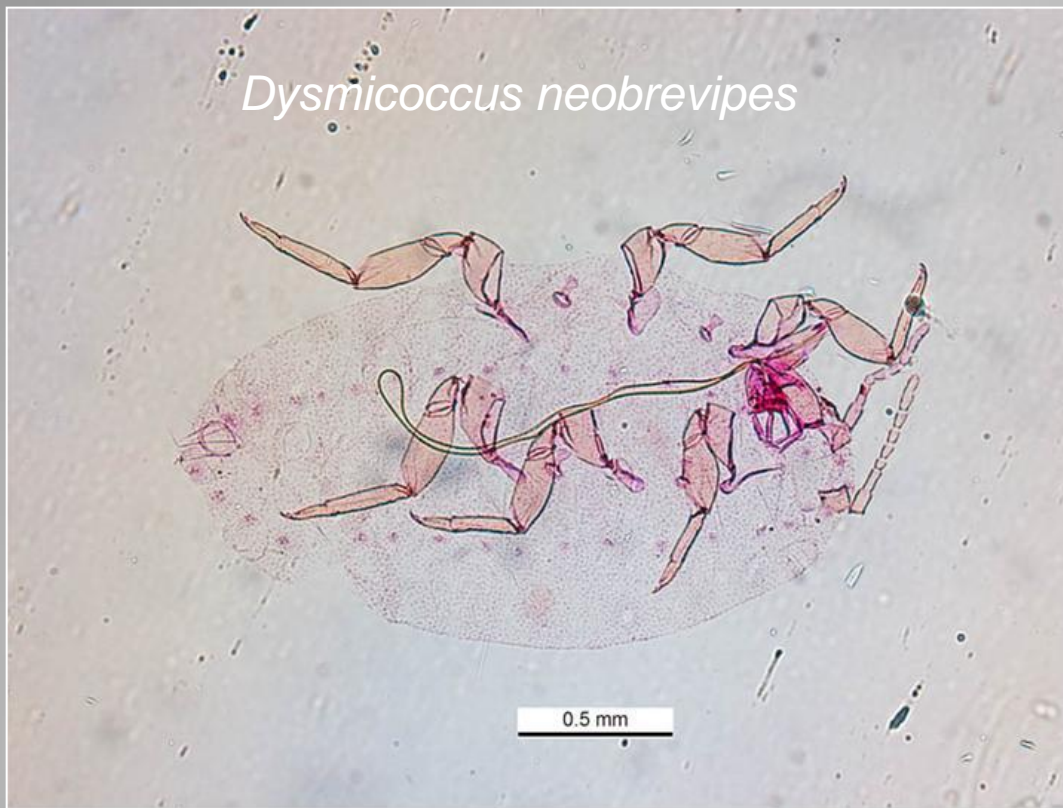
Mealybug-based screening for CSSV resistance

- *species identification vital*

Mealybugs so far identified from cacao in West Africa:

<i>Delococcus (Formicococcus) tafoensis</i> (Strickland)	Ghana/Togo
<i>Delottococcus (Pseudococcus, Paracoccus) sp. nr proteae</i> (Hall)	Ghana/Nigeria
<i>Dysmicoccus brevipes</i> (Cockerell) = <i>Pseudococcus bromeliae</i> (Bouche)	Ghana/Togo/Nigeria
<i>Ferrisia (Ferrisiana) virgata</i> (Cockerell)	West Africa
<i>Formicococcus (Planococcoides, Planococcus, Pseudococcus) celtis</i>	Sierra Leone/Ghana
<i>Formicococcus njalensis</i> (Laing) = <i>F. (Planococcus) lamabokensis</i>	West Africa/Zaire
<i>Geococcus coffeae</i> (Green)	Ghana/Nigeria
<i>Heliococcus sp.</i>	Nigeria
<i>Maconellicoccus (Pseudococcus) ugandae</i> (Laing)	Ghana/Togo
<i>Paraputo anomala</i> (Newstead) = <i>P. multispinosa</i> (James); <i>P. ritchei</i> (James)	Ghana
<i>Pa. (Cataenococcus, Farinococcus) loranthi</i> (Strickland)	Ghana
<i>Phenacoccus hargreavesi</i> = <i>Pseudococcus bukobensis</i> Laing	West Africa
<i>Ph. madeirensis</i> (Green)	Ghana
<i>Planococcus (Tylococcus) boafoensis</i> (Strickland)	Ghana
<i>Planococcus citri</i> (Risso)	West Africa
<i>Pl. kenya</i> (Le Pelley)	West Africa/Zaire
<i>Pseudococcus calceolariae</i> (Maskell) = <i>P. gahani</i> (Green); <i>P. fragilis</i> (Brain)	Ghana/Togo/Nigeria
<i>Ps. comstocki</i> (Kuwana)	Ghana
<i>Ps. concavocerari</i> (James)	West Africa
<i>Ps. longispinus</i> (Targioni-Tozzetti) = <i>P. (Dactylopius) adonidum</i> L.	Ghana/Togo/Nigeria
<i>Rhizoecus spelaeus</i> (Strickland) = <i>Coccidella spelaea</i> (Strickland)	Ghana
<i>Tylococcus westwoodi</i> (Strickland)	Ivory Coast/Ghana/Togo

= significant vector of CSSV



- Discoidal pores near eye;
- dorsomedial setae on segment VIII about same length as on VI & VII;
- abdominal cerarii anterior of anal-lobe pair, usually with > 2 conical setae;
- ventral multiloculars normally confined to posterior 3 abdominal segments;
- ventral oral collars absent from laterad of anterior coxa;
- 17 pairs of cerarii;
- translucent pores on hind femur and tibia.

Challenges:

- Taxonomic expertise on which species diagnosis depends is disappearing
- Conventional histology disrupts most specimens
- Keys generally restricted to adult females
- Morphological plasticity leads to numerous misdiagnoses

This study combines detailed microscopy of reference samples with a DNA barcoding approach to support CSSV resistance breeding and quarantine

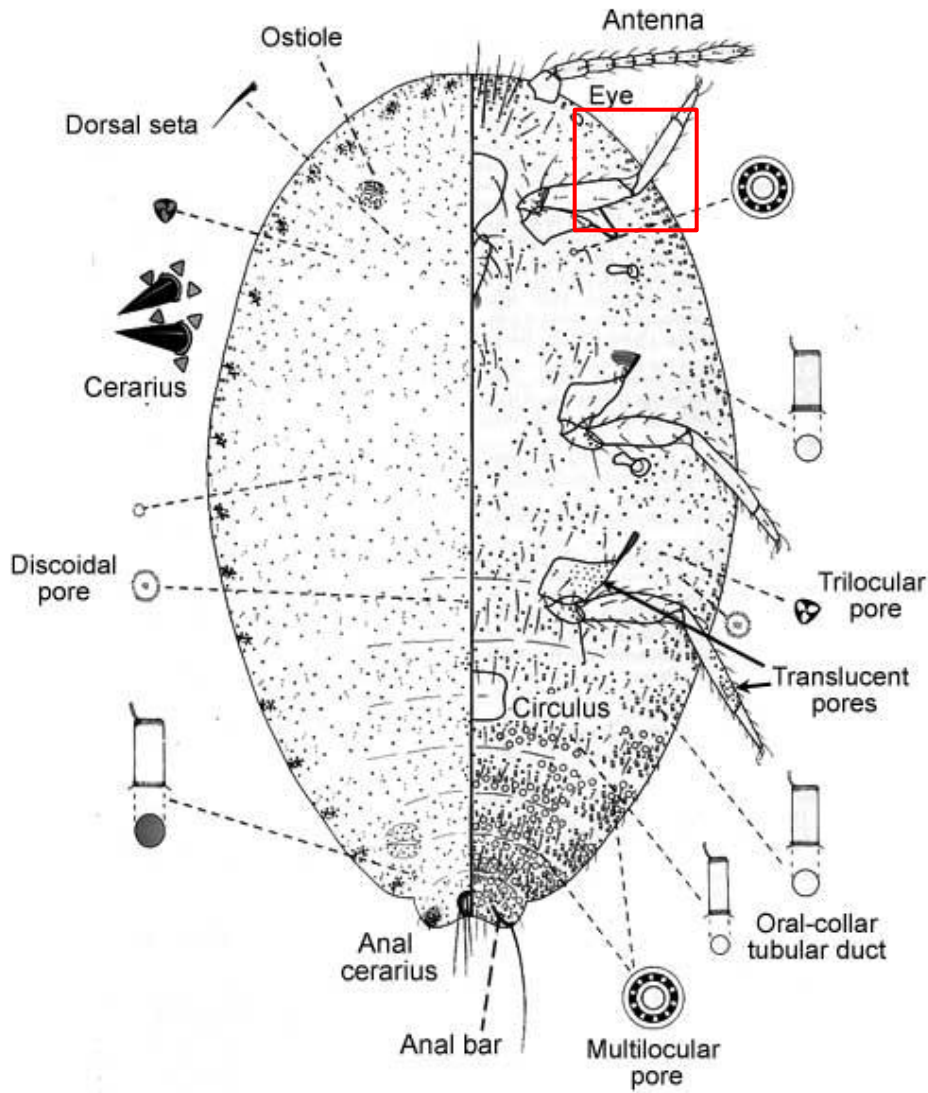
Environmental Scanning Electron Microscopy

- *Permits detailed study of fresh specimens*
- *Minimal artefacts*

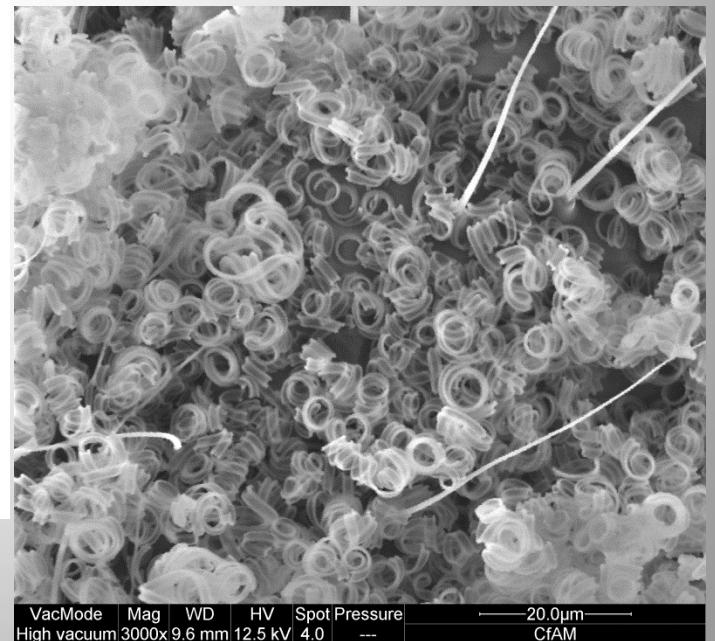
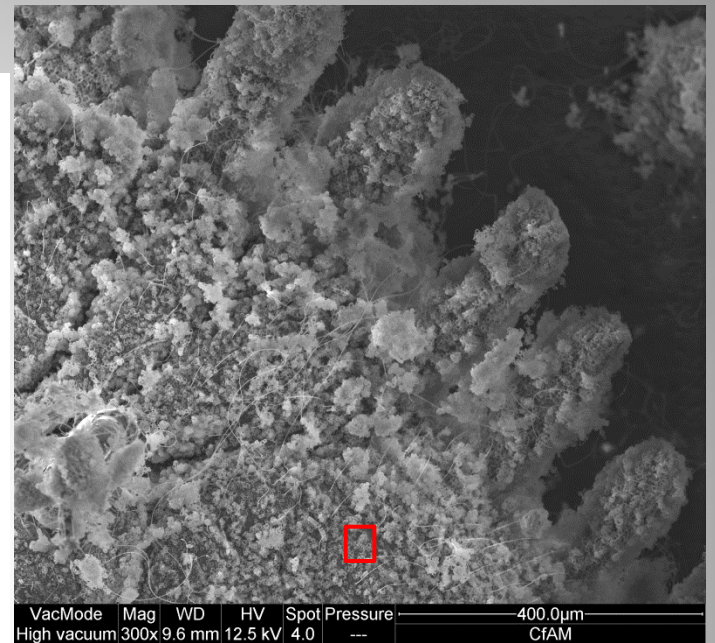


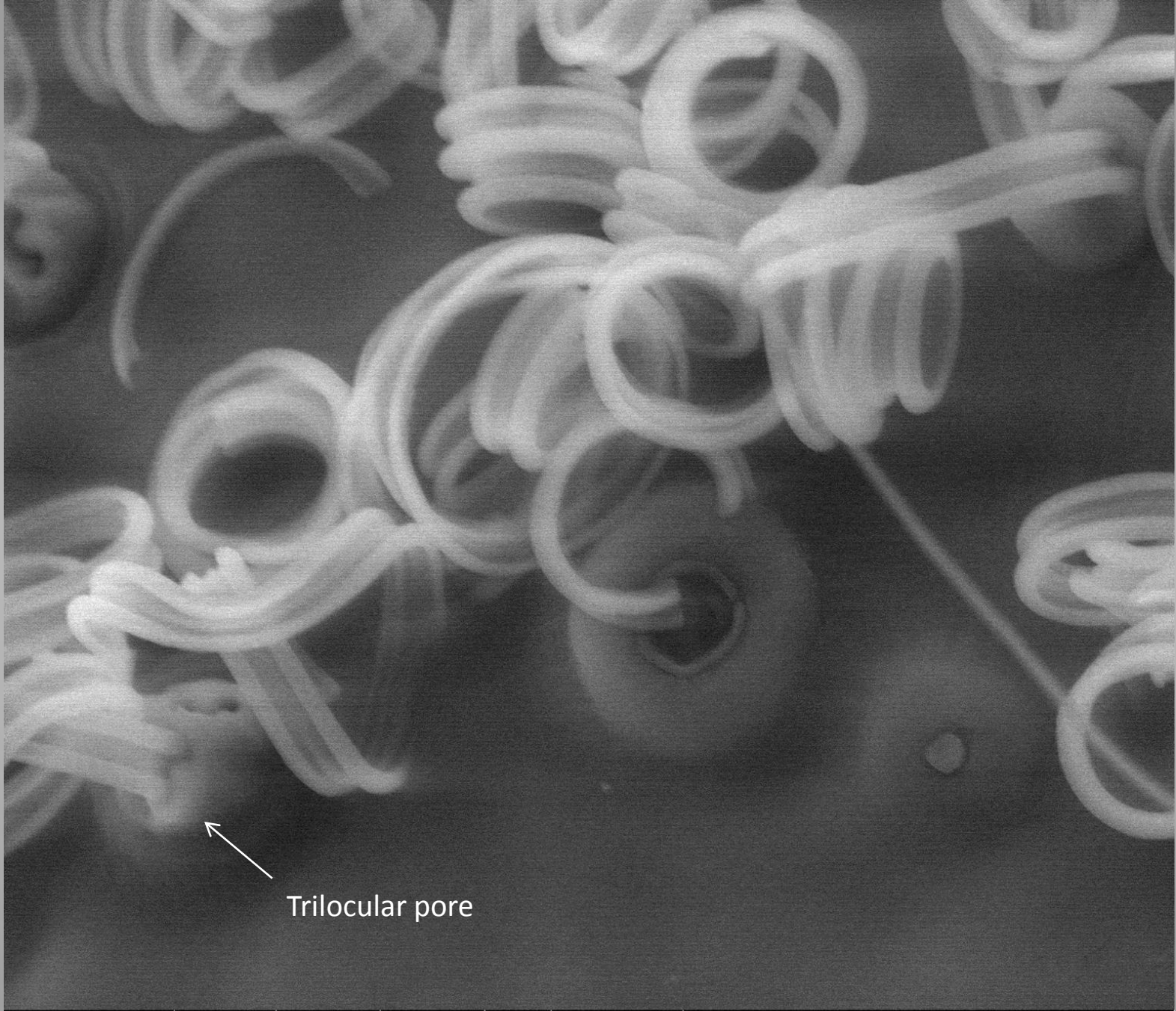
VacMode	Mag	WD	HV	Spot	Pressure
Low vacuum	140x	9.6 mm	12.5 kV	4.0	0.65 Torr

500.0µm
CfAM



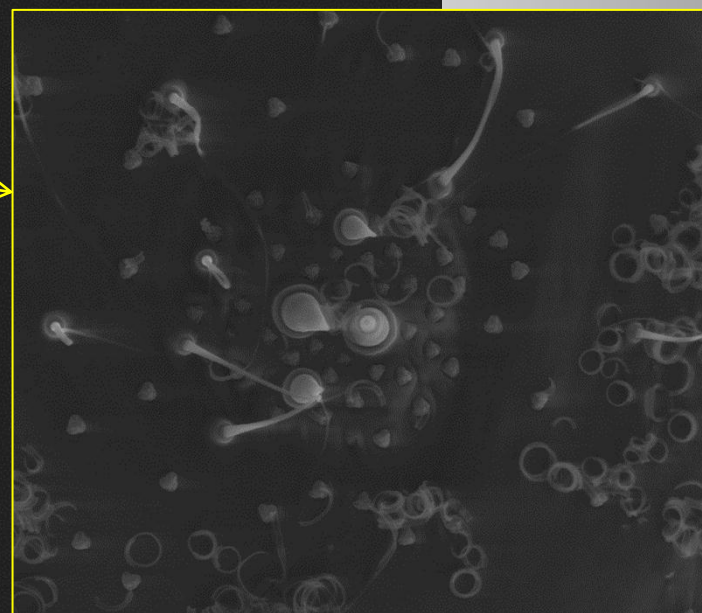
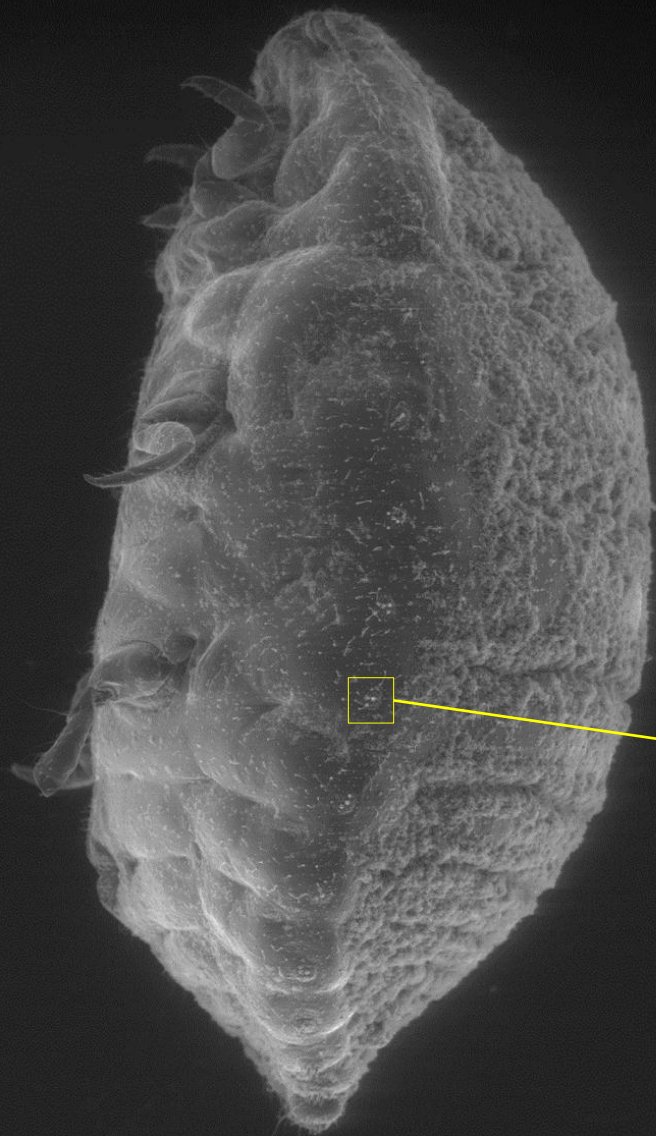
Planococcus citri





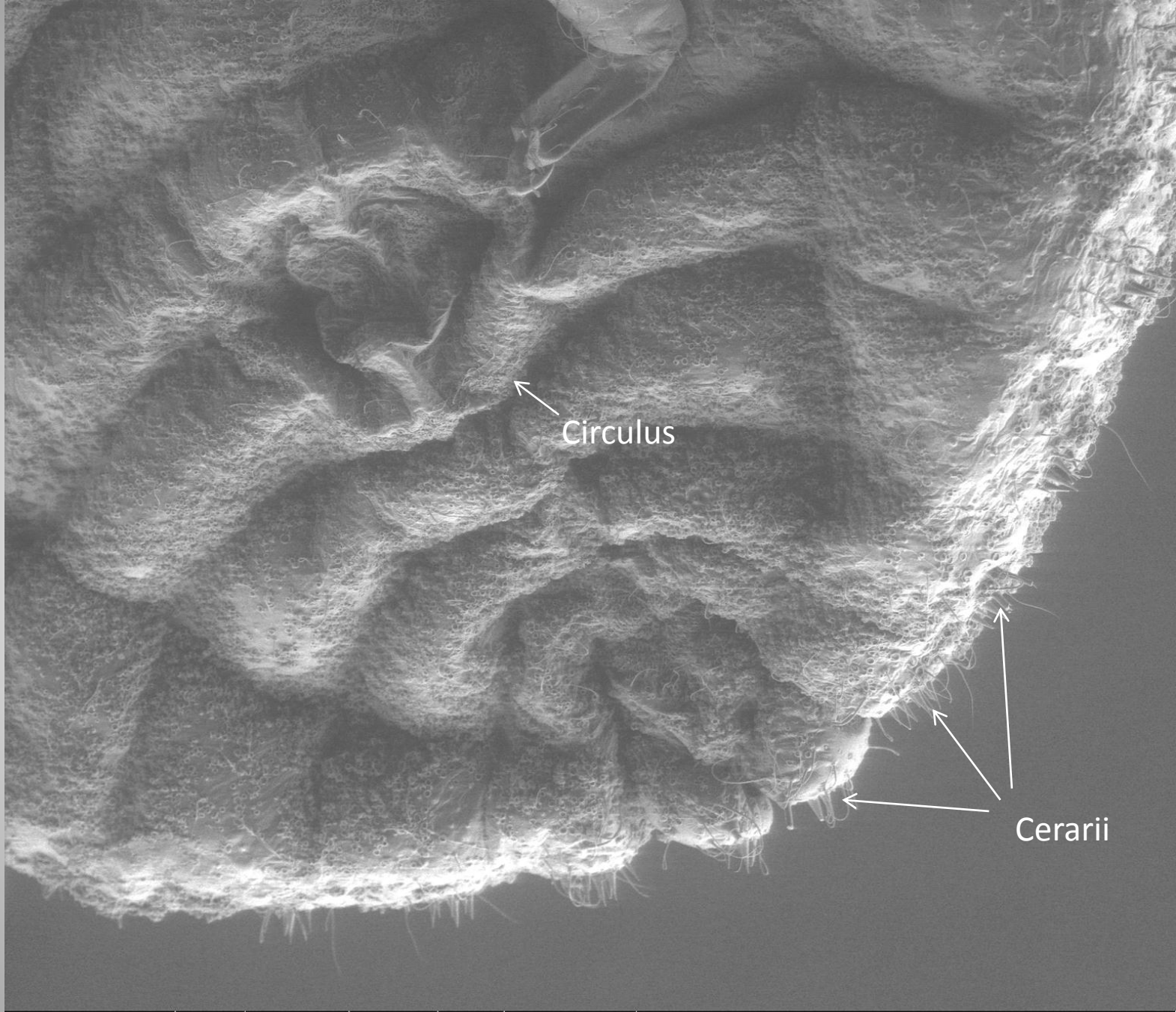
Trilocular pore

VacMode	Mag	WD	HV	Spot	Pressure	10.0µm
Low vacuum	10000x	9.6 mm	10.0 kV	3.0	0.65 Torr	CfAM

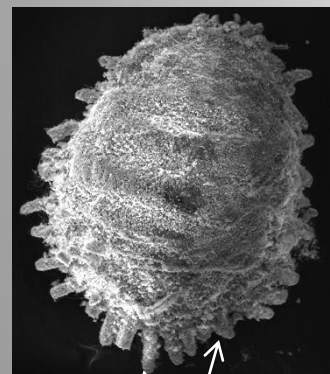


VacMode	Det	Mag	WD	HV	Spot	Pressure
Low vacuum	LFD	84x	8.0 mm	12.5 kV	4.0	0.65 Torr

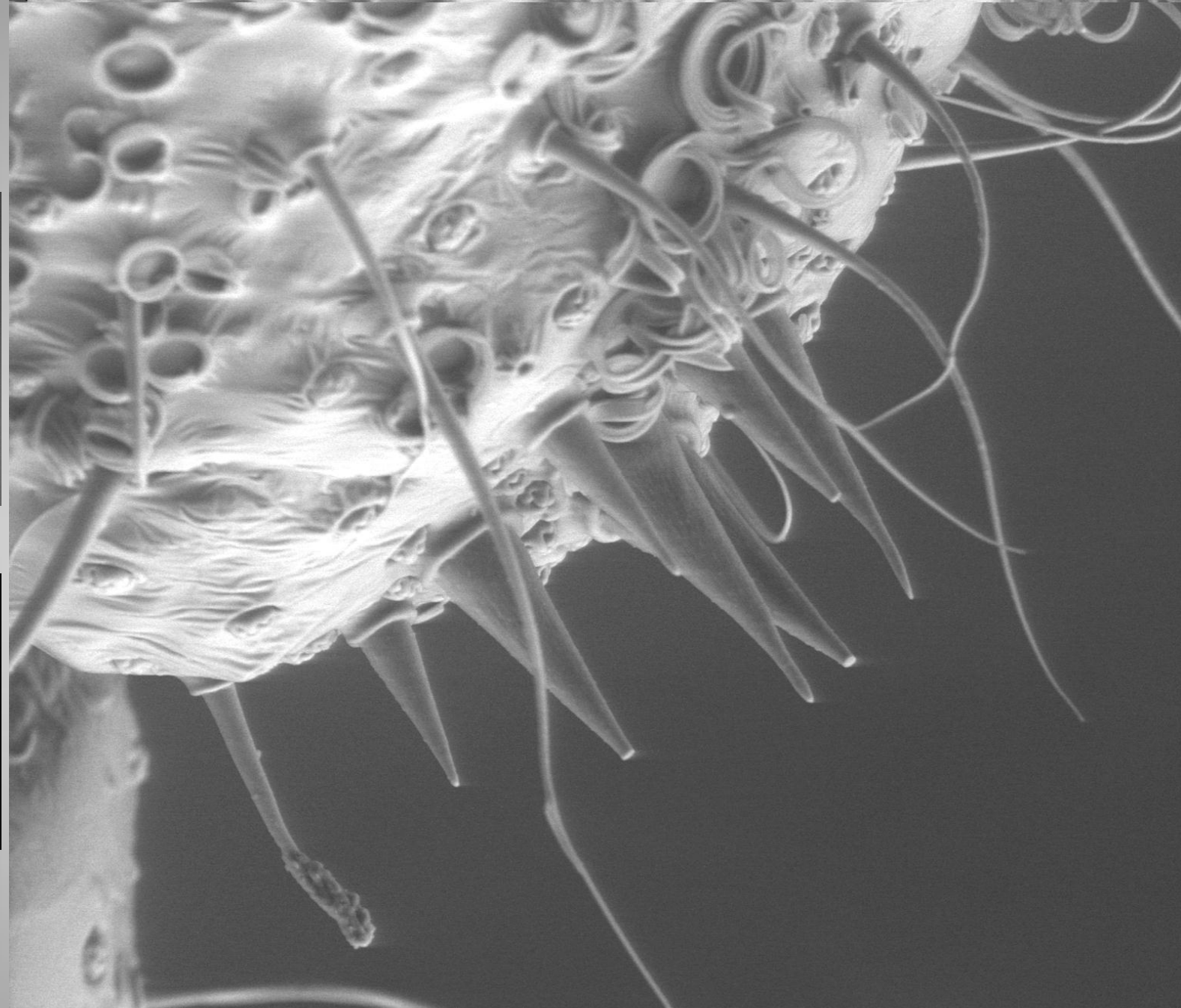
1.0mm
CfAM



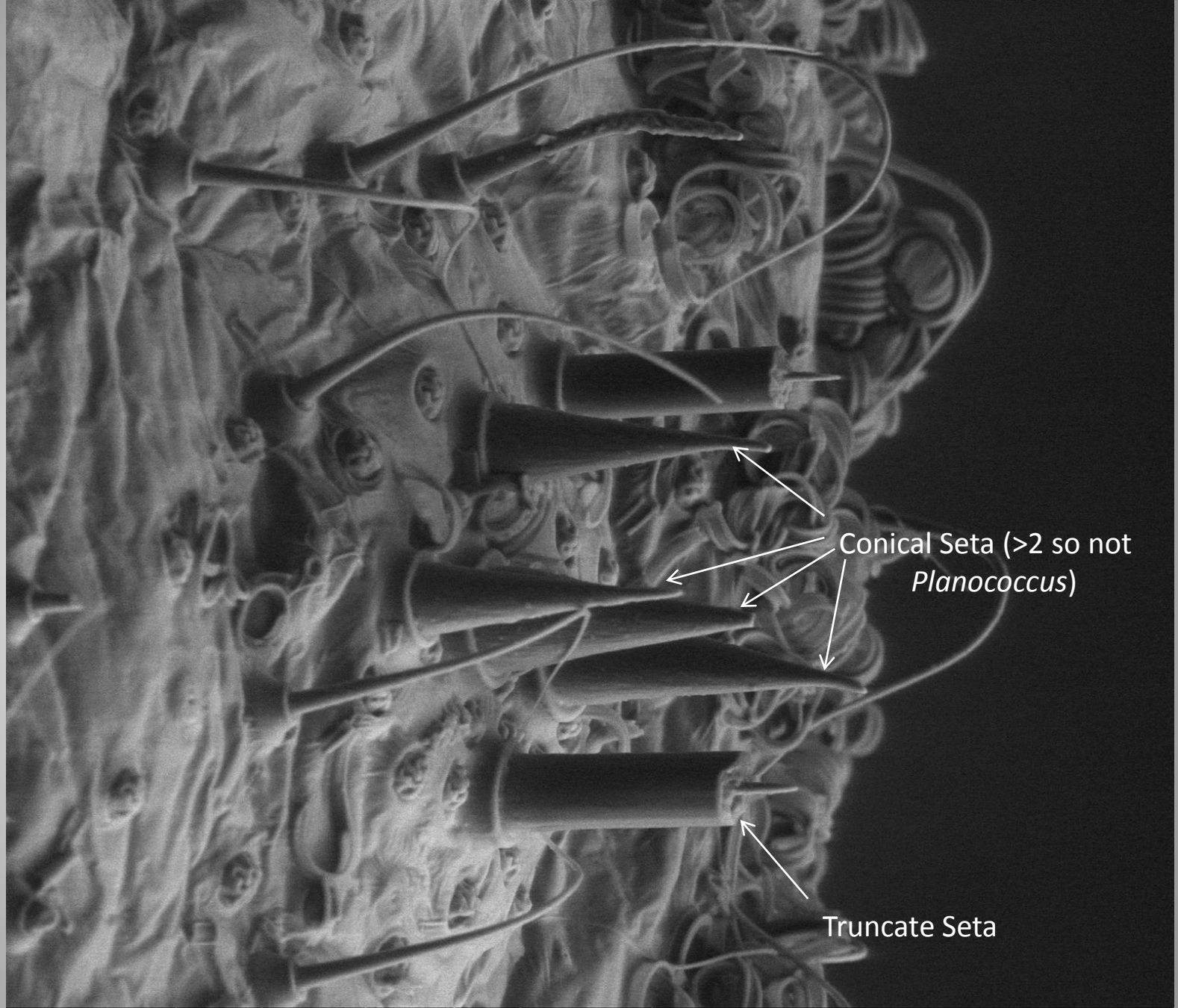
VacMode	Mag	WD	HV	Spot	Pressure	400.0µm
Low vacuum	300x	7.3 mm	5.0 kV	4.0	0.65 Torr	CfAM



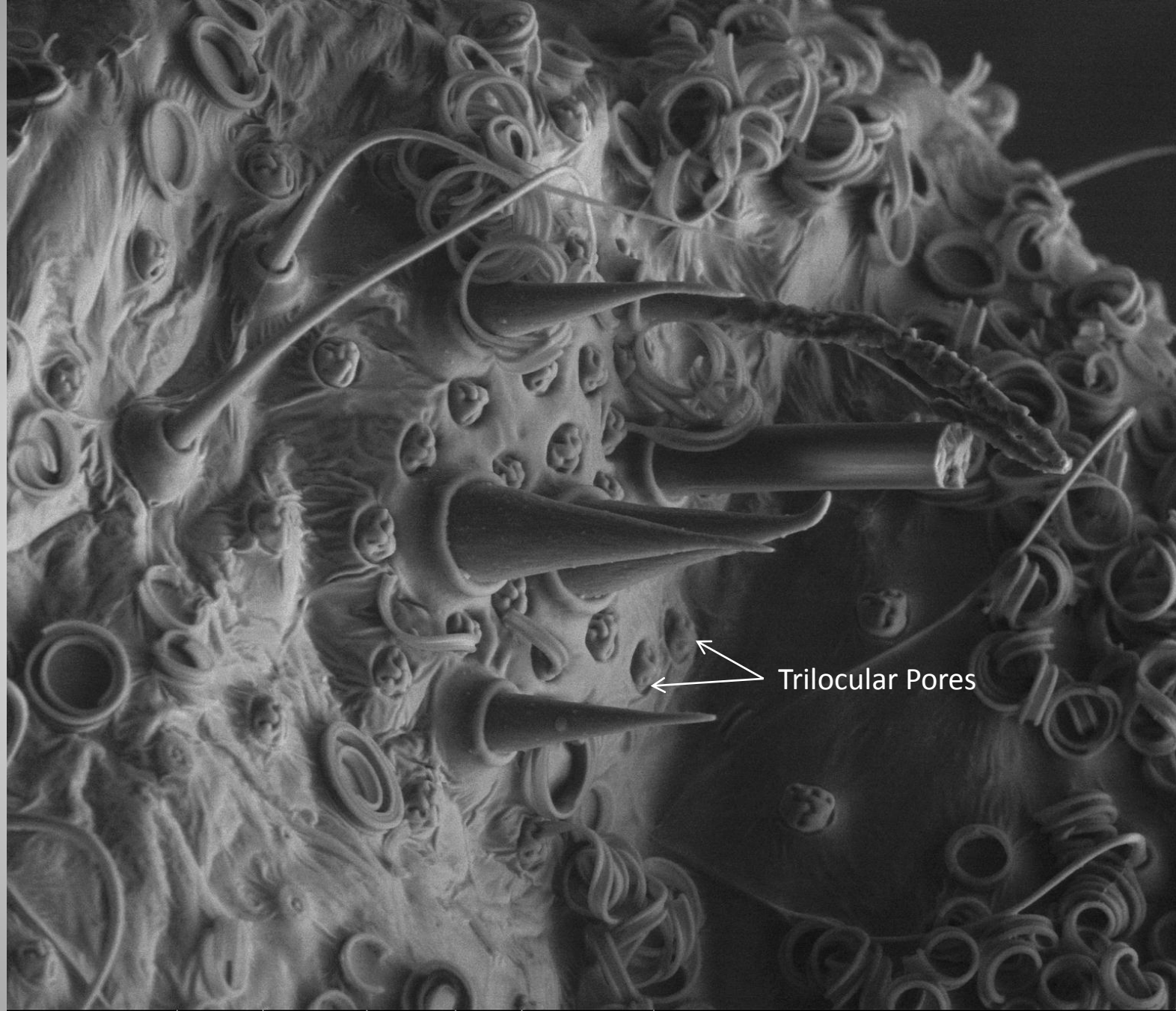
Lateral wax filaments



VacMode	Mag	WD	HV	Spot	Pressure	20.0µm CfAM
Low vacuum	3000x	7.4 mm	5.0 kV	4.0	0.65 Torr	



VacMode	Mag	WD	HV	Spot	Pressure	20.0µm CfAM
Low vacuum	3000x	7.2 mm	5.0 kV	4.0	0.65 Torr	



VacMode	Mag	WD	HV	Spot	Pressure
Low vacuum	3000x	7.4 mm	5.0 kV	4.0	0.65 Torr

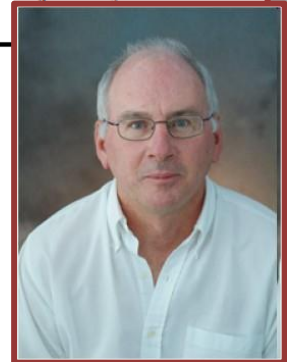
—20.0µm—
CfAM

DNA barcoding

Biological identifications through DNA barcodes

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and Jeremy R. deWaard

Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1, Canada



Although much biological research depends upon species diagnoses, taxonomic expertise is collapsing. We are convinced that the sole prospect for a sustainable identification capability lies in the construction of systems that employ DNA sequences as taxon ‘barcodes’. We establish that the mitochondrial gene cytochrome *c* oxidase I (COI) can serve as the core of a global bioidentification system for animals. First, we demonstrate that COI profiles, derived from the low-density sampling of higher taxonomic categories, ordinarily assign newly analysed taxa to the appropriate phylum or order. Second, we demonstrate that species-level assignments can be obtained by creating comprehensive COI profiles. A model COI profile, based upon the analysis of a single individual from each of 200 closely allied species of lepidopterans, was 100% successful in correctly identifying subsequent specimens. When fully developed, a COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification. Its assembly will also generate important new insights into the diversification of life and the rules of molecular evolution.

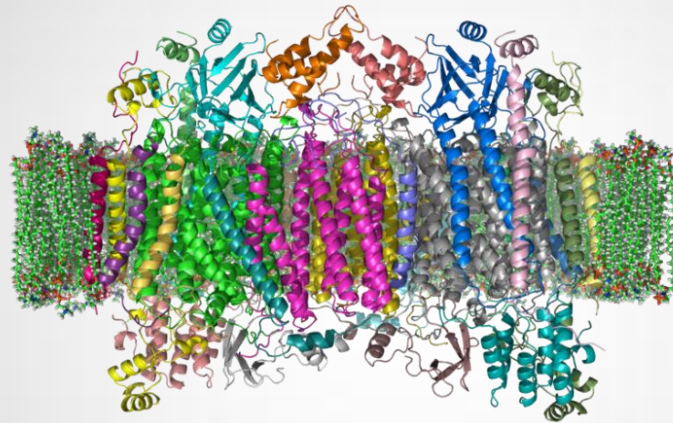
Keywords: molecular taxonomy; mitochondrial DNA; animals; insects; sequence diversity; evolution

1. INTRODUCTION

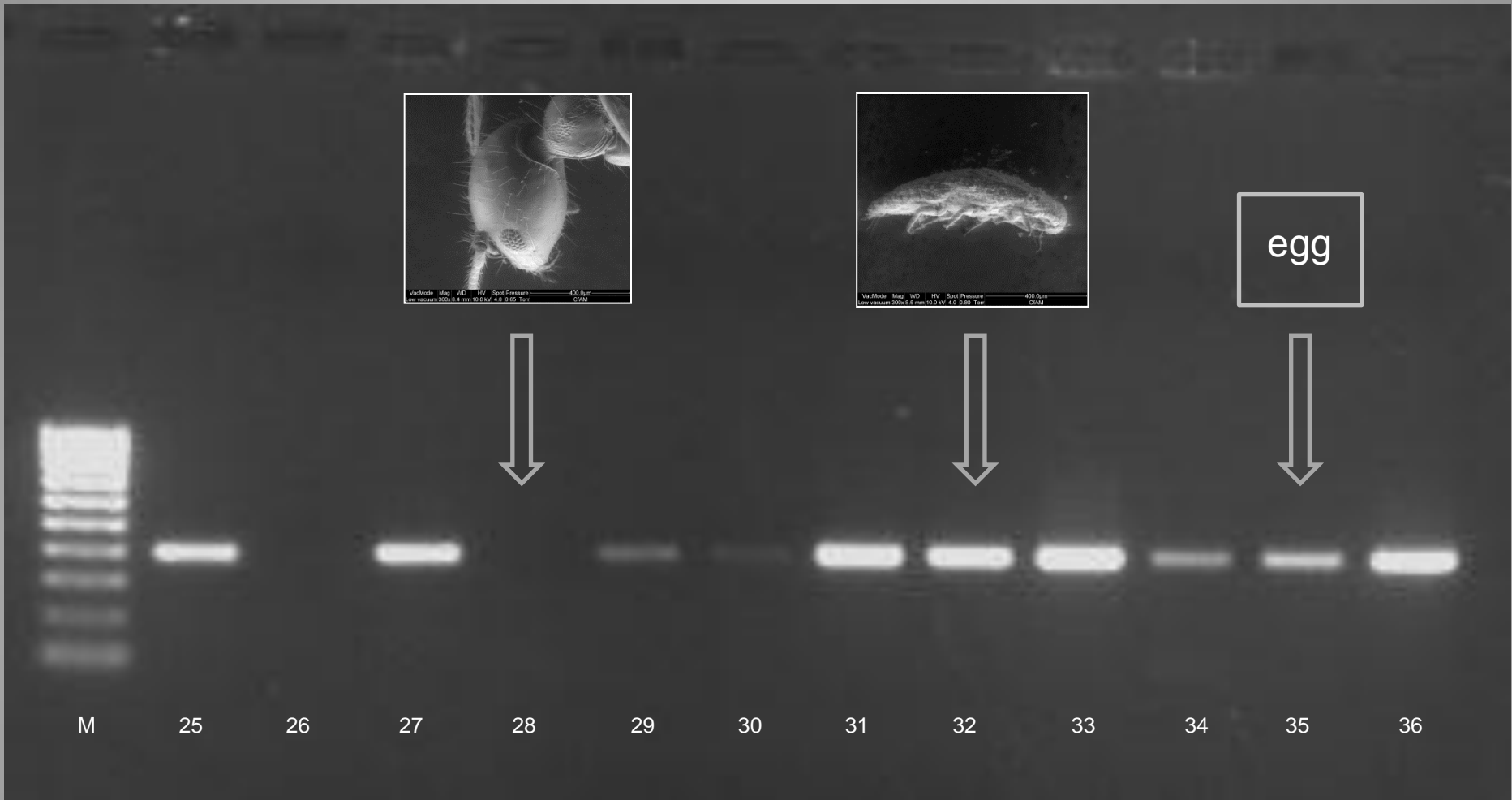
Allander *et al.* 2001; Hamels *et al.* 2001). However, the problems inherent in morphological taxonomy are general

Barcoding with mitochondrial cytochrome c oxidase I (COI)

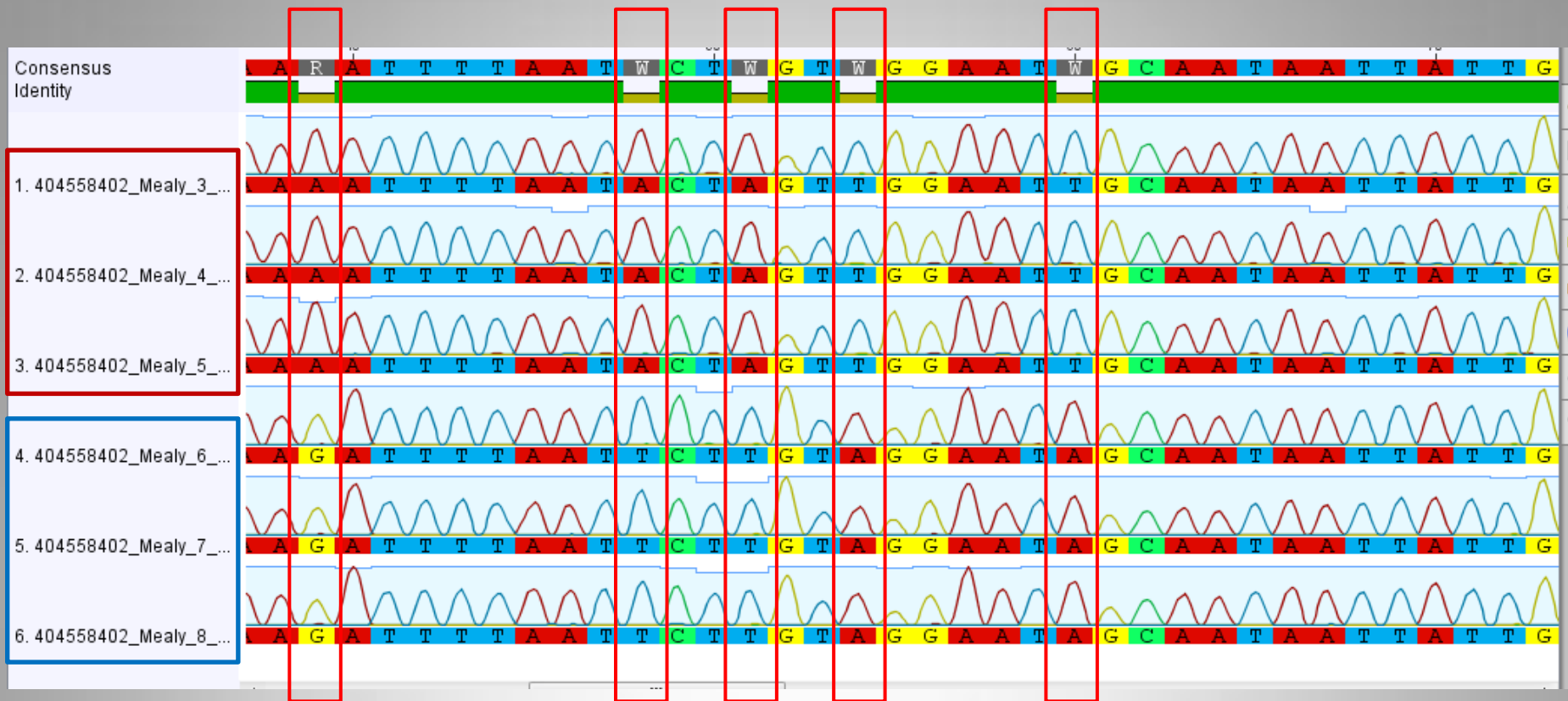
- COI – transmembrane protein gene widely used for animal barcoding



- Mitochondrial location therefore high copy number – *sensitive*
- Respiratory gene therefore highly conserved – *good for species separation*
- But sufficiently high mutation rate to allow geographical separation



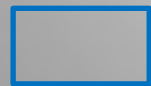
COI PCR products (400 bp) of Ivory Coast mealybugs available for DNA sequencing



Mealybug COI DNA sequence alignments reveal presence of 2 distinct species:

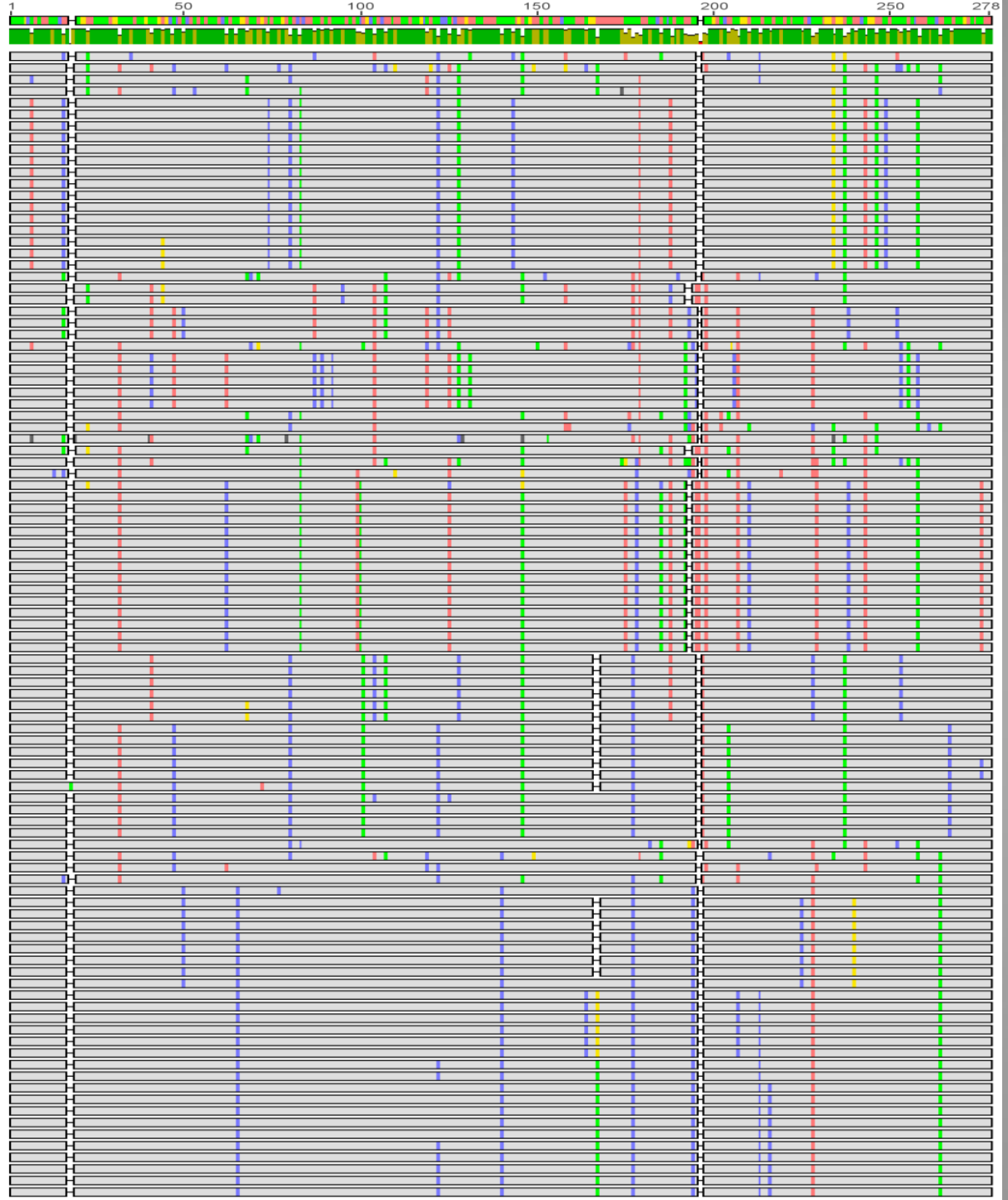


Pseudococcus longispinus



Planococcus citri

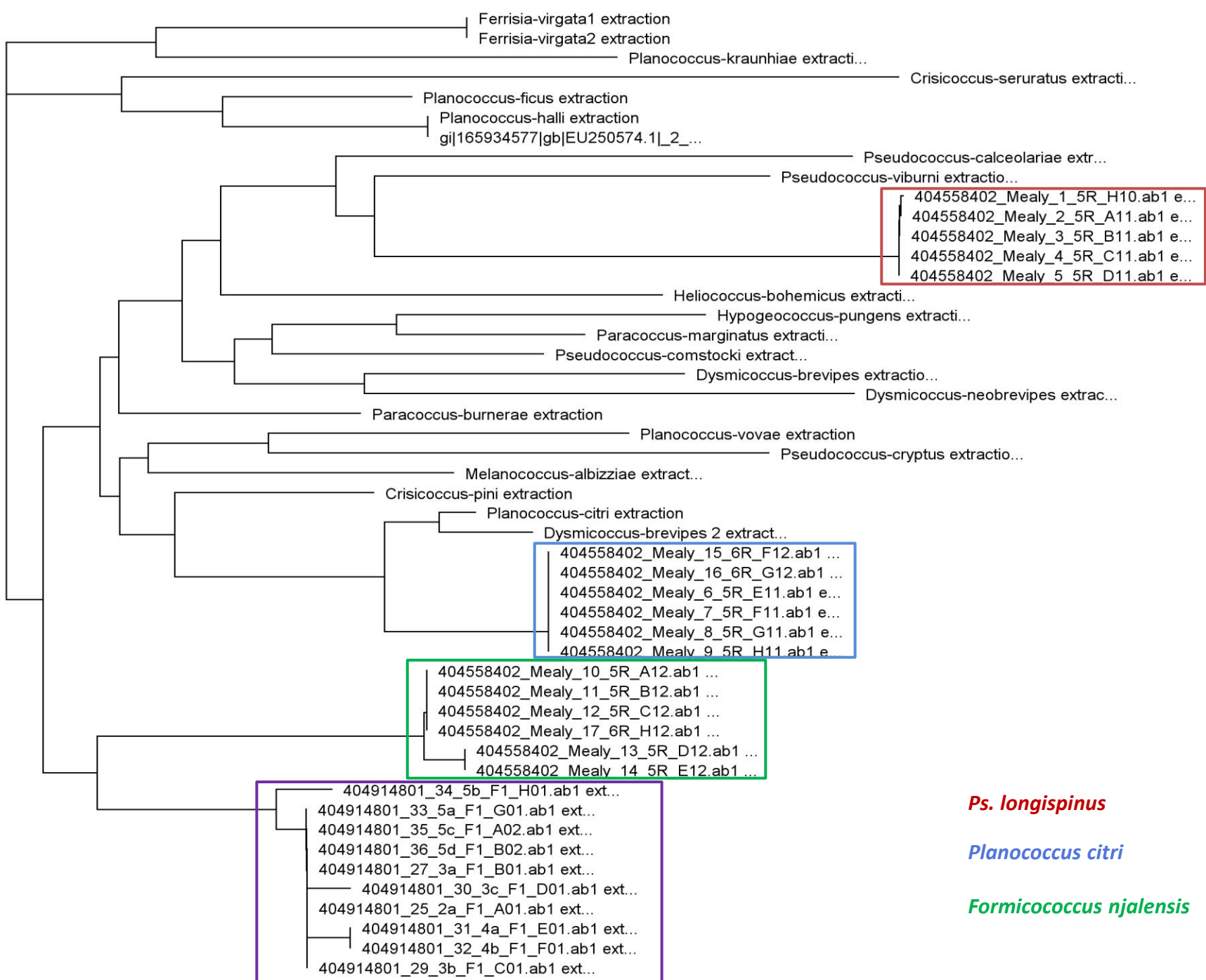
Consensus Identity



- 1. *Planococcus-kraunhia*
- 2. *Crusicoccus-seruratus*
- 3. *Planococcus-ficus*
- 4. *Planococcus-halli*1
- 5. 68
- 6. 67
- 7. 56
- 8. 55
- 9. 54
- 10. 53
- 11. 52
- 12. 51
- 13. 44
- 14. 43
- 15. 42
- 16. 41
- 17. 40
- 18. 39
- 19. 38
- 20. *Heliococcus-bohem...*
- 21. *Ferrisia-virgata*1
- 22. *Ferrisia-virgata*2
- 23. 33
- 24. 32
- 25. 31
- 26. *Pseudococcus-calc...*
- 27. 30
- 28. 29
- 29. 28
- 30. 27
- 31. 26
- 32. 25
- 33. 24
- 34. 23
- 35. 22
- 36. *Pseudococcus-viburni*
- 37. *Dysmicoccus-brevip...*
- 38. *Dysmicoccus-neobr...*
- 39. 21
- 40. 20
- 41. 19
- 42. 18
- 43. 17
- 44. 16
- 45. 15
- 46. 14
- 47. 13
- 48. 12
- 49. 11
- 50. 10
- 51. 9
- 52. 8
- 53. 7
- 54. 6
- 55. 5
- 56. 4
- 57. 3
- 58. 2
- 59. 1
- 60. 0
- 61. 0
- 62. 0
- 63. 0
- 64. 0
- 65. 0
- 66. 0
- 67. 0
- 68. 0
- 69. *Melanococcus-albizz...*
- 70. *Planococcus-vovae*
- 71. *Paracoccus-burnerae*
- 72. *Crusicoccus-pini*
- 73. *Planococcus-citri*
- 74. 75
- 75. 74
- 76. 73
- 77. 72
- 78. 71
- 79. 70
- 80. 69
- 81. *Dysmicoccus-brevip...*
- 82. 11
- 83. 9
- 84. 7
- 85. 6
- 86. 5
- 87. 4
- 88. 3
- 89. 2
- 90. 1
- 91. 0
- 92. 0
- 93. 0
- 94. 0
- 95. 0
- 96. 0
- 97. 0
- 98. 0
- 99. 0
- 82

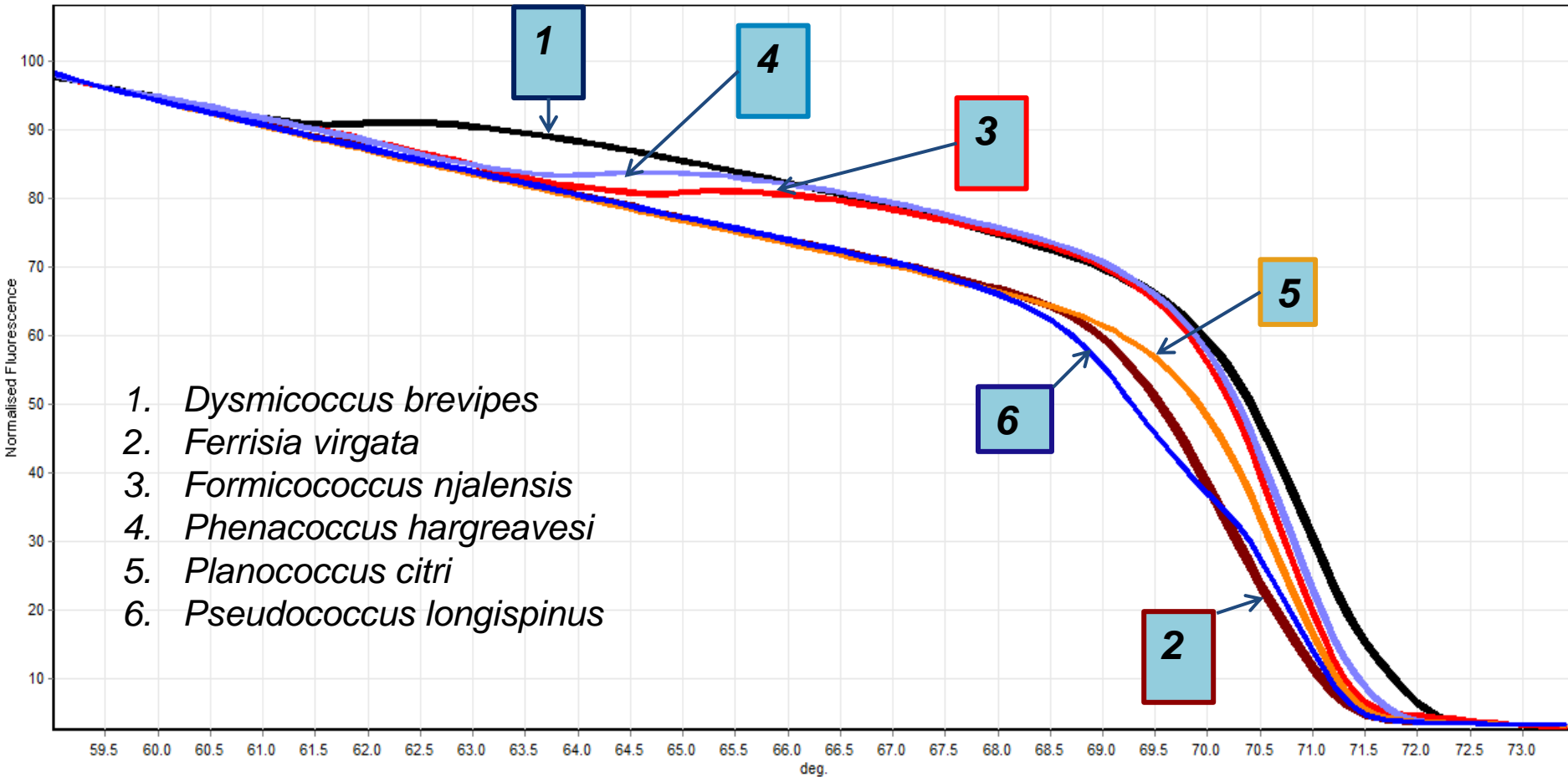
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Sequencing-free species identification by High Resolution Melt analysis



Acknowledgements:

- Research funding from CRUK & Mars
- Chris Stain – *Reading Centre for Advanced Microscopy*
- CNRA & CRIG staff & many colleagues who have helped supply mealybug samples