

3.3 *In vitro* pairings of isolates on *Eucalyptus* leaves

Mycelial growth and black necrotic lesions were observed on all leaf treatments. Additionally, asexual pycnidia were observed on the abaxial side of the wounded leaf treatments (Figure S5). Pycnidia were removed from the stomata, crushed and observed under the microscope, revealing asexual conidia (Figure S5). Crushed pycnidia were grown on MEA, and the mating type and genotype were identified as described in Section 2.1 and Section 2.2. The population genetic analyses confirmed that these structures belonged to isolate CMW48624 (*MAT1-2*). Asexual pycnidia were not found for the *MAT1-1* isolate. No sexual structures were observed.

4 DISCUSSION

This study presents the first confirmation of leaf blight caused by *T. destructans* in Malaysia. The Malaysian population had an even distribution of mating types and the highest observed genetic diversity for any *T. destructans* population studied to date. All 20 genotypes were unique to Malaysia, and population genetic analysis suggested the presence of a cryptic sexual cycle. These results were surprising, since they contradict what is known for other non-native populations of *T. destructans* (Havenga et al., 2020b; Havenga, Wingfield, Wingfield, Roets, et al., 2020), as well as for the related pathogens (Aylward et al., 2019; Taole et al., 2015), all of which appear to reproduce only asexually. The sexual state of *T. destructans* has never been found. Evidence of recombination in this study therefore suggests the existence of a cryptic sexual state in the Malaysian plantation sampled.

The Malaysian population of *T. destructans* had the highest genetic diversity (20.6%) of any known population of this pathogen. *Eucalyptus* plantations have been established in Malaysia relatively recently (Yahya, 2020). In contrast, the industry in Indonesia has been established for a much longer time period (Nambiar et al., 2018) and has the longest history of a *T. destructans* presence (Wingfield et al., 1996). Despite this fact, the genetic diversity of the pathogen in Malaysia was nearly double that found in Indonesia. It is also relevant that the diverse Malaysian population of isolates was collected from a single plantation, whereas the studied North Sumatra population in Indonesia was comprised of samples collected at different times and from different geographical locations (Havenga et al., 2020b).

Native populations of pathogens typically have higher genetic diversities and a greater number of private alleles compared with their introduced counterparts (McDonald, 1997). This is because introduced populations usually emerge from a small number of individuals, whereas native populations have had long periods of time to evolve (McDonald, 1997). Despite the high level of genetic diversity of the Malaysian population and the presence of both mating types, Malaysia is unlikely to represent the native range of this pathogen. According to current knowledge, this pathogen is specific to *Eucalyptus* (Andjic et al., 2019) and there are no *Eucalyptus* species native to Malaysia (Rejmánek & Richardson, 2011).

The first *Eucalyptus* hybrid plantation in Sabah was established with seeds obtained from southern China (Yahya, 2020), but the population from southern China (Havenga et al., 2020b) did not share any genotypes with the Malaysian isolates in

the present study. Malaysian and Chinese isolates, however, shared alleles that were previously thought to be limited to China (Havenga et al., 2020b). The hypothesis that *T. destructans* was introduced into Malaysia from China was therefore not conclusively supported. *Teratosphaeria destructans* causes widespread destruction in China (Burgess & Wingfield, 2017; Dell et al., 2008), but Havenga et al., (2020b) considered only a single site in southern China. An in-depth study of *T. destructans* in China would be required to further test this hypothesis.

High genetic diversities in the populations of two other exotic *Teratosphaeria* pathogens are believed to be the result of multiple introductions. *Teratosphaeria zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.J. Wingf. & Crous populations have an uneven distribution of mating types (Aylward et al., 2020), whereas *T. epicoccoides* (Cooke & Masee) Rossman & W.C. Allen genotypes are present in multiple populations with only a few unique alleles in the global collection (Taole et al., 2015). Although the global collection of *T. destructans* is primarily characterized by highly skewed mating type ratios and low genetic diversities, the Malaysian population had an even distribution of mating types, unique genotypes that grouped in the MSN and evidence that identical genotypes emerged through separate sexual events. This suggests that sexual recombination, rather than multiple introductions, is responsible for the genetic diversity in Malaysia.

The Malaysian population also consisted of a high number of clones. Only the asexual state of this pathogen is known (Burgess et al., 2006; Wingfield et al., 1996), and it is also the dominant form for many leaf-infecting *Teratosphaeria* species, including *T. eucalypti*, *T. pseudoecalypti*, *T. epicoccoides* and *T. cryptica* (Cooke) Crous & U. Braun (Andjic et al., 2019; Burgess & Wingfield, 2017; Taole et al., 2015). Regardless of sexual reproduction, our results indicated that the asexual state remains important in the reproduction and dispersal of this pathogen.

The Malaysian population had the highest recorded genotypic diversity of *T. destructans* populations investigated to date. It also displayed evidence of recombination, strong genetic structure and a high number of clones. We conclude that both the sexual and asexual states serve as modes of reproduction for this pathogen in Malaysia. Exclusively asexual species are rare (Sun & Heitman, 2011) because sexual reproduction produces genetically unique offspring that may be better adapted to certain conditions (Ni et al., 2011). Sexually driven evolution of *T. destructans* in Malaysia is concerning, because it may lead to a more adaptable pathogen (McDonald, 1997) and one that is less responsive to disease management practices. The extent to which *T. destructans* has spread within Malaysia is unknown. An in-depth study, investigating multiple sampling sites, would be required to evaluate the current distribution of *T. destructans* in Sabah, Malaysia and Borneo as a whole. Care must be taken to prevent further spread, which could easily occur if new plantations are established with plant material from Sabah.

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REFERENCES

- Agapow, P. M., & Burt, A. (2001). Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, 1, 101– 102. <https://doi.org/10.1046/j.1471-8278.2000.00014.x>.
- Andjic, V., Carnegie, A. J., Pegg, G. S., Hardy, G. E. S. J., Maxwell, A., Crous, P. W., Pérez, C., Wingfield, M. J., & Burgess, T. I. (2019). 23 years of research on *Teratosphaeria* leaf blight of *Eucalyptus*. *Forest Ecology and Management*, 443, 19– 27. <https://doi.org/10.1016/j.foreco.2019.04.013>.
- Aylward, J., Dreyer, L. L., Havenga, M., Roets, F., Wingfield, B. D., & Wingfield, M. J. (2020). Genomic characterization of mating type loci and mating type distribution in two apparently asexual plantation tree pathogens. *Plant Pathology*, 69, 28– 37. <https://doi.org/10.1111/ppa.13094>.
- Aylward, J., Roets, F., Dreyer, L. L., & Wingfield, M. J. (2019). *Teratosphaeria* stem canker of *Eucalyptus*: two pathogens, one devastating disease. *Molecular Plant Pathology*, 20(1), 8– 19. <https://doi.org/10.1111/mpp.12758>.
- Barber, P. A., Thu, P., Hardy, G. E., & Dell, B. (2012). *Emerging disease problems in Eucalypt plantations in LAO PDR*. Paper presented at the The Impacts of Climate Change to Forest Pests and Diseases in The Tropics, Yogyakarta, Indonesia.
- Billiard, S., Lopez-Villavicencio, M., Devier, B., Hood, M. E., Fairhead, C., & Giraud, T. (2011). Having sex, yes, but with whom? Inferences from fungi on the evolution of anisogamy and mating types. *Biological Reviews*, 86, 421– 442. <https://doi.org/10.1111/j.1469-185X.2010.00153.x>.
- Bruvo, R., Michiels, N. K., D'Souza, T. G., & Schulenburg, H. (2004). A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology*, 13, 2101– 2106. <https://doi.org/10.1111/j.1365-294X.2004.02209.x>.
- Burgess, T. I., Andjic, V., Hardy, G. E. S. J., Dell, B., & Xu, D. (2006). First report of *Phaeophleospora destructans* in China. *Journal of Tropical Forest Science*, 18(2), 144– 146. <https://doi.org/10.1071/DN07056>.
- Burgess, T. I., & Wingfield, M. J. (2017). Pathogens on the move: A 100-year global experiment with planted Eucalypts. *BioScience*, 67(1), 14– 25. <https://doi.org/10.1093/biosci/biw146>.
- Dell, B., Hardy, G., & Burgess, T. (2008). Health and nutrition of plantation eucalypts in Asia. *Southern Forests: a Journal of Forest Science*, 70(2), 131– 138. <https://doi.org/10.2989/south.for.2008.70.2.8.536>.

- Falush, D., Stephens, M., & Pritchard, J. K. (2003). Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics*, 164, 1567– 1587. PMID: 12930761. PMID: 12930761.
- Fourie, A., Wingfield, M. J., Wingfield, B. D., Thu, P. Q., & Barnes, I. (2016). A possible centre of diversity in South East Asia for the tree pathogen, *Ceratocystis manginecans*. *Infection, Genetics and Evolution*, 41, 73– 83. <https://doi.org/10.1016/j.meegid.2016.03.011>.
- Greyling, I., Wingfield, M. J., Coetzee, M. P. A., Marincowitz, S., & Roux, J. (2016). The *Eucalyptus* shoot and leaf pathogen *Teratosphaeria destructans* recorded in South Africa. *Southern Forests*, 78(2), 123– 129. <https://doi.org/10.2989/20702620.2015.1136504>.
- Havenga, M., Wingfield, B. D., Wingfield, M. J., Dreyer, L. L., Roets, F., & Aylward, J. (2020a). Diagnostic markers for *Teratosphaeria destructans* and closely related species. *Forest Pathology*, 50(6), e12645. <https://doi.org/10.1111/efp.12645>.
- Havenga, M., Wingfield, B. D., Wingfield, M. J., Dreyer, L. L., Roets, F., & Aylward, J. (2020b). Low genetic diversity and strong geographic structure in six introduced populations of the *Eucalyptus* foliar pathogen *Teratosphaeria destructans*. *Plant Pathology*, 69, 1540– 1550. <https://doi.org/10.1111/ppa.13235>.
- Havenga, M., Wingfield, B. D., Wingfield, M. J., Roets, F., Dreyer, L. L., Tatham, C. T., Duong, T. A., Wilken, P. M., Chen, S. F., & Aylward, J. (2020). Mating strategy and mating type distribution in six global populations of the *Eucalyptus* foliar pathogen *Teratosphaeria destructans*. *Fungal Genetics and Biology*, 137, 103350. <https://doi.org/10.1016/j.fgb.2020.103350>.
- Hii, S. Y., Ha, K. S., Ngui, M. L., Penguang Jnr, S. A., Duju, A., Teng, X. Y., & Meder, R. (2017). Assessment of plantation-grown *Eucalyptus pellita* in Borneo, Malaysia for solid wood utilisation. *Australian Forestry*, 80(1), 26– 33. <https://doi.org/10.1080/00049158.2016.1272526>.
- Jimu, L., Kemler, M., Wingfield, M. J., Mwenje, E., & Roux, J. (2016). The *Eucalyptus* stem canker pathogen *Teratosphaeria zuluensis* detected in seed samples. *Forestry*, 89(3), 316– 324. <https://doi.org/10.1093/forestry/cpv037>.
- Jombart, T. (2008). adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, 24, 1403– 1405. <https://doi.org/10.1093/bioinformatics/btn129>.
- Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A., & Mayrose, I. (2015). CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, 5, 1179– 1191.
- Lee, S. S. (2018). Observations of the successes and failures of *Acacia* plantations in Sabah and Sarawak and the way forward. *Journal of Tropical Forest Science*, 30, 468– 475. <https://doi.org/10.26525/jtfs2018.30.5.468475>.
- McDonald, B. A. (1997). The population genetics of fungi: tools and techniques. *Phytopathology*, 87, 448– 453. <https://doi.org/10.1094/PHYTO.1997.87.4.448>.

- McDonald, B. A., Miles, J., Nelson, L. R., & Pettway, R. E. (1994). Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. *Phytopathology*, 84, 250– 255. <https://doi.org/10.1094/Phyto-84-250>.
- Nambiar, E. K. S., Harwood, C. E., & Mendham, D. S. (2018). Paths to sustainable wood supply to the pulp and paper industry in Indonesia after diseases have forced a change of species from acacia to eucalypts. *Australian Forestry*, 81(3), 146– 161. <https://doi.org/10.1080/00049158.2018.1482798>.
- Ni, M., Feretzaki, M., Sun, S., Wang, X., & Heitman, J. (2011). Sex in fungi. *Annual Review of Genetics*, 45, 405. <https://doi.org/10.1146/annurev-genet-110410-132536>.
- Old, K. M., Wingfield, M. J., & Yuan, Z. Q. (2003). *Phaeophleospora* leaf diseases. In M. Dudzinski (ed.), *A Manual of Diseases of Eucalypts in South-East Asia* (pp. 25- 31). Center for International Forestry Research.
- Peakall, R., & Smouse, P. E. (2006). GenAIEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288– 295. <https://doi.org/10.1111/j.1471-8286.2005.01155.x>.
- Pielou, E. C. (1975). *Ecological diversity*. Wiley, New York, 165. <https://doi.org/10.4319/lo.1977.22.1.0174b>.
- R Core Team (2013). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. <http://www.R-project.org/>.
- Rejmánek, M., & Richardson, D. M. (2011). Eucalypts. In D. Simberloff, & M. Rejmánek (Eds.), *Encyclopaedia of Biological Invasions* (pp. 203– 209). University of California Press.
- Shannon, C. E. (2001). A mathematical theory of communication. *ACM SIGMOBILE Mobile Computing and Communications Review*, 5, 3– 55. <https://doi.org/10.1145/584091.584093>.
- Simpson, E. H. (1949). Measurement of diversity. *Nature*, 163, 688. <https://doi.org/10.1038/163688a0>.
- Stenberg, P., Lundmark, M., & Saura, A. (2003). MLGsim: a program for detecting clones using a simulation approach. *Molecular Ecology Resources*, 3, 329– 331. <https://doi.org/10.1046/j.1471-8286.2003.00408.x>.
- Stoddard, J. A., & Taylor, J. F. (1988). Genotypic diversity: estimation and prediction in samples. *Genetics*, 118, 705– 711.
- Sun, S., & Heitman, J. (2011). Is sex necessary? *BMC Biology*, 9(56), 1– 4. <http://www.biomedcentral.com/1741-7007/9/56>.
- Taole, M., Bihon, W., Wingfield, B. D., Wingfield, M. J., & Burgess, T. I. (2015). Multiple introductions from multiple sources: invasion patterns for an important Eucalyptus leaf pathogen. *Ecology and Evolution*, 5(18), 4210– 4220. <https://doi.org/10.1002/ece3.1693>.
- Tarigan, M., Roux, J., van Wyk, M., Tjahjono, B., & Wingfield, M. J. (2011). A new wilt and die-back disease of *Acacia mangium* associated with *Ceratocystis*

manginecans and *C. acaciivora* sp. nov. in Indonesia. *South African Journal of Botany*, 77, 292– 304. <https://doi.org/10.1016/j.sajb.2010.08.006>.

- Wingfield, M. J., Crous, P. W., & Boden, D. (1996). *Kirramyces destructans* sp. nov., a serious leaf pathogen of *Eucalyptus* in Indonesia. *South African Journal of Botany*, 62(6), 325– 327. [https://doi.org/10.1016/s0254-6299\(15\)30673-6](https://doi.org/10.1016/s0254-6299(15)30673-6).
- Yahya, A. Z. (2020). Planting of *Eucalyptus* in Malaysia. *Acta Scientific Agriculture*, 4(2), 0 1–0 2. <https://doi.org/10.31080/ASAG.2020.04.0785>.
- Zaiton, S., Paridah, M. T., Hazandy, A. H., & Azim, R. A. R. A. (2018). Potential of *Eucalyptus* plantations in Malaysia. *The Malaysian Forester*, 81(1), 64– 72, ISSN 0302–2935.