Yeasts of the Cryptococcus neoformans/gattii species complexes are human pathogens mostly in immune compromised individuals, and can cause infections from dermal lesions to fungal meningitis. Differences in virulence and antifungal drug susceptibility of species in these complexes indicate the value of full differentiation to species level in diagnostic procedures. MALDI-TOF MS has been reported to sufficiently discriminate these species. Here, we sought to re-evaluate sample pre-processing procedures and create a set of publicly available references for use with the MALDI Biotyper system. Peak content using four different pre-processing protocols was assessed, and database entries for 13 reference strains created. These were evaluated against a collection of 153 clinical isolates, typed by conventional means. The use of decapsulating protocols or mechanical disruption did not sufficiently increase the information content to justify the extra hands-on-time. Using the set of 13 reference entries created with the standard formic acid extraction, we were able to correctly classify 143/153 (93.5%) of our test isolates. The majority of the remaining ten isolates still gave correct top matches; only two isolates did not give reproducible identifications. This indicates that the log score cut-off can be lowered also in this context. Ease to identify cryptococcal isolates to the species level is improved by the workflow evaluated here. The database references are freely available from https://github.com/oliverbader/BioTyper-libraries for incorporation into local diagnostic systems.

Keywords: MALDI-TOF MS, identification, capsule, Cryptococcus neoformans complex, Cryptococcus gattii complex
INTRODUCTION

The group of basidiomycetous yeast of the Cryptococcus neoformans/gattii complexes hosts a variety of human pathogenic species, causing infections from skin lesions to fatal meningitis [reviewed in (Kronstad et al., 2011)]. This mainly contributes to morbidity and mortality in patients with underlying immune deficiencies (e.g. HIV), but can also affect immunocompetent hosts. Species of the C. neoformans/gattii complexes are readily found in the environment, living, for example, on eucalyptus tree bark, and bird droppings.

The most prominent diagnostic feature of these species are the large capsules of most isolates [reviewed in (O’Meara and Alspaugh, 2012)], which can easily be visualized by, e.g., microscopy. The polysaccharides shed from the cell also give rise the capsule and thus creates a halo around the cells visible in displacement of India ink stain. India ink does not penetrate varieties:

- serotypes based on antigenicity of the capsule, forming three through serum detection of galactomannan.

...
mass spectra did not reveal any additional mass signals, or major capsules in all strains. However, subsequent measurement of
positively in B(acetonitrile extraction protocol. Both pre-processing protocols, would deplete the capsule prior to the regular formic acid/
is soluble in DMSO, we devised pre-processing protocols that disrupted cells resulted in more evenly distributed peak intensities

**MALDI-TOF MS Preprocessing Protocols**

For regular harvest and formic acid-extraction [preprocessing protocol (A) (Bader, 2017)], cells were taken from agar plates by scraping approximately a 1µl loop full of cells and re-suspending them in 300 µl water. 700 µl absolute ethanol was added to a final concentration of 70% (v/v) and vortexes. Cells were spun down at 8500xg for 5 min, the supernatant completely discarded and the cells lysed first with 50 µl 70% (v/v) formic acid, and 50 µl pure acetonitrile. Modifications to this protocol tested were for preprocessing protocol (B) that cells were collected in 300 µl 5% (v/v) DMSO, for preprocessing protocol (C) that DMSO was included in the 70% ethanol washing step to a final volume of 5% (v/v), and for preprocessing protocol (D) that cells were collected in 300 µl water already including an equivalent of ~100 µl glass beads (0.5 mm diameter, Roth, Karlsruhe, Germany). Here, cells were mechanically disrupted in a FP120 fast prep machine (Bio101, Thermo Savant) at setting 4, for 30 sec during the formic acid step.

**Generation of MALDI Biotyper Database References**

MSP references for the MALDI Biotyper were generated according to the manufacturer’s guidelines (Kostrzewa and Maier, 2017), using preprocessing protocol A. Spectra from 24 individual spots were gathered on a freshly calibrated (BTS reference standard) Autoflex III system (Bruker Daltonics, Bremen, Germany) using the automated acquisition mode of the Biotyper 3.1. Spectra were processed using the inbuilt MSP generation method, using the standard parameters.

**RESULTS AND DISCUSSION**

**Method Optimization**

The literature reports that both, removal of cryptococcal capsule can (Thomaz et al., 2016) or does not (Hagen et al., 2015) positively influence spectrum quality. Since the capsule material is soluble in DMSO, we devised pre-processing protocols that would deplete the capsule prior to the regular formic acid/ acetonitrile extraction protocol. Both pre-processing protocols, B (Figures 1A, B) and C (not shown), efficiently removed capsules in all strains. However, subsequent measurement of mass spectra did not reveal any additional mass signals, or major differences in spectrum quality (Figure 1C).

Next, we tested if mechanical disruption of the cells yielded more informative spectra using mechanical disruption (preprocessing protocol D). Indeed, mass spectra recorded from mechanically disrupted cells resulted in more evenly distributed peak intensities across the major mass signals. However, no additional mass signals of high intensity were found (Figure 1D).

In our hands removal of the capsule did not result in spectra with higher information content, at any time. Mechanical disruption did reveal some additional masses, but in favor of the lower hands-on-time the original pre-processing protocol A was subsequently used for MSP creation and testing.

**Creation of Single Species MSPs**

Next, we created MSPs for 13 reference strains encompassing seven molecular types of the C. neoformans/gattii complexes (Meyer et al., 2003; Hagen et al., 2015), using the standard extraction procedure (pre-processing protocol A). Cluster analysis of the MSPs generated suggested sufficient distance to clearly distinguish between C. neoformans complex molecular types VNI (C. deneoformans) and VNI/II, and possibly also between VNI and VII/II themselves, but less so among molecular types within the C. gattii complex (Figure 2).

**Identification Performance**

Mass spectra for all test isolates were obtained using preprocessing protocol A. Were MALDI-TOF results using the new MSP set deviated from previous data, URAS-RFLP typing was repeated as the gold standard (Figure 3A). All but two deviations could be resolved (see below). To discriminate between C. tetragattii and potential C. decagattii strains, we sequenced the URAS-amplicon obtained from CBS 11687 (C. decagattii, deposited at Genebank under the accession number MH605184) and compared it to the respective sequence of CBS 10101 (C. tetragattii, gene bank accession AY973155). Restriction with Stul I was found, and experimentally confirmed, to discriminate the two species (Figure 3B). However, there were no further C. decagattii isolates among our strains. C. decagattii remains a rare species, and only a single isolate of this molecular type (CBS 11687) was available for this study, which was already included in the reference set. Therefore, the final test collection encompassed only six of the seven species used for generation of references.

From the test collection, we were able to correctly identify 143/153 (93.5%) of the isolates on species-level using duplicate spots, with the top log score ≥ 2.000 (Figure 3C), as recommended by the manufacturer. Of the remaining ten isolates, eight still gave correct species matches at scores between 1.700 and 1.999, considered only genus-level by the manufacturer. Among the negative control set, there were no results higher than a log score of 1.300, indicating no false positives are to be expected under routine diagnostic conditions (Figure 3C). Inconsistent identifications were only observed for two C. tetragattii isolates where repetitively top matches of different spots of the same preparation were C. tetragattii, C. gattii, or C. deuterogattii, all at values above 1.999.

Because of this, and the close relations found during cluster analysis (Figure 2), we also inspected the log score difference from the correct to the highest scoring false match for each spot (Figure 3D) for those tests where a second species matched above the significance threshold. Only 3% of all tested spots (14 out of 428) matched a second MSP with a log score > 1.999. As expected from the cluster analysis, these “best false” second matches were found only among species in the C. gattii
FIGURE 1 | Optimization of sample pre-processing procedures. Phase contrast microscopy of ink-stained cryptococcal cells (A) without and (B) with DMSO treatment depleting capsular material (protocol B). (C) Spectra obtained from de-capsulated cells by protocols B and C were not different from those generated by formic acid extraction alone. (D) Spectra obtained from mechanically disrupted cells (protocol D) had similar masses, but differed in relative signal intensity for some, as compared to those obtained by formic acid extraction. Example results shown here for CBS 10485 are valid for all isolates. Signal intensities on y-axes in panels (C, D) mainly rely on number of spectra gathered in sum buffer. Spectra have been manually re-scaled on the y-axis for better visual comparison between different experimental conditions, and scaling has been omitted to reflect this fact.

FIGURE 2 | Dendrogram reference MSPs.
complex. This was the case for three \textit{C. bacillisporus} isolates giving a second best match with \textit{C. decagattii}, with a log score difference between 0.1 to 0.4. In addition to the two inconsistent \textit{C. tetragattii} isolates discussed above, one additional \textit{C. tetragattii} isolate also gave a second best match with \textit{C. decagattii}. The score values for both matches were near 2.000. The close relationships of the different species will likely also have implications on properly identifying hybrid isolates.

**CONCLUSION**

Cryptococcal typing and species identification is complicated by the ongoing discovery of new species (Farrer et al., 2019), and the formation of inter-species hybrids (Hagen et al., 2015). Nevertheless, our data confirms that proper routine identification of clinically relevant non-hybrid \textit{C. neoformans/gattii} complex molecular types using MALDI-TOF is possible with the current algorithms and standard workflows. In our hands, the only exception was distinguishing the rarer types \textit{C. tetragattii} and \textit{C. decagattii}, which was not sufficiently possible. This may be due to the fact, that only low numbers of isolates of these lineages were available for testing.

The MSP sets generated in this study are freely available from https://github.com/oliverbader/BioTyper-libraries for use with the molecular type- (Meyer et al., 2003) or the species nomenclatures (Hagen et al., 2015).

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

**AUTHOR CONTRIBUTIONS**

Performed experiments: MB, NW, MK, OB. Contributed typed strains: NW, MK, CB, WT, AC. Wrote the manuscript: MB, AC, OB. Prepared the revision: MB, OB. Supervised the study: MW, UG, AC, OB. All authors contributed to the article and approved the submitted version.

**FUNDING**

This study received funding from Thai-German mobility scheme “CryptoType” to AC and OB (grant number 01DP13001). Article publishing fees were covered by the Open-Access-publications funds of the Universitätsmedizin Göttingen.

**ACKNOWLEDGMENTS**

The authors would like to thank Agnieszka Goretzki for expert technical assistance. This study was mainly funded by the Thai-German mobility scheme “CryptoType” to AC and OB.
REFERENCES

Bader, O. (2017). Fungal Species Identification by MALDI-ToF Mass Spectrometry. Methods Mol. Biol. 1508, 323–337. doi: 10.1007/978-1-4939-6515-1_19

Boekhout, T., Theelen, B., Diaz, M., Fell, J. W., Hop, W. C., Abeln, E. C., et al. (2001). Hybrid genotypes in the pathogenic yeast Cryptococcus neoformans. Microbiology 147, 891–907. doi: 10.1099/00221287-147-4-89

Carricione, F., Gilgado, F., Arthur, I., Ellis, D., Malik, R., Van De Wiele, N., et al. (2011). Clonality and alpha-a recombination in the Australian Cryptococcus gattii VGII population—an emerging outbreak in Australia. PloS One 6, e16936. doi: 10.1371/journal.pone.0016936

Caglioti, M., Prigitano, A., Esposto, M. C., Romano, L., Grancini, A., Zani, A., et al. (2018). Epidemiological trends of cryptococcosis in Italy: Molecular typing and susceptibility pattern of Cryptococcus neoformans isolates collected during a 20-year period. Med. Mycol. 56, 963–971. doi: 10.1093/brain/mbx152

Fang, L. F., Zhang, P. P., Wang, J., Yang, Q., and Qu, T. T. (2020). Clinical and microbiological characteristics of cryptococcosis at an university hospital in China from 2013 to 2017. Braz. J. Infect. Dis. 24, 7–12. doi: 10.1016/j.bjid.2019.11.004

Ferrar, R. A., Chang, M., Davis, M. J., Van Dorp, L., Yang, D. H., Shea, T., et al. (2019). A New Lineage of Cryptococcus gattii (VGG) Discovered in the Central Zambezean Miombo Woodlands. mBio 10 (6), e02306-19. doi: 10.1128/mBio.02306-19

Firacative, C., Trilles, L., and Meyer, W. (2012). MALDI-TOF MS enables the rapid identification of the major molecular types within the Cryptococcus neoformans/C. gattii species complex. PloS One 7, e37566. doi: 10.1371/journal.pone.0037566

Hagen, F., Khayhan, K., Theelen, B., Kolecka, A., Polycheck, I., Sionov, E., et al. (2015). Recognition of seven species in the Cryptococcus gattii/Cryptococcus neoformans species complex. Fungal Genet. Biol. 78, 16–48. doi: 10.1016/j.fgb.2015.02.009

Herbert, P. F., Dos Santos, J. C., Hagen, F., Ribeiro-Dias, F., Queiroz-Telles, F., Netea, M. G., et al. (2018). Differential In Vitro Cytokine Induction by the Species of Cryptococcus gattii Complex. Infect. Immun. 86 (4), e00958-17. doi: 10.1128/IAI.00958-17

Jin, L., Cao, J. R., Xue, X. Y., Wu, H., Wang, L. F., Guo, L., et al. (2020). Correlation of the major molecular types within the Cryptococcus neoformans/C. gattii species complex. Proc. Natl. Acad. Sci. U.S.A. 117, 17258–17263. doi: 10.1073/pnas.2004298110

Kostrzewa, M., and Maier, T. (2017). “Criteria for Development of MALDI-TOF Mass Spectral Database,” in MALDI-TOF and Tandem MS for Clinical Microbiology, one ed. Eds. H. N. Shah and S. E. Garbia (Hoboken, New Jersey:John Wiley & Sons Ltd), 39–54.

Kronstad, J. W., Attarian, R., Cadieux, B., Choi, J., D’souza, C. A., Griffiths, E. J., et al. (2011). Expanding fungal pathogenesis: Cryptococcus breaks out of the opportunistic box. Nat. Rev. Microbiol. 9, 193–203. doi: 10.1038/nrmicro2522

Kwon-Chung, K. J., Boekhout, T., Fell, J. W., and Diaz, M. (2002). Proposal to conserve the name Cryptococcus gattii against C. hondurianus and C. buccilliporus (Basidiomycota, Hymenomycetes, Tremenomycetidae). Taxon 51, 804–806. doi: 10.2307/1555045

Lee, G. A., Arthur, I., Merritt, A., and Leung, M. (2019). Molecular types of Cryptococcus neoformans and Cryptococcus gattii in Western Australia and correlation with antifungal susceptibility. Med. Mykol. 57 (8), 1004–1010. doi: 10.1093/nymj/myy161

McTaggart, L. R., Lei, E., Richardson, S. E., Hoang, L., Fothergill, A., and Zhang, S. X. (2011). Rapid identification of Cryptococcus neoformans and Cryptococcus gattii by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 49, 3050–3053. doi: 10.1128/JCM.00651-11

Meyer, W., Castaneda, A., Jackson, S., Huynh, M., Castaneda, E. Beroamerican Cryptococcal Study Group (2003). Molecular typing of IberoAmerican Cryptococcus neoformans isolates. Emerg. Infect. Dis. 9, 189–195. doi: 10.3201/eid0902.020246

O’Meara, T. R., and Alspaugh, J. A. (2012). The Cryptococcus neoformans capsule: a sword and a shield. Clin. Microbiol. Rev. 25, 387–408. doi: 10.1128/CMR.00001-12

Posteraro, B., Vella, A., Cogliati, M., De Carolis, E., Florio, A. R., Posteraro, P., et al. (2012). Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry-Based Method for Discrimination between Molecular Types of Cryptococcus neoformans and Cryptococcus gattii. J. Clin. Microbiol. 50, 2472–2476. doi: 10.1128/JCM.00737-12

Springer, D. J., Billmyre, R. B., Filler, E. E., Voelz, K., Pursall, R., Mieczkowski, P. A., et al. (2014). Cryptococcus gattii VGIII isolates causing infections in HIV/AIDS patients in Southern California: identification of the local environmental source as arboreal. PLoS Pathog. 10, e1004285. doi: 10.1371/journal.ppat.1004285

Tangwattanachuleeporn, M., Somparn, P., Polpool, K., Gross, U., Weig, M., and Bader, O. (2013). Prevalence and Antifungal Susceptibility of Cryptococcus neoformans Isolated from Pigeon Excreta in Chon Buri Province, Eastern Thailand. Med. Mycol. 54, 303–307. doi: 10.3109/13693786.2014.876297

Voelz, K., Johnston, S. A., Smith, L. M., Hall, R. A., Idnurm, A., and May, R. C. (2014). ‘Division of labour’ in response to host oxidative burst drives a fatal Cryptococcus gattii outbreak. Nat. Commun. 5, 5194. doi: 10.1038/ncomms6194

Worasilchai, N., Tangwattanachuleeporn, M., Meesilpavikij, K., Folba, C., Kangogo, M., Gross, U., et al. (2017). Diversity and Antifungal Drug Susceptibility of Cryptococcus Isolates in Thailand. Med. Mycol. 55, 680–685. doi: 10.1093/nymj/myw130.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Bernhard, Worasilchai, Kangogo, Bit, Trzaska, Weig, Groß, Chinampon and Bader. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.