



Genetic population structure of the agaric *Blastosporella zonata* (Lyophyllaceae) reveals cryptic species and different roles for sexual and asexual spores in dispersal

L.J.J. van de Peppel^{1,*}, T.J. Baroni², A.E. Franco-Molano³, D.K. Aanen¹

Key words

basidiomycetes
Blastosporella
clonality
conidia
cryptic species
genetic population structure
insect-association
Lyophyllaceae
population genetics

Abstract *Blastosporella zonata* is one of the few basidiomycete fungi that produce asexual spores (conidia) on the mushroom. The role of these conidia in the fungal lifecycle is not known. We tested whether conidia are being utilized in local dispersal by looking for signatures of clonality in 21 samples from three localities separated by about three kilometres in Murillo, Colombia. To identify clonally related individuals, we sequenced three polymorphic markers at two unlinked loci (nuclear rRNA: ITS and LSU, and *TEF1α*) for all collections plus three herbarium samples. We identified two sets of clonally related individuals growing closely together in one of the three localities, and only one pair shared between localities. In all three localities we observed multiple non-clonally related dikaryons showing that sexual reproduction is also important. Our results indicate that the conidia on the mushroom are primarily important for local dispersal. Unexpectedly, our results also indicate two reproductively isolated populations, possibly representing cryptic biological species.

Citation: Van de Peppel LJJ, Baroni TJ, Franco-Molano AE, et al. 2022. Genetic population structure of the agaric *Blastosporella zonata* (Lyophyllaceae) reveals cryptic species and different roles for sexual and asexual spores in dispersal. *Persoonia* 49: 195–200. <https://doi.org/10.3767/persoonia.2022.49.06>. Effectively published online: 1 December 2022 [Received: 2 December 2021; Accepted: 29 April 2022].

INTRODUCTION

The basidiomycete fungus *Blastosporella zonata* (family: *Lyophyllaceae*), currently the only species in this genus, is found in the neotropics in the Dominican Republic and Colombia (Baroni et al. 2007). A recent study showed that, within the *Lyophyllaceae*, *B. zonata* is part of a termitomycetoid clade which contains the termite-associated fungus *Termitomyces* (Van de Peppel et al. 2021). The same study also showed that *B. zonata* is insect-associated as it grows on faecal pellets, probably of an unidentified Coleopteran larva.

The mushroom of *B. zonata* is characterized by its zonate, cup-shaped pileus, on the centre of which dark ornamented blastoconidia are being produced (Fig. 1). In basidiomycetes the formation of asexual spores on the sexual structures is considered rare (Kües et al. 2016); it has been observed only in a small number of species including other members of the *Lyophyllaceae*, such as the arthroconidia-producing genus *Arthromyces* (Baroni et al. 2007) and chlamydospore-producing mycoparasites in the genus *Asterophora* (Buller 1924, Redhead & Seifert 2001). Another arthroconidia-producing agaric which shows resemblance to *Arthromyces* is *Arthrosporella ditopa* (Singer 1970), but after examination of the type collection it remains inconclusive whether these two species are closely related (Baroni et al. 2007). Other non-lyophylloid agarics

that produce asexual spores on the mushroom include the mycoparasites in the genera *Squamanita* (Bas 1965) and *Dendrocollybia* (Hughes et al. 2001). Furthermore, the species *Pleurotus cystidiosus* is known to produce arthroconidia on both the mycelium and the mushroom (Zervakis et al. 2004) and two species of *Cystoderma* also produce conidia on the mushroom (Saar et al. 2009).

The function of the conidia on the mushroom has been studied in some mycoparasites such as in the genus *Asterophora*. Two researchers in the 1800s already did experiments in which they infected host mushrooms with chlamydospores (Krombholz 1831, Brefeld 1889, summarized by Buller 1924). They observed that *As. lycoperdoides* mushrooms emerged on host mushrooms within three weeks after infection. This clearly demonstrates that *As. lycoperdoides* is able to complete its lifecycle with just the chlamydospores and that these spores can be utilized for host infection. A similar test has not been conducted for species in the genus *Squamanita*, but the conidia probably serve a similar function in the host infection process (Redhead et al. 1994, Griffith et al. 2019). The utilization of dikaryotic asexual spores instead of monokaryotic sexual spores by these mycoparasites makes sense as host mushrooms are ephemeral and decay quickly. Therefore, their lifecycle is significantly shortened by not having to find a compatible mate.

No studies have been conducted on the role of the conidia in the lifecycle of *B. zonata*. It has been suggested that conidia are important in substrate colonization, potentially vectored by insects (Van de Peppel et al. 2021). *Blastosporella zonata* produces both basidiospores and conidia, and it seems likely that the wind-dispersed basidiospores more often are used for long-range dispersal, while the non-wind dispersed conidia are mostly used for local dispersal (Van de Peppel et al. 2021). In

¹ Laboratory of Genetics, Wageningen University & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands; corresponding author e-mail: lennartvdpeppel@gmail.com.

² Department of Biological Sciences, State University of New York, College at Cortland, PO Box 2000, Cortland, New York 13045, USA.

³ Laboratorio de Taxonomía y Ecología de Hongos (TEHO), Instituto de Biología, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia.



Fig. 1 *Blastosporella zonata* (TJB10425) a. Dark blastoconidia located at the centre of the pileus; b. excavated specimen showing the aggregated faecal pellet substrate (TJB10433); c. ornamented blastoconidia which cover the pileus (ZT6333). — Scale bars: a–b = 10 mm, c = 10 μ m.

addition, the thick-walled conidia may also serve as a survival structure as an insect faecal substrate is most likely only seasonally available. A similar survival function has also been suggested for the conidia of the mycoparasitic genera *Asterophora* and *Squamanita* (Redhead et al. 1994).

If *B. zonata* used the conidia for local dispersal we would expect to see signatures of clonality within and not between populations while different populations would normally contain different clones that originate from independent colonisations by sexual spores. To test this hypothesis, we collected *B. zonata* mushrooms at three different sites near Murillo, Colombia. To test for genetic variation among our 21 collections of *B. zonata* we obtained DNA sequence data of three highly polymorphic genetic markers.

MATERIAL AND METHODS

Sampling and culturing of B. zonata

Blastosporella zonata is a very peculiar basidiomycete fungus, with a very specific ecology growing on isolated substrates of patches of insect faeces. The species is rare, has a patchy distribution and has only been documented from a few countries in South America, growing in cloud forests (Baroni et al. 2007).

We collected *B. zonata* mushrooms at three different sites (all < 4 km apart; Fig. 2c) in a cloud forest close to the village of Murillo, Colombia. Collecting took place between 31 October and 2 November 2016. A total of 21 mushrooms were collected. GPS coordinates were recorded for most collections except for four (Bzo2, Bzo5, Bzo18, and Bzo19), for which the coordinates were estimated based on the position of nearby collections. Mushrooms were stored in 25 ml tubes and tissue and conidial cultures were made on the same day. Tissue cultures were made by using a small piece of tissue from either the stipe or pileus of a mushroom. Tissue from the pileus was taken using sterile forceps from the exposed tissue area that was left after the removal of the stipe. The stipe was cut longitudinally using a sterile scalpel and a tissue fibre was removed from the inside of the stipe using sterile forceps. Tissue fragments were directly placed on a Petri dish containing malt yeast extract agar with streptomycin (MYA; per litre demi water: 20 g malt extract, 2 g yeast extract, 15 g agar and 100 ppm streptomycin added after autoclaving). Conidial cultures were made by dipping a sterile needle into the mass of conidia from the pileus of the mushroom and spreading this on a MYA Petri dish. Cultures were first stored at room temperature at the field location and back in the laboratory at 19 °C. Subsequent sub-culturing was

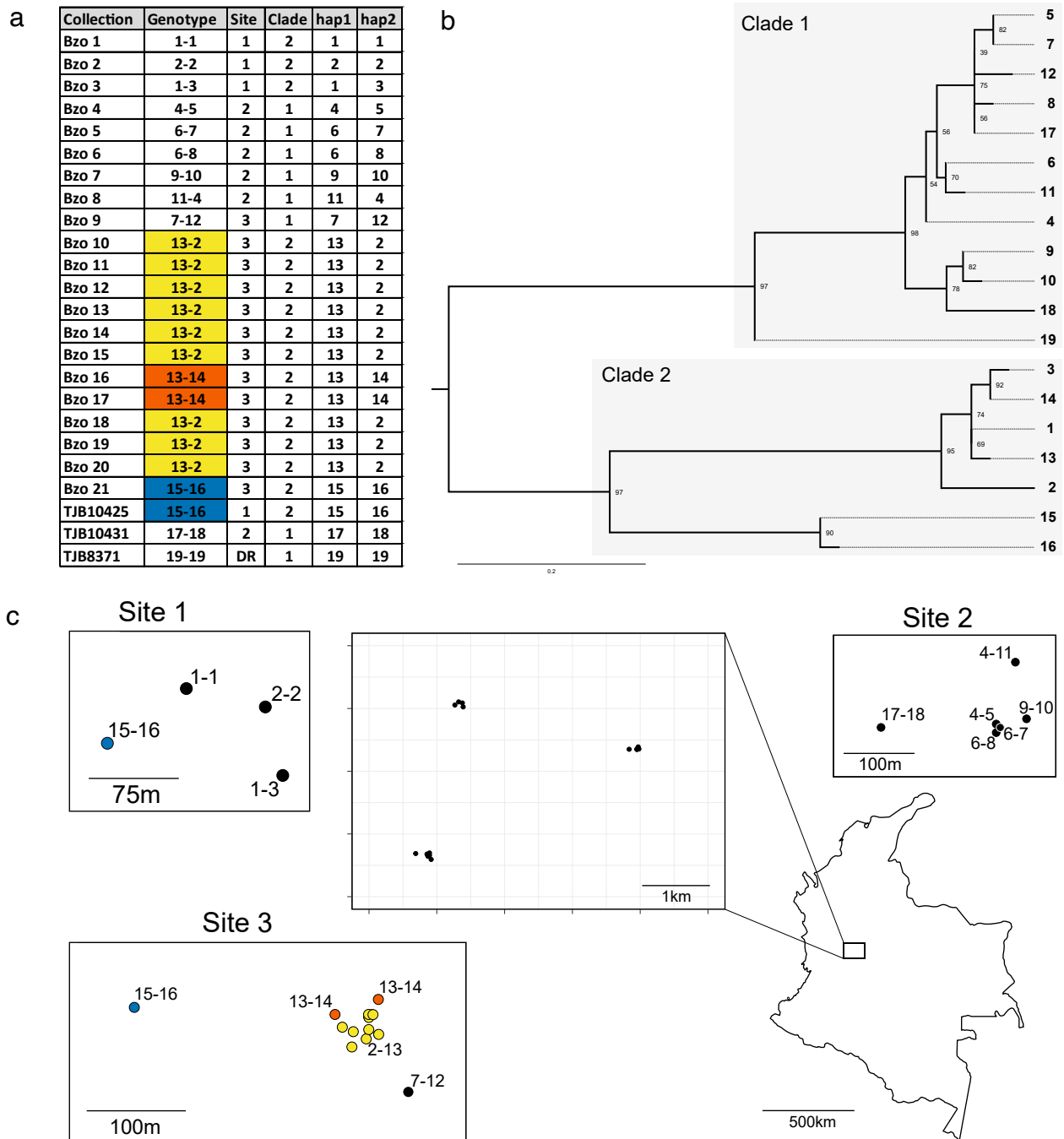


Fig. 2 *Blastosporella zonata*. a. Collection with their corresponding genotypes (predicted haplotype 1 – predicted haplotype 2), clade, haplotypes and collection site (Site 1, 2, 3 or Dominican Republic (DR)). Collections with identical genotypes are highlighted in blue, red or yellow; b. maximum-likelihood tree of predicted haplotypes with midpoint rooting, numbers at the nodes indicate ultrafast bootstraps; c. map of the collections of *B. zonata* at the three different sites around Murillo, Colombia. Presumed clones are highlighted using the same colours used in panel a.

done to obtain axenic cultures. Small leftover fragments from mushrooms were stored in 1.5 ml Eppendorf tubes in 100 % ethanol to serve as backup in case tissue or conidial cultures would not grow (Table S1). The fungal cultures obtained during this study could not be submitted to a public culture collection as the permit under which the material was collected does not allow sharing of biological material with commercial third parties. Two dried fungarium vouchers collected at the same location and one from the Dominican Republic were also added to the dataset.

Optimal growth temperature test and germination of basidiospores of *B. zonata*

To test the optimal growth temperature for *B. zonata* we grew replicate cultures of the same heterokaryotic strain. Bzo9 (genotype

7-12, clade 2), at six different temperatures (4 °C, 10 °C, 15 °C, 19 °C, 25 °C and 30 °C) on 20 ml of MA (per litre demi water: 8 g malt extract, 15 g agar). The inoculum was standardized to a 3 mm diam circle by using a sterile corkborer. Cultures were grown for 44 d and scans were made of each culture at day 5, 12, 19, 26 and 44 using an Epson Perfection V600 scanner. The entire experiment was done in triplicate.

The change in total area of a fungal colony over time was used as a measure for growth. As fast growth could go at the cost of the density of the mycelium we recorded white values of the colony as a measure for mycelial density. The area and white value of the fungal colony in each scan were measured using a script in ImageJ (Supplemental file 1).

We attempted to acquire homokaryotic isolates by germinating basidiospores of mushroom Bzo7. Basidiospores were collected by attaching the pileus of the mushroom to the lid of a Petri dish. The lid was placed on a Petri dish with MYA medium and turned after intervals of five seconds, 30 seconds, five minutes and ten minutes. Plates with basidiospores were incubated at room temperature first and later moved to 19 °C. Individual colonies were quickly subcultured to prevent mating with neighbouring colonies.

DNA isolation, PCR and sequencing

DNA was extracted from a small piece of cultured mycelium (0.5 g). In case a tissue or conidial culture was unsuccessful a small piece similar in size of the back-up mushroom stored in ethanol was used instead. All DNA extractions were done using a cetyltrimethylammonium bromide (CTAB) protocol (Nieuwenhuis et al. 2019).

Two partial sequences of the nuclear ribosomal DNA and one fragment of the single-copy nuclear elongation factor 1 alpha (*TEF1α*) were amplified using regular polymerase chain reaction (PCR) protocols. The first nuclear ribosomal marker, containing the internal transcribed spacer 1 (ITS1), 5.8S and the internal transcribed spacer 2 (ITS2) was amplified using the fungal specific forward primer ITS1f and general reverse primer ITS4 (White et al. 1990, Gardes & Bruns 1993). The second nuclear ribosomal marker, a partial sequence of the large ribosomal subunit (LSU, 28S) was amplified using the primers LR0R and LR5 (Vilgalys & Hester 1990). A partial sequence of translation elongation factor 1-alpha (*TEF1α*) was amplified using the primer pair EF595F and EF1160R (Kausarud & Schumacher 2001). The PCR programs were conducted according to Van de Peppel et al. 2018.

Chromatograms, alignment, haplotype reconstruction and phylogenetic reconstruction

The secondary mycelium, regenerated from a mushroom or conidium, contains two genetically different nuclei, showing a number of heterozygous sites in some of the sequences that we generated. We manually checked the chromatograms to identify double peaks using Geneious v. 10.0.9 (www.geneious.com) (Kearse et al. 2012). Double peaks were assigned with the letter according to the standard International Union of Pure and Applied Chemistry (IUPAC) notation for DNA. Some *TEF1α* sequences contained a large poly-T repeat of up to ten bases in length in some of the strains. The signal after the poly-T repeat was very distorted so the reverse complement sequence of the reverse primer was used to obtain the remaining part of the sequence. The poly-T region did not affect the outcome of the analyses and was therefore not removed for the final analysis. Alignments were created for each individual marker. An overview of the sequences and their corresponding GenBank accession numbers can be found in Table S1. Sequences were aligned using the web server of MAFFT Multiple Sequence Alignment software version 7 using the default settings (Katoh & Standley 2013). After trimming, the alignments for each marker were concatenated using Geneious v. 10.0.9. After manual inspection in Unipro UGENE variable positions were extracted (Okonechnikov et al. 2012). Haplotypes for each unique genotype were reconstructed using DnaSP 6 (Rozas et al. 2017). Phylogenetic reconstruction for unique haplotypes was conducted using the webserver of IQ-tree with defaults settings and 1000 ultrafast bootstrap replicates (Trifinopoulos et al. 2016).

RESULTS

Optimal growth temperature test and germination of conidia and basidiospores of *B. zonata*

The results of our growth experiment show a clear pattern, both total area and white value were highest at 19 °C (Fig. S2). At 4 °C, 10 °C and 15 °C the culture was still able to grow but the total area was smaller, and white values were very similar suggesting similar mycelial densities at these temperatures. At 25 °C colony growth was much lower and also white values were lower compared to the other treatments. At 30 °C none of the replicates showed any radial growth.

Our attempts at germinating the blastoconidia of *B. zonata* were highly successful with very low rates of bacterial or fungal infection. Basidiospores had a much lower germination success and took up to several weeks to germinate. We obtained seven homokaryotic cultures from a single parent, Bzo7 (Fig. S1).

Population study

We found that the number of genetic polymorphisms was lowest for the nuclear ribosomal marker (seven variable positions: four in ITS, three in LSU); based on the concatenated alignments of ITS and LSU we could recover five different genotypes. The *TEF1α* marker showed a much higher number of polymorphisms (44 variable positions) and yielded a total of 14 different genotypes. Theoretically, the difference in the number of variable sites between the nuclear ribosomal marker and *TEF1α* could indicate the presence of a pseudogene. We can reject this hypothesis for the following reasons. First, the majority of the variable positions (26) were in the intronic regions of the gene. Second, the variable positions in the exons were silent mutations as they did not alter the encoded protein. Third, the chromatograms of the *TEF1α* sequences that we obtained from the homokaryons did not show any double peaks, indicating that only a single copy of the gene is present. We therefore conclude that there is only a single and functional copy of *TEF1α* per nucleus.

Concatenation of the ribosomal marker alignments and the *TEF1α* alignment did not result in additional combinations other than the 14 genotypes that were already recovered using just the *TEF1α* marker. We could reconstruct 19 different haplotypes from the 14 different genotypes (Fig. 2a). Phylogenetic reconstruction of those haplotypes revealed two distinct clades (Fig. 2b). Strikingly, the two individual haplotypes of a genotype always belonged to only one of those two clades. Assuming a single, randomly mating population, the probability that the eight genotypes from clade 1, and the six genotypes from clade 2, all contain haplotypes of their respective clades is:

$$p = \frac{\binom{14}{8}}{\binom{28}{16}} = 9.87 * 10^{-5}$$

This strongly suggests that the two clades are reproductively isolated. Since our sampling sites are very close, and since one of the two clades contains samples from multiple sampling sites, it seems highly unlikely that the reproductive isolation is caused by current geographical distance. We therefore conclude that the two clades likely represent two cryptic biological species within *B. zonata*. The biological species concept defines a species based on the criterion of reproductive isolation (Mayr 2000), and has been applied to mushroom-forming basidiomycetes by testing for dikaryon formation (Vilgalys & Sun 1994, Petersen 1995, Aanen et al. 2000).

In total, we found three genotypes that occurred more than once, two twice (15–16 and 13–14), and one nine times (2–13).

We found three unique genotypes at site 1 and one genotype (15–16) at this site also occurred at site 3 (Fig. 2c). All genotypes at site 3 belonged to clade 2. Site 2 harboured six unique genotypes all belonging to clade 1 (Fig. 2c). Site 3 contained one individual of clade 1, this genotype was shared with site 1 (15–16), the larger group of nine individuals from clade 2 and the smaller group of two individuals belonged to clade 2.

Focusing on the clade where we found identical genotypes (clade 2), we calculated expected genotype frequencies under random mating and compared the expected frequencies with the observed frequencies. This showed a statistically highly significant difference ($\chi^2 = 61.55$; 36 degrees of freedom; $p = 0.001$; Table S2), demonstrating that this population deviates from free recombination. This is most likely due to clonality of some of the samples, particularly since those are also mostly located within one site. Theoretically, an alternative explanation would be inbreeding (although this would lead to a higher frequency of homozygous samples, which we did not find). Therefore, we also tested for deviations within the site where we found the identical genotypes (site 3). This analysis showed an even lower p value ($\chi^2 = 36.00$; 16 degrees of freedom; $p = 0.0009$; Table S2). We therefore conclude that the genetically identical samples are clonally related.

DISCUSSION

Our experiment on the optimal growth temperature of *B. zonata* shows that this species grows best at 19 °C, while it is also able to grow at lower temperatures as low 4 °C. At 25 °C growth speed is severely reduced while at 30 °C cultures were unable to grow. These findings show that *B. zonata* is well-adapted to grow at the colder temperatures in cloud forests (Jarvis & Mulligan 2011). This is in sharp contrast with the termite-associated genus *Termitomyces* of which a number of species have an optimal growth temperature around 29 °C (Thomas 1981).

At the two sites with the fewest collections, sites 1 and 2, we found a similar pattern with mostly unique genotypes, and the samples at each site belonging to one of the two clades, and even mushrooms growing close to each other (< 5 m apart) differ genetically (Fig. 2c). At site 3 the majority of individuals belonged to clade 2 with only a single collection from clade 1 (Fig. 2c). At this site we found two groups with identical sequences for all three markers; genotype 13–14 (two individuals) and genotype 2–13 (nine individuals), and one individual had an identical genotype (15–16) to a collection at site 1. Given the total genetic diversity we found using the three genetic markers, the identical sequences for all markers for two groups of individuals strongly indicates clonality. Inbreeding would lead to increased homozygosity, which is not what we see, only three of our samples were completely homozygous for the three markers. This suggests that mating between sibling homokaryons due to local dispersal of sexual spores does not play an important role in this species.

Our phylogenetic analysis on the predicted haplotypes for each genotype revealed that both predicted haplotypes of a single genotype always belonged to the same clade which indicates reproductive isolation between the two clades. Morphological examination of voucher specimens TJB8371 (clade 1), TJB10425 (clade 2) and TJB10431 (clade 1) did not reveal any morphological differences between the two clades. Therefore, we conclude that clade 1 and 2 represent two cryptic biological species within *B. zonata*. We do not know whether there is any ecological differentiation between the two, for example due to differences in host-specificity.

All of the mushrooms we collected were attached to isolated patches of aggregated faecal pellets, which shows the depend-

ence of *B. zonata* on this substrate to complete its life cycle. As the substrate is heterogeneous and may only be seasonally available, depending on the life cycle of the insect, we expect that the blastoconidia from *B. zonata* may enhance successful substrate colonization. Dispersal via conidia provides important reproductive assurance for a fungus that grows on a rare and seasonally ephemeral substrate because only a single dikaryotic conidium is needed for colonization and completion of the sexual and asexual lifecycle via the production of mushrooms. Colonization of such temporary substrates via sexual spores is less certain in heterothallic species, as it always requires two sexually compatible basidiospores to form mushrooms. The clonality we find at site 3 suggests that blastoconidia on the mushroom are particularly important for local dispersal. Local dispersal and substrate colonization may be facilitated by the coleopteran larvae producing the pellets (Van de Peppel et al. 2021). However, long-range dispersal via conidia might occasionally happen as we found two genetically identical individuals, which likely are clonally related, at sites 1 and 3, separated by about three kilometres. We did not observe any substantial mycelial growth extending outwards from the faecal substrate into the soil, and substrates were physically separated by soil, so colonization via mycelial outgrowth is unlikely and mycelial structures such as mycelial cords as found in *Armillaria* are unknown in this species. Occasional insect-mediated long range dispersal is an alternative explanation for this observation, for example by adult beetles.

The blastoconidia of *B. zonata* are thick-walled and pigmented, which indicates that they are adapted for long-term survival (Cléménçon 1997, Kües et al. 2016). The conidia may remain dormant in the soil until new faecal pellets are produced by the host insect. The thick-walled chlamydospores of *Squamanita* and *Asterophora* may have a similar role as the spores remain dormant in the soil until new host mushrooms appear (Redhead et al. 1994). It has been assumed that *Asterophora* mainly uses the chlamydospores to complete its lifecycle (Buller 1924). This is supported by the observation that basidiospores are often completely absent in mature mushrooms of these fungi (De Bary 1859, Buller 1924, Thompson 1936), but occasionally mushrooms with basidiospores also have been reported (Brefeld 1889, Ingold 1940). Studies on three species of *Squamanita* found no polymorphisms in the ITS1 sequence within each species, indicating predominantly asexual reproduction (Mondiet et al. 2007, Griffith et al. 2019). In our study on *B. zonata* we observe ample genetic diversity, especially at sites 1 and 2, indicating that basidiospores play an important role in shaping the genetic population structure. We also did not observe malformed or absent lamellae in mature mushrooms of *B. zonata*, which is sometimes the case for mushrooms of *Asterophora* (Redhead & Seifert 2001). In one case (Bzo7) we managed to germinate basidiospores of *B. zonata*, confirming that they are viable. In the case of *Asterophora*, both wind dispersal (Buller 1924) and arthropod dispersal have been suggested (Redhead et al. 1994). Since *B. zonata* grows on insect pellets, a potential insect dispersal agent of the blastoconidia seems likely and deserves further investigation.

Acknowledgements We want to thank Martha Cárdenas for her assistance in the lab. We thank Alex Grum-Grzhimaylo and the Colombian students for helping us spot and collect mushrooms, Ben Auxier for his assistance with creating Fig. 2 and Hilje Doekes for advice on a statistical analysis. We want to thank Natascha Oosterwijk for conducting the growth temperature experiment. The Colombian biological material used in this work was collected under the permission given to the University of Antioquia according to resolution 0524 of May 27, 2014, issued by the National Environmental Licensing Authority - ANLA. This research was funded by the Netherlands Organisation for Scientific Research (VICI; NWO 86514007).

Declaration on conflict of interest The authors declare that there is no conflict of interest.

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Supplementary material

Fig. S1 Chromatograms of partial *TEF1a* sequences of *B. zonata* (Bzo7) showing the dikaryotic (G/T) parent (top sequence) and the homokaryotic offspring (either a G or T).

Fig. S2 Increase of fungal colony area (top) and white value (bottom) over time of *B. zonata* Bzo9 (genotype 7–12, clade 2). Error bars at each data point represent standard deviation. Temperature treatments are given at the far right next to each graph. Both total area and white value were highest after 44 days for cultures grown at 19 °C. None of the cultures kept at 30 °C showed any growth.

Supplemental file 1 Script for processing of scans using ImageJ.

Table S1 Overview of the samples collected and used during this study with their corresponding GenBank accession numbers, collection site, genotype, haplotypes, type of culture available, collection date and location.

Table S2 Calculated expected genotype frequencies under random mating compared to observed frequencies with subsequent statistical analysis.