Pathogenic Allodiploid Hybrids of *Aspergillus* Fungi

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**In Brief**
Steenwyk, Lind, et al. identify clinical isolates of *Aspergillus latus*, an allodiploid species of hybrid origin. These isolates exhibit phenotypic heterogeneity in infection-relevant traits and are distinct from closely related species. The results suggest that allodiploid hybridization contributes to the evolution of filamentous fungal pathogens.

**Graphical Abstract**

- Sequencing of clinical isolates of *Aspergillus* fungi
- Evidence of allodiploid hybridization and genome stability among clinical isolates
- Hybrid isolates differ from each other & parents in diverse traits, including virulence

**Highlights**
- *Aspergillus latus* clinical isolates were previously misidentified as *A. nidulans*
- *A. latus* is an allodiploid that arose via hybridization of two *Aspergillus* species
- *A. latus* hybrid isolates exhibit heterogeneity for several infection-relevant traits
- *A. latus* is phenotypically distinct from parental and closely related species

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INTRODUCTION

Interspecific hybridization can result in the formation of new species that substantially differ in their genomic and phenotypic characteristics from either parental species. One common mechanism by which interspecific hybrids can originate is allopolyploidy, which merges and multiplies the parental species’ chromosomes [1, 2]. Allopolyploid hybrids may be more similar to one parent in some traits, reflect both parents in others, or may differ from both in the rest. Hybrids’ distinct phenotypic profiles mean that they can potentially colonize new habitats [3, 4], whereas their polyplody means that they can quickly become reproductively isolated from both parental species, forming a new species in the process [2]. Allopolyploids are relatively common in plants but are also found in several other lineages, including in animals [5] and fungi [6, 7].

Among fungi, the most well-known example of allopolyploidy is the whole-genome duplication in an ancestor of the baker’s yeast Saccharomyces cerevisiae [8–10]. Importantly, allopolyploidy is known from both fungal pathogens of plants [6, 11] and of animals [12, 13]. For example, the crucifer crop pathogen Verticillium longisporum and Verticillium dahliae are allopolyploid hybrids, as is the onion pathogen Botrytis allii [6, 14, 15]. Among fungal pathogens that infect humans, allopolyploidy has been reported in the ascomycete budding yeasts Candida metapsilosis [16] and Candida orthopsilosis [17] and in the basidiomycete yeast Cryptococcus neoformans x Cryptococcus deneoformans [18]. To our knowledge, allopolyploidy

SUMMARY

Interspecific hybridization substantially alters genotypes and phenotypes and can give rise to new lineages. Hybrid isolates that differ from their parental species in infection-relevant traits have been observed in several human-pathogenic yeasts and plant-pathogenic filamentous fungi but have yet to be found in human-pathogenic filamentous fungi. We discovered 6 clinical isolates from patients with aspergillosis originally identified as Aspergillus nidulans (section Nidulantes) that are actually allopolyploid hybrids formed by the fusion of Aspergillus spinulosporus with an unknown close relative of Aspergillus quadrilineatus, both in section Nidulantes. Evolutionary genomic analyses revealed that these isolates belong to Aspergillus latus, an allopolyploid hybrid species. Characterization of diverse infection-relevant traits further showed that A. latus hybrid isolates are genomically and phenotypically heterogeneous but also differ from A. nidulans, A. spinulosporus, and A. quadrilineatus. These results suggest that allopolyploid hybridization contributes to the genomic and phenotypic diversity of filamentous fungal pathogens of humans.

Article
Pathogenic Allopolyploid Hybrids of Aspergillus Fungi

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A. nidulans, all are similar in appearance when grown in standard laboratory conditions (Figure S1), and two were analyzed as A. nidulans isolates in previous experimental studies [28]. Examination of DNA content revealed that 6/7 isolates were more similar to the diploid *A. nidulans* R21/R153 strain than to the haploid *A. nidulans* A4 strain, suggesting that these 6 isolates are diploid (Figure 1A). The volume of asexual spores (conidia) is frequently proportional to the DNA content of the nucleus [29], and examination of their size showed that the same 6 isolates and the diploid *A. nidulans* strain have significantly larger spores than isolates with haploid genomes (p < 0.001, respectively; Dunn’s test with Benjamini-Hochberg method of multi-test correction for both tests; Figure 1B).

To gain further insight into the genomes of the 6 diploid isolates and 1 haploid isolate, we sequenced them and compared their genome size and gene number with those of representative *Aspergillus* species known to be haploid (*A. clavatus* NRRL 1, *A. flavus* NRRL 3357, *A. fumigatus* Af293, *A. nidulans* A4, *A. niger* CBS 513.88, *A. sydowi* CBS 593.65, and *A. versicolor* CBS 583.65) [31–36]. We found that the genomes and gene numbers of the diploids were significantly larger (average genome size = 69.09 ± 5.68 Mb; average gene number = 21,321.57 ± 2,342.13) than those of the haploid representative *Aspergillus* species (average genome size = 32.62 ± 3.05 Mb; average gene number = 11,330.75 ± 1,838.70; Figure 1C; figshare image 1, 10.6084/m9.figshare.8114114; p < 0.001; Wilcoxon rank sum test for both tests; Table S1). Similarly, examination of gene content completeness revealed a significantly higher number of duplicated near-universally single-copy (BUSCO) genes in the diploids relative to representative *Aspergillus* species (Figure 1C; p = 0.001; Wilcoxon rank sum test). Thus, we concluded that 6/7 clinical isolates are diploids.

**Diploid Clinical Isolates Are Aspergillus latus, a Species of Hybrid Origin**

To examine the evolutionary origin of the clinical isolates, we retrieved their calmodulin and β-tubulin sequences and performed molecular phylogenetic analysis in the context of sequences of the two genes from all available taxa in the section *Nidulantes* phylogeny [37]. We found that the haploid clinical isolate 4060 had nearly identical calmodulin and β-tubulin sequences to other strains of *A. spinulosporus* and formed a monophyletic group with them, suggesting that it belongs to *A. spinulosporus* (Figure 2A; figshare: 10.6084/m9.figshare.8114114). Notably, we found that all 6 diploid clinical isolates contained two different copies of the calmodulin and β-tubulin genes; one copy was nearly identical to *A. spinulosporus* sequences, whereas the other was nearly identical to *A. latus* ones (Figure 2A; figshare: 10.6084/m9.figshare.8114114), raising the hypothesis that the diploids originated from interspecific hybridization between *A. spinulosporus* and *A. latus*.

To test this hypothesis, we analyzed the genome of *A. spinulosporus* strain NRRL 2395 [30] and sequenced the type strain NRRL 200 of *A. latus*. Examination of the DNA content and asexual spore size of these two species’ genomes showed that *A. spinulosporus* NRRL 2395 had similar values as clinical isolate 4060 (Figure 1) and was also placed in the same phylogenetic clade (Figure 2A); these findings confirm that clinical isolate 4060 belongs to *A. spinulosporus* and that
**Table 1. Isolates Used in This Study**

<table>
<thead>
<tr>
<th>Strain/Isolate</th>
<th>Isolation Source</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. spinulosporus soil, Buenos Aires, Argentina</td>
<td>[30]</td>
<td></td>
</tr>
<tr>
<td>A. nidulans A4</td>
<td>E. Yuill, Birmingham, UK</td>
<td>Fungal Genetics Stock Center</td>
</tr>
<tr>
<td>A. quadrilineatus soil, New Jersey, USA</td>
<td>USDA</td>
<td></td>
</tr>
<tr>
<td>4060</td>
<td>clinical isolate from patient with chronic granulomatous disease</td>
<td>[28]</td>
</tr>
<tr>
<td>A. latus NRRL 200T</td>
<td>unknown origin</td>
<td>USDA</td>
</tr>
<tr>
<td>MM151978</td>
<td>clinical isolate from patient with chronic obstructive pulmonary disease (COPD)</td>
<td>laboratory of Dr. Donald Sheppard, McGill University, Canada. Patient sex: unknown. Year isolated unknown.</td>
</tr>
<tr>
<td>NIH</td>
<td>clinical isolate from patient with chronic granulomatous disease</td>
<td>[28]</td>
</tr>
<tr>
<td>ASFU1710</td>
<td>clinical isolate from patient with primary immunodeficiency</td>
<td>sputum material, clinical laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium. Patient sex: female. Isolated in year 2011</td>
</tr>
<tr>
<td>ASFU1908</td>
<td>clinical isolate from patient with asthma, allergic bronchopulmonary aspergillosis (ABPA), and IgG3 deficiency</td>
<td>sputum material, clinical laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium. Patient sex: female. Isolated in year 2011. Isolated from the same patient as ASFU2033</td>
</tr>
<tr>
<td>ASFU2033</td>
<td>clinical isolate from patient with asthma, ABPA, and IgG3 deficiency</td>
<td>sputum material, clinical laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium. Patient sex: female. Isolated from the same patient as ASFU1908 in year 2012</td>
</tr>
</tbody>
</table>

**A. spinulosporus** is one of the parental species involved in the interspecific hybridization event that gave rise to the 6 clinical isolates.

In contrast, the DNA content and spore size of the genome of the type strain of *A. latus* NRRL 200T were similar to those of the 6 diploid clinical isolates (Figure 1). Furthermore, like the 6 clinical isolates, *A. latus* NRRL 200T also had two copies of the calmodulin and β-tubulin gene sequences; one copy was nearly identical to *A. spinulosporus* sequences, and the other copy was closely related to but distinct from *A. quadrilineatus* sequences (Figure 2A; figshare: 10.6084/m9.figshare.8114114). These results suggest that the 6 diploid clinical isolates belong to *A. latus* and that *A. latus* is an allodiploid hybrid species that originated via interspecific hybridization between *A. spinulosporus* and a species closely related to *A. quadrilineatus*.

We tested this hypothesis by performing two different sets of analyses. In the first set of analyses, we sequenced, assembled, and annotated the genome of the type strain (NRRL 201T) of *A. quadrilineatus*. Consistent with our hypothesis that *A. latus* is an allodiploid hybrid, we found that the *A. quadrilineatus* genome contains a single copy of the calmodulin and β-tubulin gene sequences, that these sequences form a monophyletic group with their orthologous sequences retrieved from the genome of a different *A. quadrilineatus* strain (strain CBS 853.96; https://www.ncbi.nlm.nih.gov/sra/SRX5010607), and that the *A. quadrilineatus* sequences form a sister group with one of the two sets of the *A. latus* sequences (Figure 2A).

In the second set of analyses, we estimated the sequence divergence of each gene in the genomes of the 7 clinical isolates as well as of *A. latus* NRRL 200T and *A. quadrilineatus* NRRL 201T from *A. spinulosporus* NRRL 2395T. Under this analysis, the genomes of non-hybrids are expected to show a unimodal distribution (e.g., see control non-hybrid, *A. fumigatus*; Figure 2Bi, left), whereas the genomes of hybrids are expected to show a bimodal distribution whose two modes correspond to the distributions of gene sequence divergence values from each parental genome (e.g., see control hybrid, *Zygosaccharomyces parabailii*; Figure 2Bi, right). We found that the haploid *A. spinulosporus* 4060 clinical isolate and *A. quadrilineatus* NRRL 201T had unimodal distributions reflecting a history devoid of hybridization, and the 6 diploid clinical isolates and *A. latus* NRRL 200T contained nearly equal percentages of *A. spinulosporus* and *A. quadrilineatus*.

**Table 1. Continued**

<table>
<thead>
<tr>
<th>Strain/Isolate</th>
<th>Isolation Source</th>
<th>Reference/Source</th>
</tr>
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<tbody>
<tr>
<td>MO46149</td>
<td>clinical isolate from patient with myelodysplastic syndrome with pneumonia</td>
<td>sputum material, clinical laboratory of Dr. Catarina Lameiras, Department of Microbiology, Portuguese Oncology Institute of Porto, Porto, Portugal. Patient sex: male. Isolated in year 2011</td>
</tr>
</tbody>
</table>

The superscript “T” indicates that the strain is the type strain of the species. See also Figures 1 and S1 and Table S3.
A. quadrilineatus-like genes (51.43% ± 0.74%) A. spinulosporus: 48.57% ± 0.74% A.-quadrilineatus-like (Figure 2B, pie charts), including nearly the full sets of A. spinulosporus and A.-quadrilineatus-like secondary metabolic gene clusters (Table S2; figshare: 10.6084/m9.figshare.8114114). Putative homologs exhibited an average nucleotide sequence divergence of 7.15% ± 0.03%, a value very similar to the average divergence of 7.14% observed between the 8,523 orthologs of A. spinulosporus and A. quadrilineatus (Figure S2). These two sets of analyses confirm that the 6 diploid clinical isolates and the 6 diploid clinical isolates and the A. latus strain A4 share a 10.6084/m9.figshare.8114114). Putative homeologs exhibited an average nucleotide sequence divergence of 7.15% ± 0.03%, a value very similar to the average divergence of 7.14% observed between the 8,523 orthologs of A. spinulosporus and A. quadrilineatus (Figure S2). These two sets of analyses confirm that the 6 diploid clinical isolates and the 6 diploid clinical isolates and the A. latus strain A4 share a similar evolutionary history. To provide more insight on whether the two parental genomes A. latus hybrids undergo recombination, we first examined whether A. latus hybrids undergo the sexual cycle to produce sexual spores (ascospores). We found that all A. latus hybrids produce sexual spores and that the viability of these spores is similar to that of the sexual spores of their parental species (figshare image 2, 10.6084/m9.figshare.8114114). We next examined whether any contigs in the genomes of A. latus isolates had evidence of recombination events. Examination of long (≥ 100 kb) contigs revealed that most genes in most contigs contained genes from one or the other parent and that very few contigs contained substantial percentages of genes from both parents (Figure S4). For example, only an average of means that we cannot exclude the possibility that the two phylogenies are not statistically significantly different. To test this, we evaluated whether the two topologies were statistically different using the approximately unbiased topology constraint test [36]. Using the A. spinulosporus data matrix, we found that we could not reject the topology inferred based on the A. quadrilineatus-like data matrix (p = 0.50 for both tests). These results are consistent with the hypothesis that the two parental genomes of A. latus share the same evolutionary history.
2.67% ± 0.71% contigs per *A. latus* hybrid genome contained substantial percentages of genes from both parental species (Figure S4). However, interpretation of these data is challenging for two reasons. First, the high sequence similarity of the two parental genomes means that identification of parent of origin for highly conserved genes is difficult and likely explains the sporadic presence of one or a handful of genes from one parent in contigs comprised mostly of genes from the other parent. Second, alignment of several of the contigs that contain large numbers of genes from both parents to the *A. nidulans* A4
The Genomes of the *A. latus* Allo diploid Hybrid Isolates Are Stable

To assess the genome stability of the *A. latus* isolates, we began by examining the gene content completeness of each parental genome. We found that each parental genome contained nearly all of the 1,315 BUSCO genes from the fungal phylum Ascomycota (93.50% ± 1.88% *A. spinulosporus* and 94.30% ± 0.40% *A. quadrilineatus* like; figshare image 3, 10.6084/m9.figshare.8114114). Considering that gene content completeness from each parent is only slightly below that from haploid representative species (average = 96.33% ± 0.78%; min = 95.70%, *A. spinulosporus*; max = 97.3%, *A. nidulans* A4), these results suggest little loss of each parental genome by either aneuploidy or loss of heterozygosity events.

To further test this observation genome-wide, we examined the fraction of orthologous genes shared between the *A. spinulosporus* NRRL 2395 strain and the parental genomes of *A. latus* isolates that stem from *A. spinulosporus*. We found that the *A. spinulosporus* parental genomes from *A. latus* hybrids shared a minimum of 9,227/9,611 orthologous genes with each parental genome (Figure S2). These results suggest that the *A. spinulosporus* parental genome of this strain may be more genetically unstable than those of the clinical isolates.

Examination of loss of heterozygosity and aneuploidy events in *A. latus* genomes revealed relatively little evidence for either. Two isolates contained loss of heterozygosity regions. The *A. latus* NRRL 200 strain contained an ~1.2-Mb region homologous to the end of *A. nidulans* chromosome VIII that contained two copies of the *A. quadrilineatus*-like parental genome and lacked a copy of the *A. spinulosporus* genome. This region contains several BUSCO genes, which explains why this strain has a higher proportion of missing BUSCO genes from the *A. spinulosporus* parental genome compared to the 6 clinical isolates (figshare image 3, 10.6084/m9.figshare.8114114). The clinical isolate MO46149 contained an ~1-Mb region homologous to the beginning of *A. nidulans* chromosome V with two copies of the *A. spinulosporus* genome and lacked a copy of the *A. quadrilineatus*-like genome (Data S1). We did not find evidence for chromosome-scale aneuploidies (Data S1).

Lastly, by comparing the gene lengths of homoeolog pairs as a signature of pseudogenization, we found evidence of pseudogenization in at least one gene among an average of 11.67% ± 0.004% homeologs (Figure S2). These results suggest that the genomes of the *A. latus* allo diploid hybrids are generally stable, that loss of heterozygosity is rare, that major aneuploidies have not occurred, and that both genes in ~88% of homoeolog pairs are intact.

Hybrids Exhibit Wide Variation for Infection-Relevant Traits

To examine variation in infection-relevant traits between the hybrid isolates, one of their known parental species (*A. spinulosporus*), the closest known relative of their other parental species (*A. quadrilineatus*), and the species they were originally identified as (*A. nidulans*), we tested the virulence of all isolates in an invertebrate disease model and phenotypically characterized them across a wide variety of infection-relevant conditions, including interactions with host immune cells, drug susceptibility, oxidative stress, iron starvation, and temperature stress (Figures 3 and S5). Principal-component analysis (PCA) and examination of the traits with the greatest contributions to the observed variance among isolates revealed two major findings. First, the 7 *A. latus* hybrids exhibit substantial heterogeneity in their phenotypic profiles (Figure 3A). Second, the *A. latus* hybrids are phenotypically distinct from *A. nidulans* and their parental species but are more similar to *A. spinulosporus* than to *A. quadrilineatus* (Figure 3A). Among the traits tested, those with the largest contributions to the observed variation among isolates and species were interactions with host immune cells, antifungal drug susceptibility, and oxidative stress resistance (figshare image 3A and 4, 10.6084/m9.figshare.8114114). Here, we discuss exemplary phenotypic traits that highlight these two major findings (see Figure S5 for other phenotypes).

Phenotypic variation or strain heterogeneity among *A. latus* hybrids was observed for nearly every trait measured (Figures 3 and S5). For example, examination of virulence in the invertebrate greater wax moth (*Galleria mellonella*) model revealed substantial variation among isolates (p < 0.001; log rank test; Figure 3B). Specifically, we observed that *A. latus* isolate AFU1710 was the most virulent and *A. latus* isolate MO46149 was the least virulent. Similarly, we found substantial strain heterogeneity in how much lytic and non-lytic NETosis (a process where neutrophils release neutrophil extracellular traps, or NETs, to kill microbes) [39] was stimulated by *A. latus* hybrid isolates (Figure 3C). For example, *A. latus* NIH did not substantially stimulate NETosis although *A. latus* AFU1710 did. Strain heterogeneity was less pronounced for other traits, such as hyphal viability, drug susceptibility, and oxidative stress, yet all exhibited variation across isolates (Figures 3D–3F). One *A. latus* isolate that was consistently different from the rest is MO46149; for example, this isolate was twice as susceptible to hyphal killing by neutrophils compared to the other *A. latus* isolates and it was the isolate most sensitive to the antifungal caspofungin, as well as the isolate most tolerant to the oxidative stressor paraquat.

Phenotypic variation was also pronounced when we compared infection-relevant traits between *A. latus*, its two parental species, and *A. nidulans* (Figure 3A). For example, we found that *A. latus* hybrids (and *A. quadrilineatus*) were less susceptible to killing by neutrophils compared to *A. spinulosporus* (Figure 3D). In contrast, we found *A. latus* isolates (and *A. spinulosporus*) differed in their susceptibility to low doses of caspofungin (Figure 3E) or to high doses of oxidative stress (Figure 3F) from *A. quadrilineatus* and *A. nidulans*. 

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Figure 3. *A. latus* Hybrids Exhibit Strain Heterogeneity and Differ from Parental Species, *A. quadrilineatus*, and *A. nidulans* in Infection-Relevant Phenotypes

(A) Principal-component analysis of diverse infection-relevant phenotypes reveals strain heterogeneity among *A. latus* hybrids and that they differ from the closest relative of the unknown parent, *A. quadrilineatus*, and the *A. nidulans* A4 strain. Data for each phenotype were scaled prior to principal-component analysis. (B and C) Wide phenotypic variation among strains of *A. latus* hybrids as well as among the various species tested was observed for virulence in the *Galleria* model of disease (B) and for stimulation of NETosis, a process where neutrophils release neutrophil extracellular traps, or NETs, to kill microbes (C). (D) Examination of the caspofungin drug susceptibility profiles among *A. nidulans* and *A. spinulosporus* and the *A. latus* hybrids revealed differences between the three species (F(3) = 24.514; p < 0.001; multi-factor ANOVA). At 1 μg/mL caspofungin treatment, *A. spinulosporus* and *A. latus* hybrids grew more than *A. nidulans* and *A. quadrilineatus* (p < 0.001 for both comparisons; Tukey honest significant differences test). We found no statistically significant differences between the various species at 8 μg/mL of caspofungin but observed a qualitative difference similar to growth in 1 μg/mL of caspofungin. (E) Examination of growth in the presence of the oxidative stress agent paraquat revealed differences among the various species (F(3) = 30.25; p < 0.001; multi-factor ANOVA). Dark gray represents *A. nidulans*; red represents *A. quadrilineatus*; blue represents *A. spinulosporus*; and purple represents *A. latus*. All pairwise comparisons shown by brackets were examined using a Tukey honest significant differences test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. See also Figure S5. Barplots are displayed with error bars that correspond to one standard deviation from the mean.
In summary, we found substantial heterogeneity among A. latus hybrid isolates as well as between A. latus and closely related or parental species for diverse infection-relevant traits. Generally, A. latus hybrids are more similar to their known parent, A. spinulosporus, compared to the closest known relative of their other parent, A. quadrilineatus. Importantly, A. latus hybrids are also phenotypically distinct from A. nidulans, the species they were originally misdiagnosed as.

**DISCUSSION**

Infections by filamentous fungal pathogens affect hundreds of thousands of humans and exhibit very high mortality rates [40], so understanding the evolutionary mechanisms underlying their pathogenicity is of great interest. We have discovered several clinical isolates previously identified as A. nidulans, an important pathogen of CGD patients, which in reality are allodiploid hybrids that arose via interspecific hybridization between A. spinulosporus and a close relative of A. quadrilineatus and belong to A. latus. In line with clinical misidentification of these species, A. nidulans, A. spinulosporus, A. quadrilineatus, and A. latus are known to be nearly indistinguishable with the exception of aspects of their ascospore micromorphology and their secondary metabolic profiles [37]. The allodiploid hybrids show strain heterogeneity in their phenotypic profiles and differ from their parental species and A. nidulans with respect to several infection-relevant traits. Below, we discuss the implications of these results for disease management and the evolution of fungal pathogenicity.

Application of molecular typing, and more recently genomic, approaches to delineate fungal species and pathogens has revealed the existence of multiple, closely related species that are morphologically indistinguishable but genomically distinct from each other [41]. This “hidden” or “cryptic” genomic diversity is found in species from many genera that harbor major fungal pathogens, including Aspergillus [42–44]. Alarming, application of molecular methods on fungal clinical isolates has too begun to reveal that a significant portion of fungal infections are caused by these cryptic species. In the case of Aspergillus, studies in both the USA and Spain report that 10%–15% of aspergillosis infections stem from cryptic species [45, 46]. Understanding the biology of these cryptic species is essential for guidance in therapy, as many show high levels of antifungal drug resistance [45–47]. Several A. fumigatus-related cryptic species exhibit decreased susceptibility (relative to A. fumigatus) to antifungal drugs [48]; similarly, we found notable differences in certain phenotypic traits, including drug susceptibility, between the allodiploid hybrids and A. nidulans (Figures 3E and S5).

A growing body of literature suggests that phenotypic heterogeneity among isolates of the same species is an under-appreciated factor in understanding fungal pathogenicity [49]. For example, several recent studies have identified phenotypic and genomic differences between A. fumigatus strains that are associated with virulence [50–52]; strain heterogeneity is also observed among A. nidulans strains [27]. In line with these studies, our work reveals considerable heterogeneity in infection-relevant traits among A. latus hybrids (Figures 3 and S5), further highlighting the importance of strain heterogeneity in understanding Aspergillus pathogenicity [49].

Allopolyploid hybrids typically have unstable genomes [12]. Evolutionary paths to achieve stability after hybridization include whole-genome duplication, total or partial chromosome loss, gene loss, and loss of heterozygosity [12]. For example, an ancient allopolyploid hybridization event in the budding yeast lineage that includes the baker’s yeast Saccharomyces cerevisiae [9] was quickly followed by rapid gene loss in the parental genomes [53], with estimates suggesting that 10% of genes were lost in the first 10 Ma following hybridization [53]. Similar rates of gene loss have been reported in plants [54, 55] and animals [56], suggesting that rapid gene loss is a common outcome of hybridization. In contrast to these studies, we found that most A. latus hybrids (with the possible exception of A. latus NRRL 200) contain both copies of most homeolog gene pairs and have relatively stable genomes (Figures S2 and S4). Consistent with our genomic analyses, A. latus isolates exhibit minimal sectoring when grown in culture (Figure S1) and have similar ascospore viability compared to their closest relatives, A. spinulosporus and A. quadrilineatus (figshare image 2, 10.6084/m9.figshare.8114114). Furthermore, laboratory studies that have created synthetic Aspergillus hybrids—including those that created interspecies hybrids from distinct species in section Nidulantes—reported that some of these hybrids are relatively stable [57–60]. Taken together, these results suggest that Aspergillus hybrids may be more stable than other hybrid allopolyploids.

Although several examples of interspecies hybridization are known in fungi [6, 8–12, 14–18], most of them are ancient. Consequently, the steps that led to hybrid formation and maintenance are harder to elucidate. As the A. latus allodiploid hybrids originated much more recently, and the mechanisms that underlie synthetic hybrid formation in Aspergillus have been extensively studied [57–60], we can propose a model to explain the origin and life cycle of A. latus (Figure 4). Under our model, the first step in the formation of the A. latus allodiploid hybrid was the cellular fusion (or plasmogamy) of an A. spinulosporus parental isolate and an A.-quadrilineatus-like parental isolate through a parasexual or a sexual cycle. In the next step, the distinct nuclei contributed by the two parental isolates underwent nuclear fusion (or karyogamy) to create a single nucleus with a diploid genome comprised from the A. spinulosporus and A.-quadrilineatus-like genomes. Once formed, the allodiploid hybrid species A. latus has been capable of undergoing both asexual and sexual reproduction and forms viable asexual spores (conidia; Figure 1) and sexual spores (ascospores; figshare image 2, 10.6084/m9.figshare.8114114SS).

Hybrids have been observed in several fungal pathogens of animals and plants [6, 12, 16, 18], suggesting that hybridization of pathogenic fungi poses threats to plant and animal health. Hybridization can result in the acquisition of new traits, such as host expansion or increased virulence. For example, two powdery mildew species that specialize in infecting different species of plants have been shown to hybridize and infect a plant species that neither parent can [61]. Similarly, hybridization is thought to contribute to virulence among human yeast pathogens, but clear examples are currently lacking [12]. To our knowledge, our results are the first report of hybrid clinical isolates in a filamentous fungal pathogen of humans (Figure 2). Like previous studies, we observe that the hybrids exhibit infection-relevant traits that differentiate them from their parental relatives and may provide a fitness...
advantage inside human hosts (Figure 3). However, it should be noted that the potential selection pressure for hybrids in patients is unknown; although we isolated the hybrids from patients with diverse pulmonary diseases, we do not know whether the hybrids’ primary lifestyle is that of a pathogen or whether they originated inside a host. Importantly, the type strain of A. latus (NRRL 200T) is not a clinical isolate, suggesting that the species is also found in the environment. More broadly, the existence of hybrids in a diverse set of pathogenic fungi infecting a diverse set of animal and plant hosts raises the hypothesis that allopolyploid hybridization contributes to the evolution and diversity of all kinds of fungal pathogens, perhaps to a greater extent than currently realized.

In summary, viewed from a medical mycology perspective, our discovery of allopolyploid hybrid clinical isolates reveals the importance of accurate isolate identification and strain heterogeneity. Viewed from an evolutionary perspective, our and previous results suggest that hybridization contributes to the genomic and phenotypic diversification of filamentous fungal pathogens of humans and argue that hybridization represents a general mechanism that can be potentially employed by all fungal pathogens to adapt to all kinds of hosts.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.04.071.

Figure 4. Proposed Model for the Evolution of A. latus via Allopolyploid Hybridization

Under the model, haploid nuclei of an A. spinulosporus isolate and of an isolate from an A.-spinulosporus-like species underwent cellular fusion (plasmogamy), forming a heterokaryotic mycelium (i.e., a mycelium where cells contain two distinct nuclei). Next, nuclear fusion (karyogamy) resulted in the merging of the two genetically distinct nuclei and their genomes into a single one, giving rise to the allopolyploid species A. latus, which is capable of undergoing asexual and sexual reproduction to produce asexual spores (conidia) or sexual spores (ascospores).
ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

J.L.S., A.L.L., G.H.G., and A.R. designed the research; J.L.S. and A.L.L. performed computational and statistical analyses; L.N.A.R., T.F.d.R., L.P.S., F.A., R.W.B., T.F.d.C.F.D.s., V.L.D.B., A.M.P., and F.R. performed experiments; K.L. contributed strains; H.A.R., S.L.K., and N.H.O. contributed strains and performed experiments; J.L.S. and A.L.L. prepared the figures with input from G.H.G. and A.R.; J.L.S., A.L.L., G.H.G., and A.R. wrote the manuscript; and all authors provided comments and input and have approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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reactive oxygen intermediates but is dispensable for pathogenicity in an intranasal mouse infection model. Eukaryot. Cell 6, 2290–2302.


## STAR METHODS

### KEY RESOURCES TABLE

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**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Antonis Rokas (antonis.rokas@vanderbilt.edu).

**Materials Availability**
This study did not generate new unique reagents. *Aspergillus* strains are available upon request.

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**Deposited Data**

- Raw reads used for genomics; See Table S3: This study, N/A
- Genomic assemblies; See Table S3: This study, N/A
- Predicted gene boundaries: This study, figshare: 10.6084/m9.figshare.8114114
- Phenotypic measurements: This study, figshare: 10.6084/m9.figshare.8114114
- FACS data: This study, figshare: 10.6084/m9.figshare.8114114
- Phylogenetic and phylogenomic data: This study, figshare: 10.6084/m9.figshare.8114114
- Parent of origin analysis: This study, figshare: 10.6084/m9.figshare.8114114
- Homeologs per hybrid isolates: This study, figshare: 10.6084/m9.figshare.8114114
- Biosynthetic gene cluster predictions: This study, figshare: 10.6084/m9.figshare.8114114

**Experimental Models: Organisms/Strains**

The greater wax moth (*Galleria mellonella*): Goldman lab, N/A

**Software and Algorithms**

- In silico Whole Genome Sequencer and Analyzer (WGS), version 1.1: [62] https://github.com/zhouxiaofan1983/iWGS
- AUGUSTUS, version 3.3: [63] http://bioinf.uni-greifswald.de/augustus/
- RAxML, version 8.2.11: [68] https://cme.h-its.org/exelixis/web/software/raxml/
- Control-FREEC, version 9.1: [70] https://github.com/BoevaLab/FREEC
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Microbe strains
All Aspergillus spp. strains were obtained from public collections, in laboratories, or hospitals. Upon receipt, these strains were grown on YAG medium and after 5 days growth at 30°C, and asexual spores (conidia) were harvested and stored in desiccators at 4°C in water for further usage or at –80°C in a 30% glycerol solution in the laboratory collection. In experiments for DNA extraction and genome sequencing, the conidia were inoculated into Erlenmeyer-flasks on an orbital shaker (150 rpm) at 30°C for 24 h. A. quadrilineatus NRRL 201T was acquired separately from the NRRL culture collection. Upon receipt of the cryopreserved vial, the contents of the frozen material were aseptically removed from the vial and transferred to 50 mL of YESD (Yeast Extract, Soy peptone, Dextrose) medium, grown at 30°C for 5 days and frozen material was used for subsequent experiments, including DNA extraction and genome sequencing. Cultures of A. quadrilineatus NRRL201T are currently preserved at room temperature on potato dextrose agar (Difco).

Primary cell cultures
To obtain macrophages for a phagocytosis assay of Aspergillus asexual spores (conidia), we used bone marrow-derived macrophages that were isolated as described previously [71]. Briefly, macrophages were recovered from femurs of male C57BL/6 (wild-type, WT, Jax 000664) at 6–8 weeks old and were incubated in an RPMI medium (GIBCO) supplemented with 30% (volume/volume) L929 growth conditioning media, 20% inactivated fetal bovine serum (GIBCO), 2 mM glutamine and 100 units/milliliter of penicillin-streptomycin (Life Technologies). Fresh media was added after 4 days of cultivation and macrophages were collected after 7 days and used for subsequent experiments. The animals were housed in the animal facility of the Ribeirão Preto Medical School, University of São Paulo, under optimized hygienic conditions.

Human polymorphonuclear cells (PMNs) were isolated from 8 mL of peripheral blood of adult male healthy volunteers by density centrifugation using Mono-Poly Resolving Medium (MP Biomedicals LLC, Irvine, CA, USA) according to the manufacturer’s instructions. The PMNs (5x10⁷ / mL) were resuspended in Hank’s Balanced Salt Solution, without calcium or magnesium, containing 5% fetal bovine serum (GIBCO®, South American, Brazil).

Animals
Galleria mellonella larvae are cultivated in a greenhouse at 27°C. The larvae are kept in glass jars, in which they are separated by stages, moths, eggs, larvae and pupa.

The larvae are fed once a week, with a feed composed of (yeast extract 1.5%; soy 1%, powdered milk 1%, honey 1% and beeswax 2%).

In the sixth week of the larval stage, the larvae are adult and larger, facilitating infection.

We separated and assembled the groups with the larvae (n = 10) in Petri dishes. The groups are composed of larvae that are approximately 300 mg in weight and 2 cm long. Moth sex was not accounted for due to the impossibility of visually determining sex at the sixth week of larval development.

Ethics statement
The principles that guide our studies are based on the Declaration of Animal Rights ratified by the UNESCO on the 27th January 1978 in its 8th and 14th articles. All protocols used in this study were approved by the local ethics committee for animal experiments from Universidade de São Paulo, Campus Ribeirão Preto (permit number 08.1.1277.53.6). All animals were cared for in strict accordance with the principles outlined by the Brazilian College of Animal Experimentation (Princípios Éticos na Experimentação Animal—Colégio Brasileiro de Experimentação Animal [COBEA]) and the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society.

METHOD DETAILS
Fluorescence-assisted cell sorting for DNA content determination
Asexual spores (conidia) were collected, centrifuged (13,000 rounds per minute for 3 min) and washed with sterile 1x phosphate-buffered saline (8 g sodium chloride, 0.2 g potassium chloride, 1.44 g disodium phosphate, 0.24 g monopotassium phosphate...
per liter of sterilized water). For cell staining, the protocol described by Almeida et al. [72] was followed with modifications. Following overnight fixation with 70% ethanol (volume / volume) at 4°C, spores were harvested, washed and suspended in 850 μl of sodium citrate buffer (50 mM sodium citrate; pH = 7.5). Spores were subsequently sonicated using four ultrasound pulses at 40W for 2 s with an interval of 1 to 2 s between pulses. Sonicated spores were treated for 1 h at 50°C with RNase A (0.50 mg / millilitre; Invitrogen, Waltham, Massachusetts, USA) and for 2 h at 50°C with proteinase K (1 mg/mL; Sigma-Aldrich, St. Louis, Missouri, USA). Spores were stained overnight with SYBR Green 10,000x (Invitrogen, Carlsbad, CA, USA) diluted 10-fold in Tris-ethylenediaminetetraacetic acid (pH 8.0), at a concentration of 2% (volume / volume) at 4°C. Finally, Triton® X-100 (Sigma-Aldrich) was added to samples at a final concentration of 0.25% (volume / volume). Stained spores were analyzed in a Fluorescence-assisted cell sorting LSRII flow cytometer (Becton Dickinson, NJ, USA) with a 488 nanometer excitation laser. Signals from a minimum of 30,000 cells per sample were captured in fluorescein isothiocyanate channel (530 nanometers ± 30 nanometers) at low flow rate of ~1,000 cells / second and an acquisition protocol was defined to measure forward scatter and side scatter on a four-decade logarithmic scale and green fluorescence on a linear scale. Fluorescence-assisted cell sorting Diva was used as the acquisition software. Results were analyzed with the R package flowViz, version 1.48.1 [73].

Asexual spore size measurements

The diameters of 100 spores for each isolate were measured under a Carl Zeiss (Jena, Germany) AxioObserver.Z1 fluorescent microscope equipped with a 100-W HBO mercury lamp using the 100 x magnification oil immersion objective and the AxioVision, software v.3.1.

DNA extraction and sequencing

Frozen mycelia of all isolates were ground in liquid nitrogen and genomic DNA was extracted as previously described [74, 75]. Standard techniques for manipulation of DNA were used [76]. The genomes of all clinical isolates and the type strain of A. latus (9 in total) were sequenced at the Genomic Services Lab of Hudson Alpha (Huntsville, Alabama, USA) on an Illumina HiSeq 2500 sequencer; the sole exception was A. quadrilineatus NRRL 201T, which was sequenced using on a NovaSeq S4 at the Vanderbilt Technologies for Advanced Genomes facility (Nashville, Tennessee, USA). All isolates were sequenced using paired-end sequencing (150 bp) with the Illumina TruSeq library kit. Additionally, the type strain of A. latus and the clinical isolates MM151978 and NIH were also sequenced using mate-pair sequencing (150 bp) using the Illumina Nextera Mate Pair Library kit with an insert size of 4 kilobases. The genome coverage of each isolate was greater than 150X. Both the raw short-read sequence data and the genome assemblies are publicly available (see Table S3 for accession numbers).

Genome assembly and annotation

All genomes were assembled with the iWGS pipeline [62] using dipSPAdes, version 1.0 [77] or SPAdes, version 3.6.2 [78]. Optimal k-mer lengths were selected using KmmerGenie, version 1.68B2 [79], and assembly quality was evaluated using QUAST, version 3.2 [80]. Protein-coding genes were predicted using AUGUSTUS, version 3.3 [63], using Aspergillus nidulans gene annotation parameters. Predicted genes in each A. latus hybrid genome were annotated by reciprocal-best-BLAST against a database of all A. nidulans and A. spinulosporus proteins.

Prediction of secondary metabolic gene clusters

Secondary metabolic gene clusters (SMGCs) were predicted in all assembled genomes using antiSMASH, version 3.0.5.1 [81]. In addition, we used literature-curated SMGC annotations from the well-characterized A. nidulans A4 genome [36] to identify SMGCs not captured by the antiSMASH software.

Assigning genes in hybrid genomes to parents of origin

To determine the most likely parent-of-origin for each gene in a hybrid genome, we measured the sequence divergence between every gene in a hybrid genome and its ortholog in the A. spinulosporus NRRL 2395T parent [82]. Specifically, for each gene in a hybrid genome, we used BlastP, version 2.3.0 from NCBI’s Blast+ package [83], to identify the putative A. spinulosporus ortholog. The resulting pair was then aligned using Mafft, version 7.294b [66], with the Blossum 62 matrix of substitutions [84], a gap penalty of 1.0, 1,000 maximum iterations, a single guide tree, and the ‘genafpair’ parameter. The associated DNA sequences were then forced onto the protein alignment using PAL2NAL, version 14 [85], and the synonymous substitution rate Ks was calculated according to LWL85m method using yn00 module in paml, version 4.9 [65]. By examining the bimodal distribution of Ks values, genes with relatively low Ks values (0 ≤ Ks < 0.05) were inferred to be from the A. spinulosporus parent genome and genes with high Ks values (0.05 ≤ Ks < 10) were inferred to stem from the A. quadrilineatus-like parent genome. We performed the same analysis on the known, non-hybrid haploid genome of Aspergillus fumigatus strain A1163 compared to A. fumigatus strain Af293 [33] and on the known hybrid diploid genome of ZygoSaccharomyces parabailii strain NBRC1047/ATCC56075 compared to their closest homologs in the known parent ZygoSaccharomyces bailii strain CLIB213 [82] as controls.

To determine the completeness of each parental genome and evaluate the efficacy of assigning genes in hybrid genomes to parent-of-origin, we created separate proteome FASTA files for genes from the A. spinulosporus and A. quadrilineatus-like regions
of the genome and evaluated how many near-universally single copy orthologous genes were present in each parental genome using the BUSCO pipeline [86] with the Ascomycota database (creation date: 2016-02-13, number of species: 75, number of BUSCOs: 1315) from OrthoDB, version 9 [57].

To determine homeolog pairs between each parental genome, we used a reciprocal best blast hit approach. Specifically, we used the FASTA files of genes created in the previous step and conducted a reciprocal best blast hit analysis between the two parental genomes using an e-value cutoff of $10^{-5}$. To determine if one gene was putatively pseudogenized, we employed a previously established approach, which compares gene lengths between the two homeologs; genes whose length is substantially shorter (we used an upper threshold of 80%) than their homeolog pair are inferred to be pseudogenes [82].

Maximum likelihood phylogenetic and phylogenomic analyses
To establish the taxonomic identity of the sequenced isolates, we retrieved their β-tubulin and calmodulin sequences by performing a nucleotide BLAST of the Aspergillus nidulans A4 β-tubulin and calmodulin sequences against each assembled genome. In addition, β-tubulin and calmodulin sequences from two strains of each of Aspergillus foveolatus, Aspergillus latus, Aspergillus nidulans, Aspergillus pachycristatus, Aspergillus rugulosus, Aspergillus spinulosporus, and Aspergillus striatus and from one strain of Aspergillus corrugatus were retrieved from GenBank. We also retrieved the same sequences from one strain of Aspergillus sydowi, which served as an outgroup for the phylogeny [37]. Sequences were aligned with MAFFT, version 7.310 [66], gaps were removed with trimAl, version 1.2.rev59 [67], using the ‘gappyout’ parameter, and the β-tubulin and calmodulin sequences for each individual strain were concatenated. A phylogeny was constructed from the concatenated sequences with RAxML, version 8.2.11 [68], with the GTRGAMMAX model and 1,000 rapid bootstrap replicates. Branches with bootstrap support less than 80 were collapsed.

To determine the number of hybridization events that gave rise to A. latus isolates, we conducted phylogenomic analyses to reconstruct the evolutionary history of the A. spinulosporus parental genome in the A. latus hybrids and of the genes of A. spinulosporus strains NRRL2395 and 4060. We first identified single-copy orthologous genes across the 9 strains using OrthoFinder, version 2.3.8 [88] with default parameters, which employs a Markov clustering algorithm [89] on gene sequence similarity information derived from an ‘all-versus-all’ approach using NCBI’s Blast+. Out of the inferred 12,596 groups of orthologous genes, 5,894 were single-copy (i.e., each of the 9 isolates was represented by a single sequence). All 5,894 sets of corresponding nucleotide sequences were aligned using Mafft, version 7.402 [66], gaps were removed with trimAl, version 1.2.rev59 [67], using the ‘gappyout’ parameter. The resulting sequences were concatenated into a single nucleotide data matrix (8,405,004 sites). The strain phylogeny was inferred using iqtree, version 1.6.1 [69], with the nbest parameter set to 10 to increase the number of best trees used during the search. Bipartition support was evaluated using 5,000 ultrafast bootstrap approximation replicates [90]. The evolutionary history of the A. quadrilineatus-like parental genome among the A. latus hybrids and two A. quadrilineatus strains (NRRL2017 and CBS 853.96) was inferred using the same approach; in this case, the nucleotide data matrix was comprised of 7,385,465 sites (from 5,079 single-copy orthologous genes out of a total of 11,814 groups of orthologous genes).

Lastly, we conducted approximately unbiased topology tests [38] using the data matrices from each parental genome with the ‘au’ parameter in iqtree, version 1.6.1 [69]. Specifically, we examined if the two topologies among the 7 A. latus hybrids inferred using the A. spinulosporus or A. quadrilineatus-like parental genomes were significantly different for either genome-scale data matrix inferred from the parental genomes.

Examination of loss of heterozygosity using copy number variation analysis
To conservatively and accurately measure loss of heterozygosity, we measured copy number variation using Control-FREEC, version 9.1 [70] and CNVnator, version 0.3.2 [95]. More specifically, we evaluated false discovery rate (FDR) and false positive rate (FPR) using reads from a “concatenated” genome of the A. spinulosporus and A. nidulans FGSC-A4 using 10 different window sizes (500, 750, 1000, 1250, 1500, 2000, 3000, 4000, and 5000 base pairs). Reads were aligned with bwa-mem, version 0.7.12 [96], and duplicates reads were removed with picardtools, version 2.1.1 (http://broadinstitute.github.io/picard/). In this way, we inferred the window size parameter that minimizes the FDR and FPR CN variable regions identified in the hybrid genomes. Specific parameters used for Control-FREEC include a minimum and maximum expected GC-content of 0.3 and 0.5, respectively, and a telocentric-tomeric parameter of 10.00. Default parameters were used for CNVnator. To identify statistically significant CN variable loci, we implemented a Wilcoxon Rank Sum test [97] and a Kolmogorov-Smirnov test [98], in the case of Control-FREEC and a t test in the case of CNVnator. Using the resulting set of significant CN variants per window size for each program, FDR and FPR were calculated using the following formulas:

$$FDR = 1 - \frac{TP}{(TP + FP)}$$

$$FPR = \frac{FP}{(FP + TN)}$$
where TP represents true positives, FP represents false positives, and TN represents true negatives. Across the 10 different window sizes, we found that Control-FREEC often, but not always, slightly outperformed CNVnator (figshare image 5, 10.6084/m9.figshare.e.8114114SSS10). More importantly, we found that Control-FREEC had an FDR and FPR of 0 (i.e., had no false negatives or false positives) when using a window size of 1000 and 1250. Therefore, we used Control-FREEC with a window size of 1000 to identify CN variable loci in all other isolates.

**Macrophage isolation**

To obtain macrophages for a phagocytosis assay of *Aspergillus* asexual spores (conidia), we used bone marrow-derived macrophages that were isolated as described previously [71]. Briefly, macrophages were recovered from femurs of C57BL/6 wild-type mice (6-weeks old) and were incubated in an RPMI medium (GIBCO) supplemented with 30% (volume / volume) L929 growth conditioning media, 20% inactivated fetal bovine serum (GIBCO), 2 mM glutamine and 100 units / milliliter of penicillin-streptomycin (Life Technologies). Fresh media was added after 4 days of cultivation and macrophages were collected after 7 days and used for subsequent experiments.

**In vitro phagocytosis by macrophages**

Phagocytosis of asexual spores (conidia) by wild-type macrophages were carried out according as previously described with modifications [99]. 24-well plates containing a 15-mm-diameter coverslip in each well (phagocytosis assay) and 2x10^5 macrophages per well were incubated with 1 mL of RPMI-FBS (RPMI medium (GIBCO) supplemented with 10% inactivated fetal bovine serum (GIBCO), 2 mM glutamine and 100 units / milliliter of penicillin-streptomycin (Life Technologies)) at 37°C, 5% carbon dioxide for 24 h. Wells were washed with 1 mL of phosphate-buffered saline before the same volume of RPMI-FBS medium supplemented with 1x10^6 spores (1:5 macrophage / spore ratio) was added in the same conditions.

To determine phagocytosis, macrophages were incubated with spores for 1.5 h before supernatant was removed and 500 μl of phosphate-buffered saline containing 3.7% formaldehyde was added for 15 min at room temperature. Sample coverslips were washed with 1 mL of ultrapure water and incubated for 20 min with 500 μl of 0.1 mg / milliliter calcein yellow white to stain the cell wall of non-phagocytosed spores. Samples were washed and coverslips were viewed under a Zeiss Observer Z1 fluorescence microscope. In total, 100 spores were counted per sample and the phagocytosis index was calculated. Experiments were performed in biological triplicates.

**Viability of Aspergillus hyphae**

Human neutrophils from fresh venous blood of healthy adult volunteers were isolated according to a previous study with slight modifications [100], through centrifugation over isotonic Percoll, lysed, and resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline solution. Since we did not observe any neutrophil-mediated killing of *Aspergillus* asexual spores, as also previously described [101], we followed the protocol previously reported for neutrophil-mediated inhibition of germination. *Aspergillus* asexual spores were incubated with neutrophils (0.5, 1.0, or 2.5x10^5 cells / milliliter; effector: target cell ratios of 1:1000, 1:500, or 1:200, respectively) in a 96-well plate overnight at 37°C in RPMI 1640 medium containing glutamine and 10% fetal calf serum (Life). The neutrophils were lysed in water / sodium hydroxide (pH 11.0) and spore germination was determined using an MTT (thiazolyl blue; Sigma-Aldrich) assay as previously reported [102]. Each isolate’s viability was calculated relative to incubation without neutrophils, which was set at 100% for each isolate and evaluated separately. To evaluate the viability of *Aspergillus* hyphae in the presence of neutrophils, we used a previously described protocol [101]. *Aspergillus* asexual spores were incubated overnight at 37°C in RPMI 1640 medium containing glutamine and 10% fetal calf serum (Life) upon formation of a monolayer, as verified by microscope. Freshly isolated neutrophils (0, 1.0, 2.0, or 3.0 x 10^5 cells / milliliter) were cultured for 1 h on the *Aspergillus* monolayer at 37°C. Subsequently, the cells were lysed in water / sodium hydroxide (pH 11.0) and the MTT assay was performed. Each isolate’s hyphal viability was calculated as a percentage of its viability after incubation without neutrophils. The experiments were repeated three times, each performed in triplicate.

**NETosis assays**

Human polymorphonuclear cells (PMN) were isolated from 8 mL of peripheral blood of adult male healthy volunteers by density centrifugation using Mono-Poly Resolving Medium (MP Biomedicals LLC, Irvine, CA, USA) according to the manufacturer’s instructions. PMN (5x10^6 / mL) were resuspended in Hank’s Balanced Salt Solution, without calcium or magnesium, containing 5% FBS (GIBCO®, South American, Brazil). For flow cytometry analysis, 100 μL (5x10^5 / tube) of PMN were seeded in sterile round-bottom polystyrene tubes, stimulated with 10 nM of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) or fungi samples (5x10^5 / tube) and then incubated for 3 h at 37°C and 5% CO2. In the last 30 min, 1000x diluted LIVE/DEAD (Invitrogen, Eugene, OR, USA) was added. After that, PMN were mixed to react with SYTOX Green Nucleic Acid Stain (1 μM) (Invitrogen) for 10 min at room temperature. Data on cells were acquired by flow cytometry using a BD FACSCanto II instrument (BD Bioscience, Franklin Lakes, NJ, USA). One hundred thousand events per sample were collected, doublet discrimination was performed using Forward Scatter Area (FSC-A) versus Forward Scatter Height (FSC-H) parameters, and the PMN were gated according to size (FSC-A) and granularity (Side Scatter Area, SSC-A). LIVE/DEAD and SYTOX positive cells were analyzed with FlowJo software (TreeStar, Ashland, OR, USA). For fluorescence microscopy, 100 μL (5x10^5 / well) of PMN were seeded on 13 mm glass coverslips in 24-wells plate and pre-incubated for 30 min at 37°C and 5% CO2. After adherence, PMN were stimulated with PMA (10 nM) or fungi samples.
(5x10⁵ / tube) and then incubated for 3 h at 37°C and 5% CO₂. PMN were made to react with SYTOX Green (1 µM) (Life Technologies) for 10 min at room temperature. The glass coverslips were removed and the slides were fixed with ProLong Gold Antifade Mountant with DAPI (Invitrogen). The images were obtained using a Leica DMi6000 Fluorescence Microscope (Leica Microsystems, Wetzlar, Germany). Details on the flow cytometry gating strategy used and microscopy images are available in the figshare repository (https://doi.org/10.6084/m9.figshare.8114114).

Growth in the presence of different stresses
To study variation in infection-relevant phenotypes between the hybrid isolates, we compared the radial growth of A. nidulans, A. spinulosporus, A. latus, and of all the clinical isolates in different temperatures (30°C, 37°C and 44°C), in the presence of increasing concentrations of oxidative stress-inducing compounds (parquat and menadione), and on iron starvation. Although the importance of oxidative stress susceptibility is well established for Aspergillus virulence [103, 104], we chose to examine these phenotypes because it is well established that hosts produce reactive oxygen species in response to infection [105]. To test for the effects of iron starvation on fungal growth, MM was prepared without any iron source and supplemented or not with 200 µM of the iron chelators Bathophenanthrolinedisulfonic acid (4,7-diphenyl-1,10-phenanthroledisulfonic acid [BPS]) (Sigma-Aldrich) and 300 µM of 3-(2-pyridyl)-5,6-bis-(4-pyridylsulfonic acid)-1,2,4-triazine (ferrozine) (Sigma-Aldrich). For radial growth, isolates were grown in triplicate from 10⁵ spores and incubated at 37°C (except for the temperature test) for 5 days. Growth results in the presence of oxidative stress were expressed as ratio, dividing colony radial diameter (cm) of growth in the stress condition by colony radial diameter in the control (no stress condition).

Hydrogen peroxide tolerance
To test the viability of Aspergillus hyphae after exposure to hydrogen peroxide, we performed the XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt) (Sigma-Aldrich) assay as described by Henriet et al. [24], but with modifications. We obtained hyphae from each strain by incubating 1x10⁵ asexual spores/well in 96-well plates containing MM. After 16 h at 37°C, the medium was removed and the wells washed twice with PBS. Subsequently, 100 µl of minimal media supplemented or not (control) with different concentrations of hydrogen peroxide (1, 3 and 5 mM) were added to each well. Hyphal viability was assayed after 90 min of incubation (37°C) to avoid overgrowth of hyphae. One hundred microliters of PBS and 100 µl of XTT-menadione solution was added to each well, obtaining a final concentration of 200 µg / milliliter XTT and 4.3 µg / milliliter menadione and the plates were incubated for 2 h in the dark. After centrifugation (3,000 x g for 10 min), the supernatants were transferred to another plate and read at 450 nm in a spectrophotometer. Fungal damage was defined as the percent reduction in metabolic activity compared to that of controls without hydrogen peroxide (viability = 100%).

Antifungal susceptibility assays
Antifungal susceptibility testing for voriconazole (Sigma-Aldrich), posaconazole (Sigma-Aldrich), itraconazole (Sigma-Aldrich) and amphotericin B was performed by determining the minimal inhibitory concentration (MIC) according to the protocol established by the Clinical and Laboratory Standards Institute (CLSI, 2017). For caspofungin (Sigma-Aldrich) susceptibility, the radial growth in MM supplemented with different concentration of the drug was carried out similarly as described before (see Growth in the presence of different stresses). The results were presented as a ratio: growth in caspofungin(cm)/growth in the control/without caspofungin (cm).

Lastly, to determine the extent of phenotypic differences across all strains tested and all traits measured, we conducted principal component analysis. To do so, we first scaled (i.e., standardized) the data to account for variables that are measured in different scales (e.g., MIC, radial growth, virulence). We then conducted principal component analysis and examined each variable’s contribution to the variance along principal components using the R, version 3.5.2, packages factoextra, version 1.0.5 [106], and FactoMineR, version 1.41 [107].

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analyses were performed in R, version 3.5.2 (https://www.r-project.org/). Throughout the manuscript, comparisons were made between A. spinulosporus, A. quadrilineatus, A. latus, and/or A. nidulans (e.g., Figures 1, 3, and S5). For these comparisons, we used a multi-factor ANOVA. If the null hypothesis was rejected, we used a Tukey’s honest significant differences test to examine pairwise comparisons. To visualize these comparisons, we used violin plots with embedded boxplots (Figure 1B) or bar graphs (Figures 3 and S5). Violin plots depict the distribution of the underlying data; boxplot hinges depict the first, second, and third quartiles and whiskers extend to the largest value no greater than 1.5 times the interquartile range from the first or third quartile hinge; bar graphs depict the mean value measured across replicates and error bars depict the standard deviation across replicates. To conduct the principal component analysis (PCA) shown in Figure 1A, we first scaled all the data. To examine heterogeneity in Galleria mellonella killing curves, we employed a log-rank test. To compare Galleria mellonella killing curves solely across Aspergillus strains, we removed the negative control (PBS) prior to conducting statistical analysis. Other statistical analyses were automatically performed by publicly available software (e.g., substitution model testing for phylogenomic analyses was automatically performed by IQ-TREE).
Supplemental Information

Pathogenic Allodiploid

Hybrids of *Aspergillus* Fungi

Figure S1. The growth and appearance of the 7 clinical isolates is similar to those of *Aspergillus nidulans*. Related to Figure 1, Table 1 and STAR Methods. Clinical isolates were originally identified as *A. nidulans*. Growth in yeast extract, agar, and glucose (YAG) media and in minimal media supplemented with glucose reveals that the growth and appearance of the clinical isolates appears to be superficially similar to those of the reference A4 strain of *A. nidulans*. We also included *A. quadrilineatus* NRRL 201\textsuperscript{T} for comparative purposes.
Figure S2. The parental genomes of *A. latus* hybrid isolates exhibit considerable sequence divergence and have undergone little pseudogenization. Related to Figure 2. (Left) Homeologous gene pairs in *A. latus* genomes were identified using a reciprocal best blast hit approach. Sequence similarity of each gene pair was measured by the percentage of identical nucleotides per homeolog. Across the 7 *A. latus* hybrids, homeolog nucleotide sequence similarity is 92.85 ± 0.03% suggesting the parental genomes are ~7.15% diverged from one another. This level of divergence is on par with the sequence divergence observed between humans and lemurs (*Homo sapiens* vs. *Microcebus murinus* divergence measured using the same reciprocal best blast hit approach is 8.36%). (Right) The average percentage of pseudogenized homeologs in *A. latus* hybrid isolates is 11.67 ± 0.004%. The percentage of pseudogenized homeologs in each hybrid isolate was calculated by comparing gene lengths between homeologs. One gene in a homeolog pair was considered pseudogenized if their length was shorter (upper threshold of 80%) than the other gene in the homeolog pair.
A. spinulosporus phylogenomic data matrix

B. A. quadrilineatus-like phylogenomic data matrix

NRRL200

100

1.0E-4 substitutions / site

0.003 substitutions / site

1E-4 substitutions / site
Figure S3. The evolutionary histories of the two parental genomes of *A. latus* hybrid isolates are consistent with each other and support genome-wide instability in the *A. latus* type strain NRRL 200. Related to Figure 2 and Data S1. Evolutionary histories were reconstructed from data matrices of single-copy orthologous genes from either the (A) *A. latus* parental genome from *A. spinulosporus* and true *A. spinulosporus* strains or (B) *A. latus* parental genome from *A. quadrilineatus*-like species and true *A. quadrilineatus* strains. While the two topologies differ, application of approximately unbiased topology constraint tests showed that the two topologies were not statistically different from each other (p-value = 0.50 for both tests). These results are consistent with the hypothesis that the two parental genomes of *A. latus* share the same evolutionary history. The long branch of *A. latus* NRRL 200 inferred using the *A. spinulosporus* phylogenomic data matrix likely reflects potential genomic instability. Branch lengths represent substitutions per site. Bipartition support was assessed using 5,000 ultrafast bootstrap approximations.
A. latus NRRL 200T

A. latus MM151978

A. latus NIH

A. latus ASFU1710

A. latus ASFU1908

A. latus ASFU2033

A. latus MO46149

Percent genes belonging to Aspi or Aqua-like
Figure S4. Lack or very low levels of recombination between the parental genomes. Related to Figure 2 and Data S1. For each genomic contig of each A. latus hybrid, we examined the percentage of genes that came from one or the other parent (left column); exemplary contigs (one for each hybrid) that are putatively the result of a recombinant event are shown on the right column. Contig of origin analysis was conducted by examining the percentage of genes on long contigs (≥ 100 kb in length) from either the A. spinulosporus parent (Aspi; blue) or the A. quadrilineatus-like parent (Aqua-like; red). Contigs that are predominantly of A. spinulosporus are shown on the left side of the distributions and contigs that are predominantly A. quadrilineatus-like on the right side. We considered contigs that contained substantial percentages of genes from both parents to be putatively recombinant. For example, 2.67 ± 0.71% of A. latus hybrid contigs contain between 35% and 65% of genes from both parents. Exemplary contigs with evidence of recombination contain genes from the A. spinulosporus parent on one side and genes from the A. quadrilineatus-like parent on the other. Contigs are represented by black lines a key for contig length is shown to the right. For each contig, the entirety of the contig is depicted and the contig identifier is also provided. Genes on different strands of DNA are depicted either above or below the black line.
Figure S5. Phenotypic characterization of diverse infection-relevant traits among *A. nidulans*, *A. spinulosporus*, *A. quadrilineatus*, and *A. latus* hybrid isolate and strains.

**Related to Figure 3.** (A) Examination of cytokine production (i.e., macrophage response) to co-culture with no *Aspergillus* species, *A. nidulans* A4, *A. spinulosporus* strain 4060, and *A. latus* strain MM151978 and NIH revealed no significant difference in cytokine production with and without diphenylene iodonium (DPI). (B and C) Examination of minimum inhibitory concentration (MIC) in amphotericin B and itraconazole revealed no statistically significant difference between the various species. (D) Examination of susceptibility to caspofungin revealed statistically significant differences in some concentrations, which are shown and discussed in Figure 3. (E) Statistically significant differences in MIC of voriconazole among the various species ($\chi^2 = 14.44$, df = 3, $p = 0.002$; Kruskal-Wallis rank sum test). (F) Examination of the MIC for posaconazole reveals significant differences among the various species ($\chi^2 = 32$, df = 3, $p < 0.001$; Kruskal-Wallis rank sum test). (G) Examination of radial growth in the presence of the oxidative stressor menadione revealed significant differences among the three species at different concentrations of menadione (F(6) = 7.01, $p < 0.001$; Multi-factor ANOVA). (H) Statistically significant differences were observed in the growth of paraquat (see also Figure 3). (I) Significant differences in radial growth among *A. nidulans*, *A. spinulosporus*, and *A. latus* hybrids in the presence of H$_2$O$_2$ (F(6) = 3.00, $p = 0.009$; Multi-factor ANOVA). (J) No statistically significant differences were observed in iron starvation assays. (K) Significant differences were observed in the growth of the various species at different temperatures (F(6) = 15.65, $p < 0.001$; Multi-factor ANOVA). (M) Significant differences were observed in asexual spore internalization by macrophages between diploid (*A. latus* MM151978, *A. latus* NIH, and *A. nidulans* R21/R53) and haploid genomes (*A. nidulans* A4 and *A. spinulosporus* 4060) ($p < 0.001$;
Wilcoxon Rank Sum test). (N) Statistically significant differences were observed in NETosis. Shown here is the percentage of Human polymorphonuclear cells surviving co-culture with the fungus (see also Figure 3). (L) Statistically significant differences were observed in hyphal killing by macrophages (see also Figure 3). Additionally, significant differences were observed in the inhibition of fungal germination by macrophages among the various species (F(3) = 20.61, p < 0.001; Multi-factor ANOVA). For multi-factor ANOVAs, all pairwise comparisons were made using a Tukey Honest Significant differences test; for Kruskal-Wallis rank sum tests, pairwise comparisons were made using a Dunn’s test with multi-test correction using the Benjamini-Hochberg procedure. * represents p-values less than 0.05 but greater than 0.01; ** represents p-values less than 0.01 but greater than 0.001; *** represents p-values less than 0.001.
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Table S1. Genome assembly size, N50, and number of predicted genes. Related to Figure 1.

*N50: the contig size where 50% of the genome assembly is contained in contigs equal or larger than its size.
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Table S2. Number of predicted secondary metabolic gene clusters per genome. Related to Figure 1.
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Table S3. NCBI accession information for each sequenced genome.