ANNUAL REVIEWS

Annual Review of Phytopathology

Key Insights and Research Prospects at the Dawn of the Population Genomics Era for *Verticillium dahliae*

Jie-Yin Chen,[1] Steven J. Klosterman,[2] Xiao-Ping Hu,[3] Xiao-Feng Dai,[1,*] and Krishna V. Subbarao[4,*]

[1]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China; email: chenjieyin@caas.cn, daixiaofeng@caas.cn

[2]Agricultural Research Service, United States Department of Agriculture, Salinas, California 93905, USA; email: steve.klosterman@usda.gov

[3]State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Yangling 712100, China; email: xphu@nwsuaf.edu.cn

[4]Department of Plant Pathology, University of California, Davis, California 93905, USA; email: kvsubbarao@ucdavis.edu

Annu. Rev. Phytopathol. 2021. 59:31–51

First published as a Review in Advance on April 23, 2021

The *Annual Review of Phytopathology* is online at phyto.annualreviews.org

https://doi.org/10.1146/annurev-phyto-020620-121925

*Corresponding authors.

Keywords

Verticillium dahliae, biological characteristics, population structure, asexual, sexual, population genomics

Abstract

The genomics era has ushered in exciting possibilities to examine the genetic bases that undergird the characteristic features of *Verticillium dahliae* and other plant pathogens. In this review, we provide historical perspectives on some of the salient biological characteristics of *V. dahliae*, including its morphology, microsclerotia formation, host range, disease symptoms, vascular niche, reproduction, and population structure. The kaleidoscopic population structure of this pathogen is summarized, including different races of the pathogen, defoliating and nondefoliating phenotypes, vegetative compatibility groupings, and clonal populations. Where possible, we place the characteristic differences in the context of comparative and functional genomics analyses that have offered insights into population divergence within *V. dahliae* and the related species.

Current challenges are highlighted along with some suggested future population genomics studies that will contribute to advancing our understanding of the population divergence in *V. dahliae*.

1. INTRODUCTION

The genus *Verticillium* encompasses ten plant-associated species in the phylum Ascomycota, many of which cause destructive Verticillium wilts (48). These ten species display remarkable diversity in both morphology and host range; therefore, clearly delineated species boundaries are required for informed disease management purposes (52). Among the ten *Verticillium* species, *Verticillium dahliae* is the most destructive and widespread (39, 65). *V. dahliae* and most species of this genus infect plants via their roots and colonize the xylem tissue, a relatively unique niche among fungal plant pathogens. Eventually, the colonization of the xylem and disruption of water flow leads to the characteristic foliar wilting and chlorosis consistent with the restricted water flow in occluded vessels (39). In some instances, disease progression can cause defoliation of the entire plant (39, 65).

Since the public release of the first genomes of *V. dahliae* and *Verticillium alfalfae* (66), technological advances combined with lower costs for whole-genome sequencing have transformed our understanding of this genus and enabled the development of molecular markers associated with virulence traits and population structure (25, 129). Early comparative genomic analyses have demonstrated significant divergence in genome structure and sequence both within and among *Verticillium* species (17, 64, 66, 102, 105). Some genomic differences within *V. dahliae* underlie key virulence characteristics such as the races affecting tomato (24) or the defoliating (D) and nondefoliating (ND) phenotypes affecting cotton, olive, and okra (129). As in other areas of the biological sciences, the full potential of these technological advances to unlock a more comprehensive understanding of this genus and its evolutionary history is only now being revealed. This review highlights advances in our understanding of *V. dahliae* and Verticillium wilt and sets the stage for additional major lines of research that can be addressed through population genomics approaches.

2. BIOLOGICAL CHARACTERISTICS OF *VERTICILLIUM DAHLIAE*

Key biological characteristics of *V. dahliae*, the type species of genus *Verticillium* (48), are highlighted in **Figure 1** and serve as a reference to understanding a variety of phenotypes and their underlying genetic bases. The establishment of a database of genomes of *V. dahliae* isolated from diverse hosts and locations has hastened the uncovering of the genomic basis of biological characteristics of other *Verticillium* spp.

2.1. Morphology

The name *Verticillium* is derived from the unique branching of conidiophores that forms whorls capped with flask-shaped, pointed phialides bearing terminal conidia (54). *V. dahliae* colonies are white but progressively darken owing to the production and maturation of melanized resting structures, which include microsclerotia. The conidia are hyaline, smooth-walled, and nonseptate, with round to oval apices (48). Among *Verticillium* species, the resting structures produced vary considerably (48), with microsclerotia forming a key morphological characteristic differentiating *V. dahliae* from other species within the genus. In *V. alfalfae*, the resting structures are dark resting mycelia rather than the discreet microsclerotia of *V. dahliae* or the elongated microsclerotia of

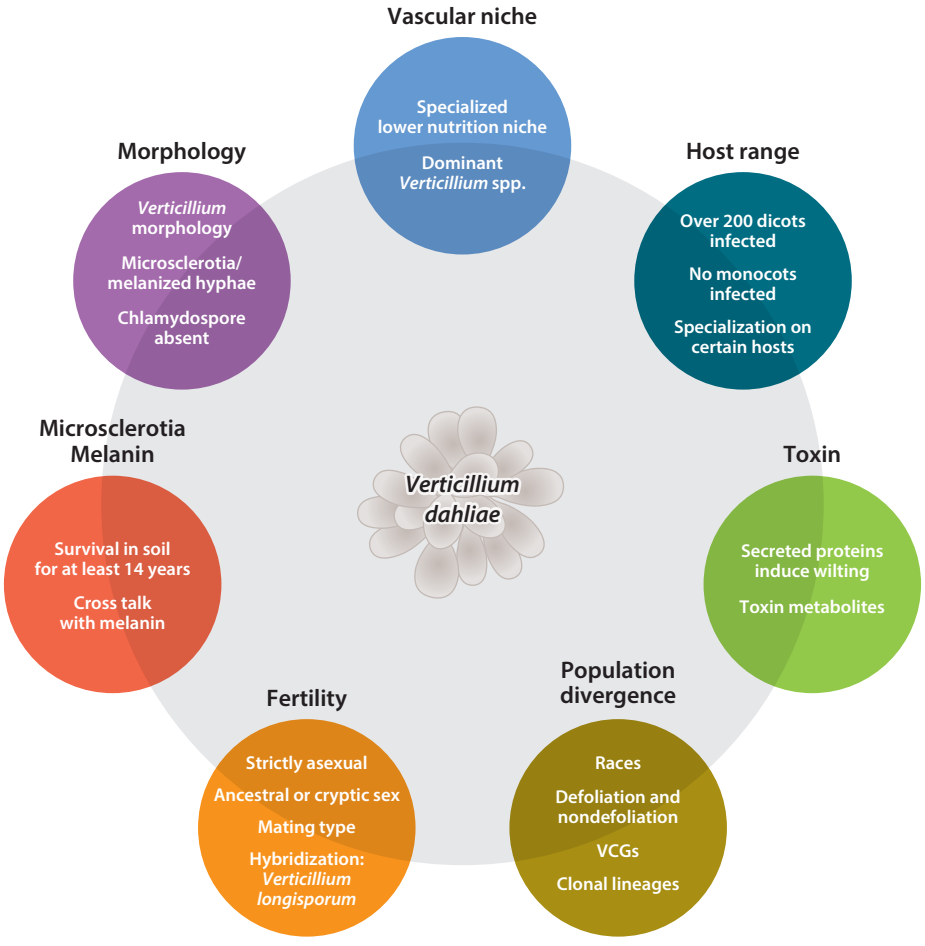

Figure 1

Main biological characteristics of *Verticillium dahliae*. Beginning at the top and moving clockwise, these include its niche in colonizing the plant vascular system, broad host range, production of toxin-like substances that influence disease symptoms, divergence of the population, reproductive strategy or fertility, production of long-lived microsclerotia as resting structures, and morphological characteristics unique to the species. Abbreviation: VCGs, vegetative compatibility groups.

Verticillium longisporum. In contrast, *Verticillium nubilum* produces chlamydospores exclusively, rather than microsclerotia, whereas *Verticillium tricorpus* produces microsclerotia, resting mycelia, and chlamydospores (48).

2.2. Microsclerotia and Melanin

V. dahliae is difficult to control, owing in part to the production of heavily melanized microsclerotia that survive in the soil for 14 or more years (127). Melanin is composed of polymerized phenolic or indolic compounds and offers protection against temperature extremes, enzymatic lysis, nutrient deprivation, ultraviolet radiation, and fungicides (12). Melanin production is tightly coupled

with later stages of microsclerotial development in *V. dahliae* (64), and its appearance implies microsclerotial maturation (34, 128). However, the synthesis of melanin is uncoupled from microsclerotia formation, as demonstrated in melanin-deficient mutants of *V. dahliae*, which produce microsclerotia (123). Although melanin-deficient mutants are pathogenic (68), certain metabolic precursors and signaling pathways that affect melanin production appear to have a role in virulence (37, 123). This role, especially the overlapping effects of signaling pathways or biochemical precursors of melanin biosynthesis regulating virulence, is currently being elucidated (37). Additional genes required for microsclerotia development in *V. dahliae* are also important for virulence, suggestive of a complex cross talk between signaling processes that govern these functions (64, 110).

2.3. Broad Host Range

Among dicots, the host range of *V. dahliae* is very broad, although it exhibits host preference in some host plants, such as cotton and mint (39, 65). Remarkably, some populations of *V. dahliae* act as both pathogens and endophytes (125). In contrast, other *Verticillium* species exhibit clear host specialization (44). For example, neither *Verticillium klebahnii* nor *Verticillium isaacii* causes disease on lettuce, eggplant, tomato, and spinach, whereas *V. dahliae* causes wilt on each of these hosts (44). Other species exhibit host specialization, such as *V. longisporum* with brassicaceous hosts (27, 77), *V. alfalfae* with alfalfa (48), and *V. tricorpus* with tomato (53). Some *Verticillium* species may have only limited distribution, exemplified by *Verticillium zaregamsianum*, which has been associated only with lettuce in Japan (48). A much-debated question has been whether the distribution of individual *Verticillium* species is delimited by host range by confinement or encounters with specific hosts or by adaptation to a specific ecological niche where the environment and host differ. The answers likely lie in the combination of these attributes.

Questions also remain as to why *V. dahliae* colonizes only the root cortex of monocots commonly grown in rotation with dicotyledonous crops but causes no symptoms in these hosts (9, 124). Potentially, this may involve structural and biochemical differences in the endodermis and xylem between monocots and dicots. Nevertheless, the production of microsclerotia in monocotyledonous crops augments the soil inoculum density and therefore is a significant concern in the management of Verticillium wilt (124).

Recent key advancements will further guide research into the genetic basis of host range. Genome comparisons among nine sequenced *Verticillium* species, excluding the hybrid species *V. longisporum*, show extensive genomic rearrangements and gene losses between species that potentially account for the differences in host range (105). However, comparative analyses of the genomes of *V. tricorpus* and *V. dahliae* documented a similar repertoire of virulence-related effectors in both, and, surprisingly, *V. tricorpus*, the species with the narrowest host range and attenuated virulence, has an expanded set of carbohydrate-active enzymes (CAZymes) relative to *V. dahliae* (102), and some CAZymes are important in virulence. Thus, the CAZyme repertoire may not correlate with the host range of individual *Verticillium* species but has offered insights into the complexities of correlating sequence attributes from individual *Verticillium* genomes with host range characteristics. Nevertheless, because *Verticillium* species exhibit clear host range differences, comparative population genomics of dozens of isolates within and between species is likely to demonstrate specific sequence differences that determine the host range of individual species. For example, comparison of broad host range *V. dahliae* and those species with limited host range, e.g., *V. alfalfae*, *V. klebahnii*, and *V. isaacii* (48, 53), is a possible strategy to decipher the genetics that govern host range.

2.4. Toxins That Cause Leaf Wilting

The term toxin in *V. dahliae* may be defined as a secreted biomacromolecule that affects symptom development or severity. Some toxins can induce host cell death and are required for virulence in *V. dahliae* (39). The most well-known toxin is the necrosis- and ethylene-inducing protein (VdNEP) that induces wilting in cotton leaves (122) and cell death in tobacco and is required for virulence on tomato and *Arabidopsis thaliana* (99, 133). An examination of the exoproteome of *V. dahliae* induced by cotton revealed hundreds of extracellularly secreted proteins particularly enriched in CAZymes that may disrupt the structural plant defenses induced by the infection (18). In addition, several members of the exoproteome play critical roles in pathogenicity by manipulating plant immunity. The various roles of the members of the exoproteome have previously been delineated (26, 42, 70, 91, 130). *V. dahliae* endoglucanases (VdEG1 and VdEG3, which belong to glycoside hydrolase family 12) display cellulase activity that is required for virulence and act as elicitors to induce immunity that can be suppressed by the carbohydrate-binding module 1 (CBM1) domain protein to promote pathogenicity (42). There is some evidence to suggest that the metabolites of *V. dahliae* contribute to virulence, although it is not thoroughly convincing because it is difficult to ascribe functions to only the secreted metabolites, separate from the fungus and its secretome (69, 129, 131). The secreted proteins are mainly intrinsic toxins causing leaf wilting, with two primary roles: disrupting host physical barriers via enzymatic activity and manipulating host plant immunity as effectors. These two activities synergistically contribute to the virulence of *V. dahliae* on the host.

2.5. Vascular Niche Adaptation

V. dahliae is xylem limited during a substantial portion of its disease cycle, an attribute that disrupts water and mineral transport, resulting in severe wilting and possible death of infected plants (81). *V. dahliae* multiplies profusely in the xylem and spreads vertically within the vessels via internally produced conidia and horizontally between vessels (39, 119). To disrupt the robust physical barriers composed of macromolecular polysaccharides, *V. dahliae* requires sugar-cleaving enzymes to degrade host cell walls, which consist mainly of cellulose microfibrils embedded in a matrix of hemicelluloses, pectic polysaccharides, and glycoproteins (15, 39). Because *Verticillium* species occupy plant xylem, genomic comparisons of this genus to nonvascular fungal pathogens provide a means to study how *V. dahliae* evolved this unique niche adaptation. CAZymes, especially the families representative of the pectinolytic machinery, are significantly enriched in *V. dahliae* relative to nonvascular pathogens that may collapse the endodermis directly and contribute to its adaptation to the plant xylem (66). Moreover, a glucosyltransferase (GT) ortholog was likely transferred horizontally from α-proteobacteria into *V. dahliae*, *V. alfalfae*, and *Fusarium oxysporum* but not into other nonvascular *Fusarium* pathogenic species and may be necessary for xylem colonization (66). However, orthologous GT sequences have been identified in at least two other broad host range fungal species (64), and thus these orthologs may have a conserved function in virulence to crack the endodermis and facilitate xylem colonization. Future searches attempting to uncover the vascular niche specialization in *V. dahliae* may also consider comparisons of other *Verticillium* species that may not occupy this specific niche. *V. nubilum* colonized only the surface of a potato tuber (84), and thus may also be a candidate species for such comparisons to uncover the genetic basis of vascular niche adaptation. Further work corroborating whether *V. nubilum* is limited to colonization of potato surfaces or whether this species is truly defective in its ability to colonize the xylem is warranted. Because the xylem is relatively limited in nutrients, comparative analyses to decipher niche adaptation and metabolomics and transport machinery among species may prove fruitful.

2.6. Does *Verticillium dahliae* Have an Extant Sexual Phase?

Most population genetic studies indicate that *V. dahliae* has a clonal population structure (6, 8, 73, 74, 92, 107). A cluster analysis of a global collection of *V. dahliae* isolates (107) differentiated them into seven independent evolutionary lineages. Furthermore, because sexual structures have not been observed under laboratory conditions or in nature, *V. dahliae* is considered an exclusively asexual fungus (107a). Nevertheless, two different mating-type loci, *MAT1-1* and *MAT1-2*, have been identified in *V. dahliae* (116). The *MAT1-1* idiomorph is observed in approximately 1% of a global collection of isolates with *MAT1-2* present in the rest (73, 107, 115). The disproportionate frequencies of the two *MAT* idiomorphs ensure that the two types seldom encounter each other, which is exacerbated by *V. dahliae*'s soilborne, nonmotile lifestyle and limited capacity to disperse (103) unless facilitated by human activities (6, 107). Furthermore, recent genomic analyses revealed extensive chromosomal rearrangements occurring in the different *V. dahliae* isolates (17, 23). The magnitude of the rearrangements could potentially prevent pairing of homologous chromosomes during meiosis (63). For these reasons, *V. dahliae* is mostly considered to be a clonally propagating organism (103).

Interestingly, however, recent studies of *V. dahliae* indicate that the potential for cryptic sexual reproduction is still an open question (4, 5, 51, 73, 107a, 116). Within the Ascomycota, the two *MAT* loci and other sex-related genes play a central role in sexual compatibility and fruiting body formation (35). Nearly all isolates of *V. dahliae* contain either the *MAT1-1* or *MAT1-2* idiomorph and clearly exhibit heterothallism (115, 116). Various genes involved in the regulation of sexual reproduction were expressed by *V. dahliae* in laboratory-grown cultures (107a). These conserved genes in *V. dahliae* are analogs of those within the Ascomycota that mediate compatibility and mating (73, 107a). Further evidence that *V. dahliae* has a cryptic or ancestral sexual cycle based on three separate hybridization events that shaped the evolution of the hybrid species *V. longisporum*. In this hybrid species, *V. dahliae* was apparently one of the parents in *V. longisporum* lineages A1/D2 and A1/D3 (27, 48, 50). Whether *V. dahliae* still retains the ability to sexually reproduce and whether the clonal populations arose by recombination remain unclear. Answers are anticipated in the population genomics era, during which the extent and significance of recombination can be investigated in large sets of sequenced data.

2.7. Diversity in *Verticillium dahliae*

Complexity within *V. dahliae*'s population structure is agriculturally significant and has been a long-standing research theme that dates back more than one hundred years. Based on the tools used to study population biology, various descriptions have emerged. Currently, *V. dahliae* can be classified into at least three pathogenic races based on the response of host differentials (32, 117, 118), and into D and ND strains by the presence or absence of defoliation in certain hosts following infection (10, 57, 101, 129). The species was further differentiated into clonal lineages or vegetative compatibility groups (VCGs) by various genetic diversity analyses employing molecular markers or by the compatibility of nutritional deficiency mutants (14, 56, 73, 107), respectively. The subpopulation structure of *V. dahliae* and how genomics can improve the understanding of this structure are the focus of the next section.

3. POPULATION STRUCTURE IN *VERTICILLIUM DAHLIAE*

Clarification of *V. dahliae*'s population structure continues to yield information that contributes to finer-scale structures, evolutionary relationships within subspecies groups, and information that can be deployed in crop management. Much of the data for this are being acquired through the

Verticilli-Omics Project (**https://db.cngb.org/Verticilli-Omics**), which to date has sequenced the genomes of >3,500 *V. dahliae* strains from diverse host plants/continents.

3.1. Race

Resistance to Verticillium wilt in tomato was first described in 1951, and this resistance was attributed to the *Ve* locus (100). *Ve*-mediated resistance was overcome by race 2 of the pathogen soon after the deployment of cultivars with resistance (1, 32, 41). This was effectively demonstrated using tomato cultivars Pakmor (race 1 resistant) and Earlypak 7 (susceptible) as host differentials that informed the replacement of race 1 with race 2 in California tomato fields (41). In 2012, the corresponding gene that confers avirulence in *V. dahliae* race 1 isolates, *Ave1*, was identified; it encodes a secreted cysteine-rich effector (25). Similarly, in lettuce, two races of *V. dahliae* were discovered using differential cultivars, LaBrillante (race 1 resistant) and Salinas (susceptible) (46, 98, 118), and an *Ave1* orthologous sequence was identified in the race 1 isolates from lettuce (90). Analysis of the *Ve* locus in tomato revealed two homologous sequences, *Ve1* and *Ve2*, that are inversely oriented, each encoding leucine-rich repeat receptor–like proteins (60). Subsequently, *Ve1*, but not *Ve2*, was determined to confer resistance to race 1 of *V. dahliae* (40). *Ve1* was further demonstrated to play a dual role in the pathogen defense and enhanced root growth in tomato. This increased root biomass allowed plants carrying this gene to outgrow infection by *V. dahliae* (76). Similarly, the *Ve* locus from lettuce also segregates with resistance to Verticillium wilt (46). However, unlike tomato, lettuce possesses three *Ve* paralogous sequences at this locus. One of these alleles, *LsVe1L*, correlated with resistance to race 1 of *V. dahliae* based on field testing of resistant and susceptible lettuce lines (49).

Since the discovery of these two races in *V. dahliae* on tomato and lettuce (1, 32, 41, 118), a third race has been described attacking tomato in Japan (117). The third race was found after deployment of race 2 resistance from wild tomato *Solanum neorickii*. The single dominant gene conferring resistance to race 2 (named *V2)* from *S. neorickii* was bred into rootstock cultivars 'Aibou,' 'Ganbarune-Karis,' and Back Attack (16, 117). Some isolates of *V. dahliae* within the race 2 population compromised the resistance in these cultivars and were designated as race 3 (117). The *V2* gene in tomato was identified only recently (117), and an analogous gene that confers resistance to race 2 in lettuce has not been detected despite extensive screening of the germplasm (46). Some cultivars of lettuce, however, do possess partial race 2 resistance (97).

Using a comparative population genomics approach, a 277-kb sequence specific to race 2 isolates of *V. dahliae* was identified that included two candidate effector genes (16). One of these genes, designated as *Av2*, was highly expressed in planta and encoded a secreted effector protein. *Av2* conferred resistance in tomato cultivars otherwise susceptible to race 3, suggesting that *Av2* is the avirulence factor in race 2 isolates on tomato. Although *V. dahliae* has diverged into races that possess or lack either *Ave1* or *Av2*, isolates that possess both *Ave1* and *Av2* or lack both cannot be entirely ruled out (16, 117).

Both races 1 and 2 of *V. dahliae* are present worldwide, although their distribution and relative frequency are influenced by geography and crop (8). For example, in Lebanon, of the 125 isolates collected from potato, only 12.1% of those were race 1, and of 78 isolates from olive, 55% were race 2. In tomato, it is well documented that race 2 supplanted race 1 isolates following the introduction of race 1 resistance in North America and other locations where tomatoes are grown (32, 41). In the case of lettuce, race 1 is dominant in central coastal California (46, 90, 118). However, cultivars with race 1 resistance have not yet been deployed, and hence the population is still dominated by race 1. Isolates from infested spinach seed planted in California show an increased proportion of race 2 (108). Because all spinach seed is imported from outside California, race

distribution may be skewed toward race 2 in the future within this region and elsewhere. It therefore remains critically important for lettuce breeding programs to develop race 2 resistance (97, 98).

Ve orthologous sequences are highly conserved across plant species (109) and confer resistance to Verticillium wilt in diverse species, including strawberry (19), sunflower (38), mint (121), and cotton (104) as well as tomato (100) and lettuce (46), as discussed above. However, the race classifications in *V. dahliae* on some hosts are ambiguous owing to the lack of genetically defined host differentials. For instance, Verticillium wilt is the most serious fungal disease in cotton, especially in China (132), and up to five races were previously identified on the basis of virulence patterns on several cultivars (85–87). However, these results have been neither universally accepted nor validated, and the resistance appears to be quantitative (69). Thus, in some hosts, *V. dahliae* cannot be clearly parsed into separate races, as qualitative resistance responses are lacking when the host is challenged with avirulence factors or pathogen isolates (109).

3.2. Defoliating and Nondefoliating

The symptoms of Verticillium wilt caused by *V. dahliae* vary among different hosts, including leaf wilting, chlorosis, stunting, vascular discoloration, necrosis, and vein clearing (39). In certain hosts, strains of *V. dahliae* can cause complete defoliation and hence are classified as D, and others that do not cause defoliation are classified as ND (10, 55, 101). Surprisingly, despite *V. dahliae* being a broad host range pathogen (39, 52), only a few hosts exhibit defoliation symptoms following infection by the D strains (74). The defoliation symptoms were first described on cotton (Mississippi, USA) in the 1950s, where plants of the susceptible cultivars shed all the leaves and bolls (88). Since then, only a few other hosts such as olive (11), poplar (61), and okra (129) have been reported to exhibit defoliation following infection by the D strains. Plant species exhibiting the defoliation phenotype are woody with high stem lignification, and the defoliation phenotype seems absent in herbaceous plants (such as tomato, potato, lettuce, etc.), suggesting that the defoliation symptom may be specific to some woody hosts of *V. dahliae*. The question of why the D phenotype is observed only in woody hosts has puzzled and continues to puzzle the research community. This natural phenotype is seldom reproduced in either the greenhouse or the laboratory, as immature cotton seedlings without mature euphyllia fail to display defoliation when challenged with the most aggressive D strains (17, 129).

Verticillium wilt represents a serious threat to a plant's longevity, with the most common symptoms of infection being early leaf chlorosis and defoliation (13, 62, 80, 120). Analysis of gene expression in susceptible hosts indicates that infection by *V. dahliae* causes the plant to divert resources away from the chlorophyll synthesis for purposes of defense (64a) and that the diversion ultimately induces leaf necrosis and defoliation (39). In the 1960s, there was speculation that a toxin was the basis for leaf necrosis in *V. dahliae*–infected plants (111), although many secreted *V. dahliae* proteins have roles as toxins, as inducers of host immunity, or in causing programmed cell death (18, 26, 64). Additionally, leaf abscission by *V. dahliae* disrupts the hormonal levels in hosts (29). Ethylene accumulation was strongly correlated with cotton defoliation, which was higher in plants inoculated with the D strain than in plants inoculated with the ND strain (126). The disruption of abscisic acid and gibberellic acid synthesis can also accelerate defoliation in cotton (75). However, fluctuations of hormones are also caused by either endogenous or exogenous sources because hormones are also synthesized by microorganisms (114). In response to *V. longisporum*, the receptor of the plant defense hormone jasmonic acid (CORONATINE INSENSITIVE1) negatively regulated the symptom of early leaf senescence in *A. thaliana* (94), implicating the hormone signals associated with leaf senescence.

Of central importance to the management of Verticillium wilt is the rapid and consistent detection of *V. dahliae* pathotypes in the field to prevent the spread of D and ND strains to new areas (83). Pathotype-associated random amplification polymorphic DNA (RAPD) markers specific to D and ND strains were developed (82), as was a nested polymerase chain reaction (PCR) for detection of early infections by the two pathotypes in olive (72) and soil (83). The use of RAPD markers was fully coincident with the pathotype assignment in biological or vegetative compatibility tests (47, 82) and facilitated an understanding of the distribution of Verticillium wilt caused by D and ND strains (95).

Genome analyses of the D strain (Vd991 from cotton) revealed the presence of a lineage-specific region (G-LSR2, which encodes 23 genes) that was absent in the genomes of the ND strains (VdLs.17 and JR2 isolates from lettuce and tomato, respectively). This region was likely transferred horizontally from the Fusarium wilt pathogen of cotton, *Fusarium oxysporum* f. sp. *vasinfectum*, another soilborne fungus that also colonizes the plant vascular niche (17). G-LSR2 also exhibited linkage to the RAPD marker specific to the D pathotype. The LSR2 region in the D strain encodes seven genes (named *VdDfs*, *VdDf1–VdDf7*) (129), and these belong to a secondary metabolite gene cluster. Genes *VdDf5* and *VdDf6* share homology with polyketide synthases, whereas VdDf7 shares homology with proteins involved in the biosynthesis of *N*-acylethanolamine (NAE12:0), a compound that upregulates cotton fatty acid amide hydrolase to enhance the sensitivity of cotton to *V. dahliae* and disturbs the abscisic acid balance (129). G-LSR2 represents a common genetic explanation for the defoliation phenotype in *V. dahliae* (17, 129), but it is unclear whether the broad host range pathogen *V. dahliae* has evolved or adapted for host specificity via the acquisition of G-LSR2 to cause defoliation only on certain hosts.

3.3. Vegetative Compatibility Groups and Clonal Populations

Frequently, the hyphae of some fungal isolates of a species are able to fuse with each other (anastomose) to form a stable heterokaryon. In this context, compatible (anastomosing) isolates are assigned to the same VCGs, whereas those that do not form a heterokaryon are assigned to a different group (59, 96). Initially, the *V. dahliae* population (86 strains from 14 countries) was divided into 16 VCGs (89). Subsequently, 15 of the 16 VCGs were collapsed into four (VCG1–VCG4) via utilization of nitrate-nonutilizing (nit) auxotrophic mutants that arise naturally in the culture medium, which contains chlorate. The four VCGs were further divided into subgroups, VCG1A, VCG1B, VCG2A, VCG2B, VCG3, VCG4A, and VCG4B (58). Since then, two new VCGs have been added (14, 112). Most of these groups are distributed among many hosts, with a few groups restricted to certain hosts only (14, 112).

Although VCGs have been useful in characterizing the population structure of *V. dahliae*, their correlation to virulence and other characteristics of *V. dahliae* has been tenuous. VCGs have not always consistently correlated with molecular genotyping using codominant markers (14, 31, 71). An example of the inconsistency is that the VCG1A and VCG1B isolates from California, Spain, and Greece shared identical haplotypes within the same VCG in contrast to VCG2 and VCG4 isolates, which shared different haplotypes. For instance, VCG2 isolates fell into multiple haplotypes, as shown by single-gene phylogenetic analysis (71). Likewise, five VCG4 isolates fell into four mitochondrial haplotypes, one of which was identical to the largest VCG2 grouping, indirectly suggesting that molecular grouping may not correlate with VCGs (71). Limited analyses have been performed to investigate the population structures within VCGs using molecular markers developed for specific VCGs, even though molecular diagnostics specific to individual VCGs are available (21, 30, 79).

In addition to VCGs, the mostly clonal nature of the populations in *V. dahliae* was further structured using molecular markers and genome resequencing (3, 4, 36, 71, 73, 107). Application

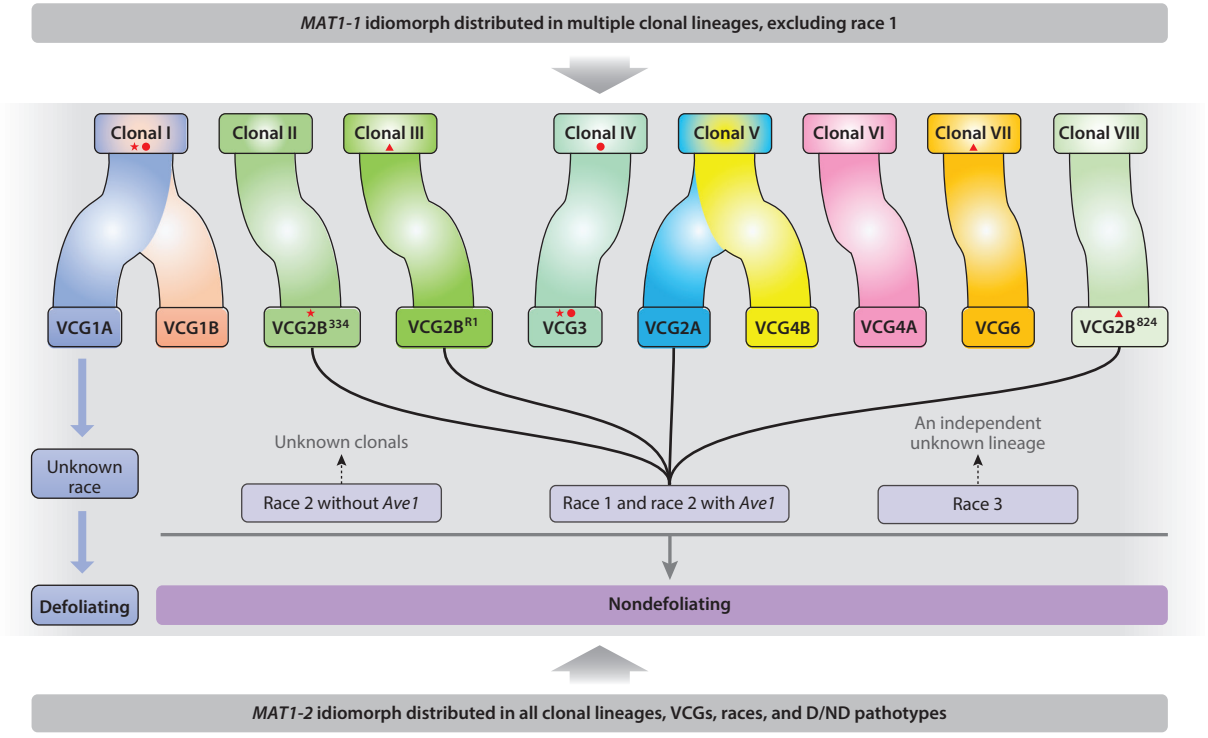

Figure 2

The population structure of *Verticillium dahliae*. Eight clonal lineages of *V. dahliae* and the distribution of vegetative compatibility groups (VCGs), races, mating-type genes, and defoliating (D) or nondefoliating (ND) pathotypes within the lineages. The relationships between the population entities were constructed based on the information from the following references (22, 28, 47, 57, 67, 71, 73, 74, 93; Verticilli-Omics Project, unpublished data). VCG designations are as shown in Milgroom et al. (73).

of amplified fragment-length polymorphism (AFLP) markers and conserved DNA regions clustered the strains into groups that reconciled with the VCGs (20), but this could be an artifact of dominant makers such as AFLP. Analysis of the genetic diversity using microsatellite markers in several studies repeatedly provided evidence that *V. dahliae* from different hosts and geographical regions fall into several lineages (4, 7, 33, 57, 73). The largest collection of isolates from 26 hosts and 10 countries assigned 1,100 isolates into at least seven major lineages (107). Genotyping by sequencing also confirmed that a relatively small population of 141 isolates from diverse geographic and host origins diverged into eight lineages (73), as seen in **Figure 2**. Thus, strains from within the same lineages exhibit low genetic diversity, and strains from different lineages display high genetic diversity.

Although some clonal groups defined by molecular markers displayed a high association with specific VCGs (7, 20, 33, 57, 74), other groups show that VCGs do not always align with the markers (57; see Section 4). In addition, chromosomal rearrangements are an important mechanism in the evolution of asexual pathogens such as *V. dahliae* (23), but chromosomal rearrangements do not provide genetic evidence to differentiate the clonal divergence. In other words, chromosomal rearrangements can occur in strains from within the clonal lineage or from different clonal lineages.

For instance, *V. dahliae* ND strain JR2 and D strain Vd991 belong to different clonal lineages, and, comparatively, both genomes show chromosomal rearrangements (17). However, JR2 and VdLs.17 belong to the same clonal lineage and also show extensive chromosomal rearrangements (23). Potentially, host or environmental factors could play critical roles in shaping population divergence, but evaluation of the global isolate collection has revealed a weak correlation between clonal divergence and geographical distribution of the strains (107).

4. INCONGRUENCIES IN THE POPULATION STRUCTURE OF *VERTICILLIUM DAHLIAE*

Characteristics of race, VCGs, and mating types of *V. dahliae* and how each maps within the clonal groupings of *V. dahliae* are complex (**Figure 2**). Clarifying the relationship between the genetics of strains versus these varied genotypes is important for understanding population structure and evolution within *V. dahliae*.

4.1. Clonal Populations versus Vegetative Compatibility Groups

V. dahliae propagates asexually, but the levels of genetic differences observed within and between *V. dahliae* populations are often incongruent with the expected molecular signatures of a strictly asexually reproducing population (4, 73, 103, 108). If VCGs evolved independently and populations remained strictly asexual, VCGs would not be anticipated to occur in multiple independent clonal lineages, as shown in **Figure 2**. VCG4A is wholly contained within a specific clonal population (e.g., VCG4A in lineage VI) (**Figure 2**). However, this observation is not always true in most VCGs (57) because the isolates grouped into any one clonal group belong to several VCGs (**Figure 2**) (57, 73), which suggests that *V. dahliae* strains from the same clonal population are also incompatible. Haplotypes determined by, e.g., microsatellite markers and mitochondrial and intergenic spacer region sequences have also confirmed that the clonal populations do not always correspond to VCGs in *V. dahliae* (57, 71).

4.2. Races versus Population Structure

The *V. dahliae* ND and D strains are correlated with race 1 and race 2, respectively (47, 129), but this correlation is not complete. For example, strain VdLs.17 exhibits the ND pathotype but does not encode *Ave1*, which characterizes race 1 strains (17, 25). In the relationship between race and clonal groups or VCGs, race 1 isolates (including some race 2 isolates and those in which *Ave1* and *Av2* co-occur) cluster in at least two different clonal groups and VCG2A and VCG2B (57, 74, 93; Verticilli-Omics Project, unpublished data) (**Figure 2**). With the identification of additional resistance genes, race 2 strains may be expected to be distributed in several clonal lineages, and race 3 may form an independent clonal group (Verticilli-Omics Project, unpublished data). However, the classification of races in *V. dahliae* is more complicated than assumed over the past 50 years, as demonstrated by the recent partition of race 2 from tomato in Japan into two different races (117).

4.3. Defoliating and Nondefoliating versus Population Structure

The much-studied relationship of D and ND pathotypes to VCGs confirmed the distribution of D pathotype only in VCG1A and its convergence into an independent clonal population; however, the ND pathotype is widely distributed in other VCGs or clonal groups (22, 28, 57, 67, 74). Except for the relationship of D and ND pathotypes with races as described above, the D pathotype may

belong to an as yet uncharacterized race, because these strains lack *Ave1* and *Av2* and also do not belong to race 3 (Verticilli-Omics Project, unpublished data).

5. INCONCLUSIVE GENETIC EVIDENCE TO SUPPORT EITHER STRICT ASEXUAL OR SEXUAL REPRODUCTION IN *VERTICILLIUM DAHLIAE*

Currently, several lines of evidence support the mainstream view that *V. dahliae* propagates asexually; for example, the mating-type distribution is heavily skewed in favor of *MAT1-2* (73, 108), extensive genomic rearrangements may prevent meiosis (23), and repeated sampling from different hosts and continents has revealed little genetic variation within clonal groups (43, 73). Although still speculative, chromosomal rearrangements have been projected as a main driving force in the divergence of clonal groups (23, 103), a concept that may be supported if isolates from the same clonal group share identical chromosome structures. Although the chromosomal rearrangements (especially translocations) significantly prevent the chromosome pairing during meiosis that should reduce or destroy fertility (103), several sexually reproducing fungi exhibit considerable chromosome polymorphisms caused by translocations (63, 134). Studies to examine the production of sexual structures in vitro are challenging at best, considering the limit of our knowledge on the induction of sex. Some fungi exhibit sexual reproduction only in specific locations or at particular times within their hosts and may also have specific nutritional requirements for it to occur (113). One remarkable example of this is the discovery of sexual reproduction in the fungal pathogen *Aspergillus fumigatus*, in which the sexual stage arose six months after incubation under specialized conditions in the dark (78).

Investigations into the clonal distribution of the *MAT1-1* idiomorph may shed light on sexual reproduction in *V. dahliae* (**Figure 3**). If the *MAT1-1* and *MAT1-2* idiomorphs are unequally distributed among clonal groups, it may indicate that *V. dahliae* had an ancestral sexual phase and that the observed population structure is due to strong selection of successful asexual genotypes (**Figure 3*a***). Conversely, if the *MAT1-1* and *MAT1-2* idiomorphs are equally distributed in different clonal groups (i.e., *MAT1-1* idiomorph accompanies *MAT1-2* idiomorph during the course of the evolution and the idiomorphs are compatible), sexual reproduction may be more prevalent (**Figure 3*b***). Identical chromosomal structure, a high proportion of orthologous genes in clonal lineages, and equilibrium in MAT1-1 and MAT1-2 idiomorphs are characteristics that are conducive to sexual reproduction (**Figure 3*c***). Although rare isolates of the *MAT1-1* idiomorph have been characterized to date, analyses of the genetic diversity of *V. dahliae* isolates with a broad geographic distribution and wide host range revealed that the isolates carrying the *MAT1-1* idiomorph are distributed in several lineages (108; Verticilli-Omics Project, unpublished data), indicating that *V. dahliae* preserved the ability for sexual reproduction to mediate the evolution of the current clonal groups (**Figure 2**) and is potentially still contributing to the diversification of the gene pool.

Horizontal gene transfer (HGT), together with chromosomal rearrangements, has substantially shuffled fungal genomes and contributed to speciation (2, 23, 25, 26, 45). Evidence for HGT is found in *V. dahliae* (17, 25, 66, 106), which has potentially contributed to the divergence of populations in *V. dahliae*. There is strong evidence to suggest that *V. dahliae* acquired *Ave1* from plants to form race 1 (25) and isolates from cotton obtained a gene cluster from *Fusarium oxysporum* f. sp. *vasinfectum* to evolve the D strains (129). However, HGT does not adequately explain the observed distribution of race 1 and D pathotypes in different clonal populations (57, 74, 93; Verticilli-Omics Project, unpublished data).

Because of the evidence for a mixed reproductive mode, it remains important to ascertain the conditions that favor or are required for sexual recombination in present-day *V. dahliae*. It is also critical to understand the historical/evolutionary conditions that have led to *V. dahliae*

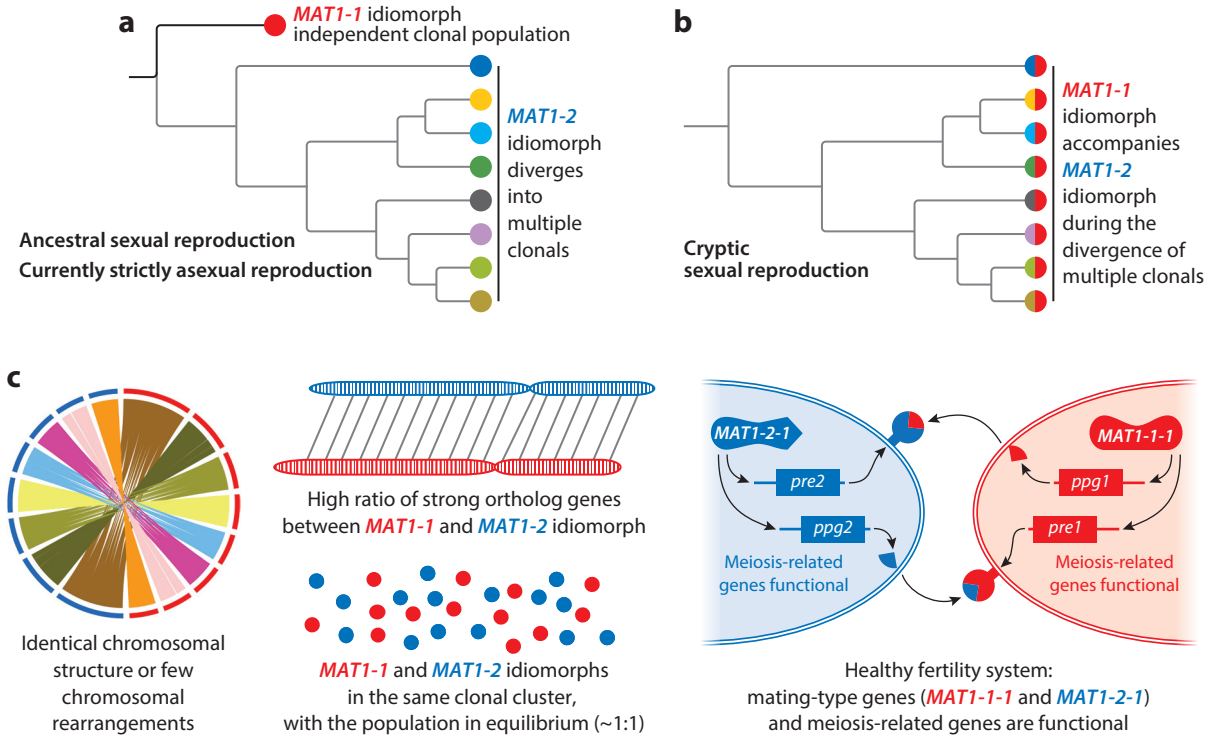

Figure 3

Tracking the theoretical distribution of mating-type loci in *Verticillium dahliae* as potential evidence of sexual or asexual reproduction. (*a*) *MAT1-1* and *MAT1-2* idiomorphs diverge independently in clonal groups. In this situation, *V. dahliae* may have undergone ancestral sexual reproduction but currently propagates strictly asexually. (*b*) *MAT1-1* idiomorph accompanies *MAT1-2* idiomorph in the same clonal lineage during evolution. Under this scenario, cryptic sexual reproduction is not detected in the current population. (*c*) A likely successful strategy to select *MAT1-1* and *MAT1-2* idiomorphs to study sexual reproduction in *V. dahliae*.

reproduction becoming so overwhelmingly asexual. Population genomics likely holds the keys to answering such questions.

SUMMARY POINTS

1. *V. dahliae* colonizes the specialized niche of the plant vascular system and produces long-lived resting structures of microsclerotia but not chlamydospores or dark resting mycelia like other *Verticillium* species.
2. *V. dahliae* is the predominant species among the *Verticillium* spp. in its distribution and the breadth of its host range and causes economic damage on hundreds of dicotyledonous hosts.
3. *V. dahliae* employs a batch of extracellular proteins (specially encoded in the lineage-specific genomic regions) to induce symptoms, but the functional roles of many of these proteins remain undetermined.

4. Currently, *V. dahliae* may be separated into three races by pathogenicity tests on tomato and classified into D and ND strains on woody host plants. Genetic diversity analyses and vegetative incompatibility tests have thus far grouped isolates into several clonal populations and VCGs, respectively.
5. *V. dahliae* behaves most commonly as an asexual pathogen that propagates clonally, but there is evidence of a mixed reproductive mode and the preservation of ancestral or rare sexual reproduction. To what extent asexual mechanisms (e.g., chromosomal rearrangements) or undetected sexual mechanisms drive genetic recombination and promote population divergence is another topic that needs further scrutiny.

FUTURE ISSUES

1. Population genomic analyses of *V. dahliae* will deliver an enhanced understanding of the genetic basis for various traits within *V. dahliae* populations in addition to their origins, divergence, and evolution. Uncovering the basis of these characteristics coincides with our goals of managing Verticillium wilt disease in plant species worldwide and providing molecular markers and other tools for effective and timely development of resistance.
2. Do host species and geographic location drive genomic changes in *V. dahliae* such as genomic features and markers underlying race, D or ND phenotype, clonal population, VCGs, and *MAT1-1* or *MAT1-2* loci? Are recombination and HGT events prevalent in select clonal populations? Within clonal populations, what are the differences in lineage-specific regions, and how do these differences correlate with virulence?
3. Was there an ancestral or a contemporary cryptic sexual phase in *V. dahliae*? Systematic investigations at the whole-genome level in populations will help explain the genetic basis for ancestral sexual reproduction and the evolutionary mechanisms associated with the loss of sexual reproduction. Studies of *MAT1-2* and *MAT1-1* idiomorphs with near-identical genome structure (without chromosomal rearrangement) are essential to examine sexual compatibility and investigations into fruiting body formation in vitro and in planta.
4. How do differences between *Verticillium* species across genome structures correlate with unique biological traits within and between species? For example, what is the genetic basis for the production of microsclerotia versus dark resting mycelia and chlamydospores among different species?
5. What is the basis of race divergence in *V. dahliae* only on certain hosts? Investigations of the genomes from different races and their populations are important in understanding the evolutionary mechanisms of race differentiation and the relationship between race divergence and chromosomal rearrangements.
6. The Verticilli-Omics Project has sequenced the genomes of more than 3,500 *V. dahliae* strains from diverse host plants/continents in an ongoing project involving an international consortium of researchers (**https://db.cngb.org/Verticilli-Omics**). Coupled with the insights gleaned from this review, many of the mysteries surrounding the origin, divergence, and evolution of *V. dahliae* and its genetic and genomic structural basis will undoubtedly be unraveled in the coming years.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this view.

ACKNOWLEDGMENTS

We apologize in advance to all the investigators whose research could not be appropriately cited owing to space limitations. We extend a special thanks to Dylan Short and Zahi Atallah for thoughtful reviews of the manuscript. This work was supported by grants from the National Key Research and Development Program of China (2018YFE0112500) and the Elite Youth Program CAAS to J.-Y.C. and the Agricultural Science and Technology Innovation Program to X.-F.D. Portions of research referred to in this article were also funded by USDA National Institute for Food and Agriculture (NIFA) grant 59-5305-4-002 and USDA-NIFA Specialty Crops Research Initiative grant 2010-51181-21069 as well as annual funding from the California Leafy Greens Research Board to K.V.S.

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