Title:

The role of hybridization in the evolution and emergence of new fungal plant pathogens

Author:

Eva H. Stukenbrock

Affiliations

Environmental Genomics, Christian-Albrechts University of Kiel, Am Botanischen Garten 9-11, 24118 Kiel, Germany and Max Planck Institute for Evolutionary Biology, August-Thienemann-Str. 2, 24306 Plön, Germany

Corresponding author

Eva H. Stukenbrock

Environmental Genomics, Christian-Albrechts University of Kiel

Am Botanischen Garten 9-11, 24118 Kiel, Germany

Email: estukenbrock@bot.uni-kiel.de

Tlf: +49 (0) 431 880 6369

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Abstract

Hybridization in fungi has recently been recognized as a major force in the generation of new fungal plant pathogens. These include the grass pathogen *Zymoseptoria pseudotritici* and the powdery mildew pathogen *Blumeria graminis triticale* of triticale. Hybridization also plays an important role in the transfer of genetic material between species. This process is termed introgressive hybridization and involves extensive backcrossing between hybrid and the parental species. Introgressive hybridization has contributed substantially to the successful spread of plant pathogens such as *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* the casual agents of Dutch elm disease and other tree pathogens such as the rust pathogen *Melampsora*. Hybridization occurs more readily between species that have previously not co-existed, so-called allopatric species. Reproductive barriers between allopatric species are likely to be more permissive allowing interspecific mating to occur. The bringing together of allopatric species of plant pathogens by global agricultural trade consequently increases the potential for hybridization between pathogen species. In light of global environmental changes, agricultural development and the facilitated long distance spread of fungal plant pathogens; hybridization should be considered an important mechanism whereby new pathogens may emerge. Recent studies have gained insight into the genetics and biology of fungal hybrids. Here I summarize current knowledge about hybrid speciation and introgressive hybridization. I propose that future studies will benefit greatly from the availability of large genome datasets and that genome data provides a powerful resource in combination with experimental approaches for analyses of hybrid species.

1. Introduction

Hybrids emerge from successful genetic crosses of non-conspecific individuals. In fungi hybrids can be generated both by sexual mating and asexual fusion of cells or hyphae (Kohn 2007). Phylogenetic analyses can identify hybrids as intermediate clades with incongruent phylogenetic topologies that reflect the different evolutionary histories of different loci in the genomes of hybrids (Schardl and Craven 2003). Phenotypically hybrids are often characterized by intermediate phenotypes of the parental species (e.g. Greig et al. 2002).

Pure hybrids are found relatively rare in nature. One reason is that hybrids exhibiting intermediate phenotypes often have a reduced fitness compared to their parents. Due to their non-optimized phenotypes they are outcompeted by their parents if co-existing in the same environment (Barton 2008). Additionally hybrids may suffer negative fitness effects due to genetic incompatibilities of parental alleles. Many allelic combinations in the hybrid will consist of alleles that have not co-evolved in the same genomic background and therefore have not been optimized in parallel by natural selection. Such genomic incompatibilities in hybrids are also referred to as negative epistatic interactions or Dobzhansky-Muller interactions (Kondrashov and Kondrashov 2002; see also Kohn 2007 for discussion about the importance of Dobzhansky-Muller interactions in speciation of fungi). The finding of pure hybrid species in nature however suggests that genomic incompatibilities and fitness effects under some conditions can be overcome (Figure 1A) (e.g. Greig et al. 2012; see also review by Yakimowski and Rieseberg 2014).

Another more common result of mating between non-conspecific individuals is introgression by which “transient” hybrids backcross with individuals of the parental species (Arnold 2004 Mallet 2005) (Figure 1B). Repeated backcrossing can “dilute” the hybrid genome to only maintain fragments of the genome of the other parental species (Baack and Rieseberg 2007). Examples from plants, animals and oomycetes suggest that introgressive hybridization can act to transfer adaptive traits between species and thereby be a mechanism that speeds up adaptive evolution (see Arnold 2004 for a review).

Genomics have increased our understanding of hybrid genetics and have provided new insights into the importance of introgressive hybridization for species evolution (Mallet 2005). In a recent paper using comparative population genomics analyses, Lamichhaney and colleagues explored the adaptive radiation in one of the most famous models in evolutionary biology: Darwins finches at the Galapagos islands (Lamichhaney et al. 2015). The authors showed evidence for extensive gene flow between species of finches. Clearly introgressive hybridization has played a role in adaptive radiation of finches together with natural selection on standing and introgressed variation. Adaptive introgression has also been identified in other species using genome analyses. Recent examples include *Heliconius* butterflies (Pardo-Diaz et al. 2012), monkeyflowers (Stankowski and Streisfeld 2015) and fungal plant pathogens causing mildew (Menardo et al. in press). Together these studies demonstrate that hybridization in diverse organismal groups has been instrumental in the formation of adaptive diversity.

Among plant associated fungi (pathogens, endophytes or symbionts) there are several well-characterized hybrid systems including the grass endophyte *Epichloe* (Schardl 2001), the grass pathogen *Zymoseptoria pseudotritici* (Stukenbrock et al. 2012), and the triticale infecting mildew pathogen *Blumeria graminis f. sp. triticale* (Menardo et al. in press). Moreover introgressive hybridization has been documented in several systems such as the Dutch Elm disease pathogens *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (Brasier et al. 1998; Brasier and Kirk 2010), and the anther smut pathogens *Microbotryum silene-dioicae* and *M. lychnidis-dioicae* (Gladieux et al. 2011).

Hybridization has been proposed a major force in the evolution of plant pathogens and in particular the emergence and adaptation of crop infecting pathogens (Brasier 2000 Brasier 2001). Global trade moves pathogens and hosts around the world and thereby brings together species, which previously did not co-exist (Brown and Hovmøller 2002). Introduced pathogens provide a threat to naïve hosts (cultivated as well as wild plants). Another concern is the potential of hybridization between introduced and native pathogen species. As will be discussed in more details below, reproductive barriers between species that

have previously not co-existed can be more permissive and thereby allow hybrids to be formed more readily (e.g. Anderson et al. 1980, Stenlid and Karlsson 1991 Dettman et al. 2003). The result can be either the emergence of new hybrid pathogen species or the transfer of genetic material between pathogen species.

In this review, I will present examples of hybrid fungal plant pathogens, and I will discuss the genetic basis of hybrid formation in the context of adaptive evolution. I propose that more studies integrating genomic and experimental data are essential to understand the biology and evolution of hybrids fungal plant pathogens in natural environments as well as agro-ecosystems.

2. How hybrids arise

A hybrid is the offspring of two non-conspecific individuals (Mallet, 2007). Normally, reproductive barriers act to maintain the genetic integrity of species and prevent the introduction of "foreign" DNA by mating with non-conspecific individuals (Orr 1995). It may however occur that hybrids can form if reproductive barriers are permissive, allowing mating between individuals of different species. To understand how reproductive barriers can be permissive, we first need to identify the underlying genetics of reproductive barriers. Detailed studies in the two ascomycete model species *Saccharomyces cerevisiae* and *Neurospora crassa* have shed light on speciation genetics and reproductive isolation in fungi (Dettman et al. 2008; Dettman et al. 2010, Turner et al. 2011). These studies have documented how both pre-zygotic and post-zygotic reproductive barriers either impair the formation of hybrid zygotes (pre-zygotic reproductive barriers) or severely decrease fitness of hybrid offspring (post-zygotic reproductive barriers). Dettman and colleagues used an experimental evolution approach to generate two diverged yeast populations adapted to distinct environments, high salinity (S) and low glucose minimal (M) media (Dettman et al. 2010). As expected, fitness of S-M hybrids was severely reduced compared to fitness of the parental populations. However, the hybrids were also impaired in meiotic efficiency suggesting additional genomic "defects". The meiotic impairment could be linked to antagonistic interactions between

diverged alleles brought together in the SM hybrids. Dettman and colleagues demonstrated how rapidly (500 cell transfers) negative interactions can arise between diverging populations. Moreover the study shows that one or few genetic incompatibilities may be sufficient to prevent mating between diverged populations and thereby be the drivers of incipient speciation.

Turner and colleagues took a different approach to assess the underlying genetic traits of incompatibility between sympatric strains of *N. crassa* and *N. intermedia* (Turner et al. 2011). The authors generated hybrids of allopatric and sympatric strains of the two *Neurospora* species and used a quantitative trait locus (QTL) approach to identify the genetic traits associated with pre-zygotic reproductive barriers. By crossing sympatric and allopatric conspecific and non-conspecific strains it was shown that hybrid fruiting bodies of sympatric crosses are aborted at a significantly higher frequency than hybrid fruiting bodies of allopatric species. This finding is in agreement with the presence of reinforced reproductive isolation between co-existing *N. crassa* and *N. intermedia* strains. Interestingly, maternal *N. crassa* colonies that aborted fruiting bodies in crosses with sympatric *N. intermedia* strains were subsequently able to mate normally with a conspecific partner. In comparison, a maternal *N. crassa* strain already fertilized by a conspecific partner (sympatric or allopatric) could not produce new fruiting bodies in a second round of fertilization with a con-specific partner. A key finding in the study by Turner and colleagues is the identification of genetic traits underlying fruiting body abortion and thereby the reinforced reproductive barriers (Turner et al. 2011). These major QTLs located on the mating type determining chromosome of *Neurospora* and associated with con-specific reproduction represent interesting candidates in future studies of speciation genomics of *Neurospora*.

Reinforced reproductive barriers between sympatric species have also been elegantly demonstrated by in vitro crossing experiments in other ascomycetes including wild populations of *Saccharomyces cerevisiae* (Kuehne et al. 2007) and in Basidiomycetes such as *Armilaria mella* (Anderson et al. 1980) and *Heterobasidion annosum* (Stenlid and Karlsson 1991).

We know very little about reproductive barriers in plant pathogenic fungi, although important insight has been gleaned from studies of the anther smut fungus *Microbotryum* (Le Gac et al. 2007a Yockteng et al. 2008; Gladieux et al. 2011). Studies of *Microbotryum* have shown an effect of pre-zygotic reproductive barriers on hybridization between the two species *M. lychnidis-dioicae* and *M. silene-dioicae* and show a central role of host-dependent effects on hybrid fitness and establishment (Büker et al. 2013). The nature of these effects is not known in the *Microbotryum* system. However, they are likely to involve an optimal repertoire of effector genes and associated regulatory machinery essential for successful host infection. An optimal intact combination of effector genes is likely not preserved in hybrid plant pathogen genomes (Le Gac et al. 2007a). Studies from *Microbotryum* and another plant associated fungus, *Epichloe,* document that specialization to distinct hosts is a primary barrier for interspecific mating between pathogens (Le Gac et al. 2007b; Schirrmann and Leuchtmann 2015). Pathogens belonging to distinct species may never encounter each other if they are not compatible on the same host. Hybridization will in this case only be possible if the sexual cycle is detached from host infection or if the life cycle of both parental species includes a saprotrophic stage where hyphal fusions can occur. For most plant pathogens, the biology of growth outside the host is still poorly known.

3. The hybrid genome: conflicts and potentials

The formation of a fungal hybrid can occur by sexual mating between parental species or fusion of hyphae or vegetative cells. Fusion of vegetative cells can be followed by parasexual mating where mitotic crossover generates recombined hybrid cells (Schardl and Craven 2003). The outcome of hybridization can be an off-spring with the same chromosome number as the parent, known as a homoploid hybrid, or it can be a hybrid with an increased chromosome number known as an alloploid or heteroploid hybrid (Mallet, 2007).

The genome structure can have a determining effect on fitness and evolution of the hybrid (Yakimowski and Rieseberg, 2014). First of all, the hybrid can be affected by genomic incompatibilities between interacting alleles. Secondly, the consequence of chromosome number changes can be reproductive isolation.

This is the case when backcrossing with the parental species is prevent due to a novel chromosome structure in the heteroploid hybrid.

We first consider genomic incompatibilities: Examples of hybrid sterility and inferior fitness observed in plants, animals, and fungi support the hypothesis that incompatibilities between individual genes with major effects, or larger numbers of genes with a cumulative effect, are responsible for deleterious effects in hybrids (Orr 1995). As found by Dettman and colleagues, divergently adaptive yeast strains rapidly evolve genetic incompatibilities, negative epistasis that lower mitotic fitness in yeast hybrid cells (Dettman et al. 2010). When the authors dissected the underlying genetic nature of the evolved incompatibilities in *Saccharomyces cerevisiae* they found that two genes involved in cell wall synthesis were underexpressed in the hybrid while two other genes involved in sporulation were overexpressed (Dettman et al. 2010; Parreiras et al. 2011). Regulatory defects in hybrids were also shown in crosses of *S. cerevisiae* and *Saccharomyces bayanus* (Lee et al. 2008) suggesting that negative epistatic interactions in hybrids often are determined by non-optimal expression of genes. Several examples of successful hybrids suggest that genomic incompatibilities in some cases, under some circumstances can be overcome (Mallet, 2007). In heteroploid hybrids, the genomic contents of both parents may be entirely or partly retained, and genomic incompatibilities in the form of negative epistasis can be reduced, when both parental alleles are expressed. Instead, it is possible that incompatibilities relating to gene dosage may become relevant. Moreover, negative fitness effects caused by genomic incompatibilities can under some circumstances be "overruled" by positive fitness effects in hybrids. As will be discussed below, hybrids can also contain new advantageous allele combinations that contribute to new phenotypic characteristics and that allow them to explore niches not occupied by the parental species (Greig et al. 2002).

As mentioned above, the chromosome content and structure of hybrids have implications for the ability to backcross with parental species. Thereby, the evolutionary potential of heteroploid and homoploid hybrids is not the same and hybrid speciation is considered more likely in heteroploid hybrids (Yakimowski and Rieseberg, 2014). This is because the altered genome structure of the hybrid

can act as a reproductive barrier and prevent backcrossing to any of the parents. In contrast, backcrossing is a more probabilistic outcome in homoploid hybrids where chromosome number is retained. In other words, introgressive hybridization is a more probably outcome with homoploid hybrids that share the same chromosome composition as their parents (Yakimowski and Rieseberg 2014).

Several studies have in recent years documented that some fungal plant pathogens can tolerate and even benefit from high levels of genomic plasticity, including rearrangements, dynamics of repetitive islands, and accessory chromosomes (see reviews by Raffaele and Kamoun 2012; Croll and McDonald 2012; Seidl and Thomma 2014). To what extent hybrid incompatibilities can be overcome in genomes that already frequently undergo rearrangements should be a central question in future studies of hybrid plant pathogens. In this context, it may be considered that fungi typically produce millions of spores; therefore, many "hybrid experiments" can be tested by natural selection. One or few spores with the right combination of genes can be enough for the establishment of a fit hybrid lineage.

4. The ecological niche of hybrids

Not only do genomic conflicts constrain hybrids, but also their level of fitness in a new niche or one co-occupied by parental species. The parents are expected to be considerably better adapted in their respective environments relative to hybrids of intermediate genotypes and phenotypes. Hybrids may however contain new gene combinations that contribute new phenotypic characteristics, allowing them to exploit other environmental niches not occupied by the parental species (Le Gac et al. 2007b; Schirrmann and Leuchtmann 2015). In yeast, experimental evolution studies revealed that hybrid progenies of *S. cerevisiae* and *Saccharomyces paradoxus* under certain intermediate environmental conditions can exhibit higher fitness than their parents (Greig et al. 2002). Similar hybrid fitness advantages in plant pathogens have, as mentioned above, been described for hybrids of *Ophiostoma*, *Verticilium*, and *Microbotryum* (Brasier and Kirk 2010; Inderbitzin et al. 2011; Gibson et al. 2014). The ability to colonize a new

host niche can be the "key" to establishment without resource competition between parental and hybrid lineages.

Also host expansion through introgressive hybridization has been reported for a number of pathogens including several oomycete species in the genera *Phytophthora* and *Albugo* (e.g. Brasier et al. 2004; Goss et al. 2011; McMullan et al. 2015). *Albugo candida* is an obligate biotrophic parasite, comprising a number of races specialized to infect distinct *Brassicaceae* host species. While the genomes of these different races are clearly distinct, they also show signatures of recurrent introgression, including the transfer of host-specific effector determinants (McMullan et al. 2015). Introgressive hybridization is thereby a mechanism by which new host specificities of *A. candida* can rapidly emerge and be transferred between species. The role of introgressive hybridization in adaptation and ecological speciation of fungal plant pathogens is only studied in a few systems (Le Gac et al. 2007b; Schirrmann and Leuchtmann 2015). The acquisition of genome data from an increasing number of species will however provide new possibilities for future studies of introgression between plant pathogens species of wild and cultivated plants.

5. The population genetics of hybrids

Different evolutionary mechanisms, including natural selection, gene flow, recombination and genetic drift shape the population genetic structure of populations of plant pathogens (McDonald and Linde 2002). The population genetic structure of a hybrid plant pathogen may differ considerably from the population genetic structure of the parental species. Consequently, populations of hybrids may evolve by mechanisms other than the parental species.

Hybridization can be associated with a substantial loss of genetic variation as observed in the grass pathogen *Zymoseptoria pseudotritici* (Stukenbrock et al. 2012). This will be the case when hybrids are formed by a single cross or by few crosses. In the absence of gene flow and backcrossing, the build up of new genetic variation will be determined by the acquisition of new mutations. In this case, the hybrid population will resemble a founder population characterized by low levels of genetic variation and strong effects of genetic drift (Barton and Charlesworth 1984). Hybrids may also emerge as clones that do not undergo

sexual recombination, e.g. *Verticilium longisporum* a pathogen of cruciferous plants (Inderbitzin et al. 2011). Comparative genome studies of fungal plant pathogens have shown that adaptive genetic diversity can be generated by other mechanisms than sexual recombination (Seidl and Thomma 2014). Adaptive genetic diversity can evolve in repeat-rich and fast evolving genome compartments (e.g. Klostermann et al. 2011, Rouxel et al. 2011). Clonal hybrids may likewise benefit from rapidly evolving repeat-rich regions.

Hybrids that undergo recurrent backcrossing will receive new genetic variation from the parental species. If the extent of backcrossing is high then the genetic differentiation between the hybrid and the parental species will decrease proportionally (Arnold, 2004). Below, I briefly consider different population genetics parameters in the context of hybridization of plant pathogens. Further detailed theoretical analyses of hybrid evolution are presented in reviews by Orr (1995), Baack and Rieseberg (2007) and Barton (2008).

Effective population size and genetic drift: The parameter "effective populations size" termed N_e reflects the amount of genetic diversity that can be passed on from one generation to the next in a population (Wright 1938). Depending on the "format" of the hybridization event, hybrids may emerge with small effective population sizes resembling that of a founder population. In species with small effective population sizes, the effect of genetic drift will be more severe. Genetic drift refers to the random loss of genetic variation and such an effect can potentially aid the negative effect of genomic incompatibilities. In populations with many alleles of a particular gene (high effective population size), the effect of genetic drift is small; however in populations with fewer alleles (low effective population size), the effect is high (Barton and Charlesworth 1984).

Gene flow: Gene flow mediates the transfer of genetic variation between populations and may be the source of new genetic variation into the hybrid population. Introgressive hybridization is the extreme case in which the hybrid is a "transient" state in the exchange of genetic material between the two parental species (Arnold 2004; Mallet 2005). As discussed above, the extent of backcrossing will be determined by the nature of reproductive barriers between

359 the hybrid and the parental species, which for example may be a difference in
360 genome composition for heteroploid hybrids. Furthermore, the ecological niches
361 occupied by the species will affect rates of gene flow (Giraud et al. 2010). Distinct
362 host compatibilities can act as reproductive barriers between plant pathogens
363 and thereby also isolate hybrids from gene flow (Giraud et al. 2010).
364 Lastly, gene flow between species can be facilitated by human mediated long
365 distance dispersal of plant pathogens (Brown and Hovmøller, 2002). This has for
366 example been the case for the ascomycete fungus *O. ulmi*, the casual agent of
367 Dutch elm disease (Brasier 2000). Intercontinental dispersal of the two species
368 *O. ulmi* and *O. novo-ulmi* led to the emergence of rare hybrids that mediated the
369 transfer of genetic material between *Ophiostoma* species by introgressive
370 hybridization (Brasier, 2001).
371
372 *Natural selection:* As already pointed out above, the hybrid genome, in contrast
373 to the genomes of the parental species, was never tested in the same extent by
374 natural selection and comprises gene combinations that did not evolve in parallel
375 (Barton 2006). To which extent hybrids can overcome such genomic
376 incompatibilities depends on multiple factors such as the genetic composition of
377 the hybrid, the nature of negative epistatic interactions, and the interplay of
378 environment, selection and genetics in the hybrid (Barton 2001). As shown
379 experimentally for yeast hybrids (Greig et al. 2002), a particular intermediate
380 "recombinant" environment can provide an optimal niche for a hybrid where it
381 has a selective advantage compared to the parental species.
382
383 *Recombination and mating systems:* Reproduction of fungal plant pathogens
384 ranges from purely clonal reproduction (e.g. *Verticilium dahliae*) to obligate
385 outcrossing (e.g. *Ustilago maydis*) (McDonald and Linde 2002). Furthermore
386 sexually reproducing species can be heterothallic (mating between compatible
387 strains of opposite mating types) or homothallic (when self-fertilization is
388 possible) (Lin and Heitman 2007). In this wide range of reproductive modes and
389 mating systems, hybrids can form in different ways and possess different types
390 of mating systems similar or distinct to the mating systems of their parents.

In hybrids that are able to undergo meiosis, recombination may be important in combining the right parental alleles required for successful establishment and propagation. Recombination can also be instrumental in breaking down genomic incompatibilities between parental traits. Furthermore, as in all sexually reproducing organisms, recombination in the hybrid can act to speed up adaptive evolution through the fixation of beneficial mutations and the removal of deleterious and non-adaptive variants from the genome (Marais and Charlesworth 2003; Goddard et al. 2005).

Sexual recombination has been a main driver in the emergence and evolution of the hybrid *Z. pseudotritici*, a close relative of the heterothallic ascomycete *Z. tritici* (Stukenbrock et al. 2012). Consistent with the mating type structure in *Z. tritici* we found both mating types present among individuals of the hybrid *Z. pseudotritici* (Waalwijk et al. 2002). Furthermore, the genome of this hybrid is shaped by recombination between conspecific strains suggesting that mating behavior resemble mating in *Z. tritici* and other non-hybrid relatives.

In contrast to *Z. pseudotritic*, hybrids of *Epichloe* are asexual while non-hybrids include both asexual and sexual species (Charlton et al. 2014). *Epichloe* hybrids are abundant and are considered to increase the diversity of alkaloids produced to enhance host protection against herbivores (Charlton et al. 2014).

Parasexuality and dikaryosis: Parasexuality refers to the transfer of genetic material between individuals without meiotic recombination and the formation of fruiting bodies. Some asexual pathogens can benefit from parasexual mating by the formation of new genotypes (Hickman et al. 2015). Parasexuality can also be instrumental in the formation of hybrids (Schardl and Craven, 2003; Roach and Heitman 2014).

The first step of parasexual reproduction in fungi is the fusion of vegetative cells or hyphae. Normally, vegetative compatibility in both Ascomycetes and Basidiomycetes is governed by genetic programs that prevent fusion with non-conspecific individuals (Glass et al. 2000; Worall 1997). In cases where vegetative compatibility systems fail to prevent inter-specific fusions, a heterokaryon will be formed. Heterokaryons of incompatible strains are often unstable and revert into homokaryons or they show reduced growth and fitness

(Kauserud et al. 2012). But in some cases the heterokaryon can convert into heteroploid or haploid cells or hyphae by parasexual nuclear fusion and mitotic cross-over of chromosomes. Recent studies of the human asexual pathogen *Candida albicans* show that parasexual mating can generate a diverse population of progenitors differing in ploidy and chromosome numbers (Hickman et al. 2015). In the same way parasexual mating between non-conspecific partners can give rise to a diverse population of heteroploid hybrid individuals from which natural selection can "pick out" fit genotypes.

6. Recent examples of plant pathogenic fungal hybrids

Zymoseptoria pseudotritici – *a one-time fit combination*

Z. pseudotritici, a close relative of the prominent wheat pathogen *Z. tritici*, was isolated from two distinct grass hosts *Elymys repens* and *Dactylis glomerata* in the north of Iran (Stukenbrock et al. 2012). Population genomic sequence data of the haploid *Z. pseudotritici* revealed a peculiar mosaic genome structure with long segments completely lacking variation (Stukenbrock et al. 2012). The non-variable segments were found interspersed by regions of unusual high sequence variation. The variable segments consistently exhibited only two haplotypes differing by 30 SNPs per 1kb on average, corresponding to 3% nucleotide variation between haplotypes.

A genomic region with comparatively low levels of genetic variation often indicates a selective sweep where a beneficial allele in a population of individuals through time has been fixed by natural selection. In the case of *Z. pseudotritici*, however, the non-variable segments were interspersed between variable regions always comprising two haplotypes. This mosaic genome structure of *Z. pseudotritici* did not support a selective sweep model. We instead propose that hybrid speciation explains this peculiar genomic mosaic in *Z. pseudotritici* and postulated that the species was formed by a single hybridization event between two individual from two closely related species or lineages about 3% divergent from each other. The non-variable segments in the genome are chromosomal regions that were transmitted from one parent to all hybrid progeny. The variable segments comprising two haplotypes are chromosomal regions that were transmitted to the hybrid swarm from both

parents. Using the correlation of the length distribution of variable segments with recombination rate and generation time of *Z. pseudotritici* it was possible to estimate the number of recombination events that have occurred since the hybridization event forming *Z. pseudotritici*. From the generation time of *Z. pseudotritici* we found that the mosaic genome of *Z. pseudotritici* results from approximately 400 recombination events between individuals of the hybrid swarm. The lack of additional polymorphism in the non-variable segments indicates that backcrossing to the parental species did not occur after the initial hybridization event.

Z. pseudotritici was found to occur frequently on grasses collected at five plots along a 500 km transect in Iran. Individuals from the parental species were not collected from grasses at the same plots at subsequent collecting trips in the same area (M. Javan-Nikkhah, M. Zala, B.A. McDonald and E.H. Stukenbrock, unpublished data) suggesting that the hybrid has invaded a completely new niche (in time or space) or outcompeted both parental species.

Z. pseudotritici is an excellent example of recent hybrid speciation of a fungal plant pathogen. A number of questions remain to be addressed in this hybrid system: which hosts did the parental species colonize? Why did only one successful hybrid clone emerge from the parental species? How has successful adaptation over multiple generations been possible following extreme loss of genetic variation? Further dissection of the genome evolution in *Z. pseudotritici* as well as more extensive sampling at the center of origin of *Z. pseudotritici* including Septoria pathogens from non-*Poaceae* hosts will provide further insight into the origin and evolution of this hybrid.

Microbotryum – *incomplete reproductive barriers and introgression*

The species complex of the anther smut fungus *Microbotryum violaceum* (Basidiomycota) provides an excellent model system to address the importance of intogressive hybridization among closely related plant pathogens (e.g. Le Gac et al. 2007a, Le Gac et al. 2007b; Giraud et al. 2008; Gladieux et al. 2011). Gladieux and colleagues addressed the extent of hybridization between the two species *M. lychnidis-dioicae* and *M. silene-dioicae* using population genetic analyses and coalescent models (Gladieux et al. 2011). *M. lychnidis-dioicae* and *M.*

silene-dioicae are ecologically isolated from reproduction as they each are specialized to distinct *Silene* hosts. In a large collection of *Microbotryum* isolates, the authors identified only a small number of hybrids (15 out of 1028 isolates) based on microsatellite data and a Bayesian clustering algorithm. Few isolates were assigned as "cross-species" isolates, i.e. isolated assigned to either *M. lychnidis-dioicae* and *M. silene-dioicae* but isolated from the original host of the other *Microbotryum* species (Gladieux et al. 2011). These "cross-species" isolates show that host-defined pre-zygotic reproductive barriers can be permissive allowing isolates from distinct hosts to cross with each other. By applying an "Isolation-with-migration" coalescence model to the dataset, the authors were able to date the divergence of *M. lychnidis-dioicae* and *M. silene-dioicae* and to estimate when gene flow between species occurred (Gladieux et al. 2011). Consistent with the low frequency of hybrids, analyses of gene flow revealed recent divergence times and low rates of gene flow between the two *Microbotryum* species. Although the two pathogen-species co-exist in the same environment, reproductive barriers determined by the host may be sufficient to prevent the long-term establishment of hybrid individuals. Nevertheless, recurrent introgressive hybridization between *Microbotrym* species may allow the transfer of new adaptive traits between species and eventually promote the evolution of new host specificities. Experimental back-crossings of *M. lychnidis-dioicae* and *M. silene-dioicae* hybrids have demonstrated a mating type effect on inter-specific conjugation formation (Büker et al. 2013). This could suggest that not only host factors determine reproductive barriers but in addition, as also observed in *N. crassa* (Turner et al. 2011), mating type related factors.

Comparative genome analyses may in the future allow the identification of introgressed traits in *Microbotryum* species. Which and how many genes determine host specificity in the *Microbotryum* system still needs to be determined. An intriguing question will be whether these genes have been introgressed between species as predicted.

Epichloe – *hybrids and the evolution of host specificities*

The ascomycete endophyte *Epichloe* is the main fungal model for studies of hybridization and the impact of hybridization on phenotypes *in planta*. Species of

Epichloe colonize the apoplast of pooid grasses. The endophyte has little effect on the vegetative tissue of its host; however, during sexual reproduction *Epichloe* can negatively affect the development of flowering shoots by formation of fungal sexual structures in these plant parts, causing the so-called "choke" disease (Schardl et al. 2004). Sexual reproduction of the pathogenic *Epichloe* species is thereby tightly associated with reproductive success of the host. *Epichloe* also includes mutualistic endophytic species that confer a fitness advantage to their host, for example by the production of anti-herbivore alkaloids (Schardl 2001). Interestingly, several mutualistic *Epichloe* species have been identified as hybrids. These hybrids do not originate from interspecific sexual mating, as mating populations in the field can be clearly distinguished according to sexual compatibility and host specificities (Schirrmann and Leuchtmann 2015). Rather, hybrids are asexual lineages that originate from vegetative fusions between hyphae of different *Epichloe* species (Tsai et al. 1994; Shoji et al. 2015).

Asexual *Epichloe* species were previously classified in the genus *Neotyphodium*, but they were recently attributed to the genus *Epichloe* (Leuchtmann et al. 2014). Interestingly, phylogenetic analyses show that asexual species frequently are heteroploid hybrids emerged from interspecific hyphal fusions of other *Epichloe* species (Moon et al. 2004; Shoji et al. 2015).

In a recent paper, Shoij and co-workers assessed the fate of nuclei and organelles during vegetative hyphal fusions in different *Epichloe* species (Shoji et al. 2015). Cytological analyses of interspecific hyphal fusions showed that distinct types of nuclei never co-existed. Viable hybrids are therefore formed by nuclear fusion and the formation of allodiploid hyphae. The authors also addressed the frequency of interspecific hyphal fusions and could with quantitative analyses show that interspecific hyphal fusions are rare. Nevertheless in nature interspecific hybrids of *Epichloe* are found frequently. The authors propose that established hybrids could have a fitness advantage through host specificities acquired by the hybridization event allowing them to rapidly increase in frequency. It is also possible that *in-planta* conditions promote hyphal fusions to an extent not reproduced experimentally.

Interestingly, non-hybrid and hybrid *Epichloe* endophytes differently affect growth and reproduction of host species. The evidence suggests that the

performance of hybrid compared to non-hybrid endophytes depends on host genotype and environmental conditions. Hybrids were shown to enhance the competitive abilities of the grass *Festuca arizonica* by increased biomass production (Saari and Faeth 2012). But in another grass species *Hordelymus europaeus* non-hybrid Epichloe endophytes were found to have more positive influence, compared to hybrid endophytes, on host fitness measured as seed production of different accessions of *H. europaeus* (Oberhofer et al. 2014). As for *Z. pseudotritrici* and *Microbotryum,* a deeper understanding of the underlying host-fungus interaction is necessary to unravel differences in the biology of hybrid and non-hybrid *Epichloe* species.

Verticilium longisporum – *multiple parents and multiple hybridizations*

Formation of the diploid hybrid *Verticilium longisporum* occurred by vegetative fusion of other haploid *Verticilium* species. Phylogenetic analyses have shown that the hybrid originated from multiple hybridization events involving the unknown species Species A1 and Species D1 and the D2 and D3 lineages of *V. dahliae* (Inderbitzin et al. 2011, Inderbitzin and Subbarao, 2014).

Both genome structure and host range of *V. longisporum* differ from those of *V. dahliae*: While *V. dahliae* is known to have a broad host range, it is, with some exceptions, not infectious on *Brassicaceae* hosts. *V. longisporum* on the other hand is mainly a pathogen of *Brassicaceae* plants (Zeise and Tiedemann 2008) indicating that new host determinants were acquired from the unknown parental species (A1 or D1) or emerged by the combination of multiple host determinants from several parents. The capacity to infect new host species may however not only have resulted from a new combination of genes but also from the doubling of chromosomes in the diploid *V. longisporum*. Populations of other heteroploid hybrids have similarly been shown to comprise an increased phenotypic variability compared to the parental species allowing the exploitation of a broader range of host species (Newcombe et al. 2001; Brasier et al. 2004). To address the genomic source of new virulence traits in heteroploid hybrids as *V. longisporum*, the underlying determinants of host infections are still to be discovered.

7. Studies of fungal hybrid species: future directions

Comparative genomics and population genomics provide new powerful approaches to recognize ongoing and past hybridization in plant pathogens (e.g. Stukenbrock et al. 2012; Menardo et al. in press). Likewise experimental evolution studies have shed light on the genetics of reproductive barriers in model ascomycetes (Dettman et al. 2010, Turner et al. 2011). Although hybridization may be an important (yet underestimated) force in the evolution of plant pathogens, we still know little about the biology and genetics of hybrid plant pathogenic fungi. A key question in evolutionary genomics of hybrids is how negative epistatic interactions of distinct parental alleles are overcome. Futures studies should combine experimental and genomics approaches to explore the genetics of hybrid incompatibilitites and to assess phenotypic and evolutionary potentials of hybrid plant pathogens.

Population genomics

I consider at least four important applications of population genomics in hybrid studies. The first is the use of population genomic data to investigate the genome wide distribution of genetic variation in hybrids. As discussed above, this may deviate significantly from the parents, but nevertheless influence the evolutionary potential of the hybrid, including the potential for dispersal, introgression and adaptation. Secondly, population genomic data can be explored to identify signatures of selection in the hybrid genome reflecting novel adaptations and the selection against incompatible allele combinations (Stukenbrock 2013). Signatures of selection can be identified as outlier loci of increased divergence (e.g. Ellison et al. 2011), regions depleted of variation (selective sweeps) (e.g. Sharpio et al., 2012 or genes with an increased proportion of non-synonymous variation (balancing selection) (e.g. Stukenbrock et al. 2011). Thirdly, introgressive hybridization will leave strong signatures of inter-specific exchanges of genetic material (Arnold 2014). Genomic data from hybrids and their parents allow the identification of these transferred and putative adaptive traits (Arnold 2004). Introgressed regions are recognized in genome scans as segments with altered levels of divergence (Menardo et al. in press). For example, divergence will be reduced in regions affected by introgressive

622 hybridization. Lastly, genomic incompatibilities in hybrids may be recognized by
623 in depth analyses of linkage disequilibrim or QTL mapping. As described above
624 experimental evolution, QTL analyses and genomics have shed light on genetic
625 incompatibilities in *S. cerevisiae* and *N. crassa* (Dettman et al. 2010; Turner et al.
626 2011).
627
628 *Experimental studies in planta and in vitro*
629 Only few experimental studies have used *in-planta* experiments to compare
630 fitness of fungal hybrids relative to non-hybrid species (Büker et al. 2013; Saari
631 and Faeth 2012; Oberhofer et al. 2014). It has been documented that
632 hybridization can change the host range of plant pathogens but it is not know
633 how (e.g. Inderbitzin et al. 2008). Is it the new combination of effectors that
634 allow hybrids to explore novel host niches? Or do changes in ploidy and gene
635 dosage alter virulence in the hybrid? Studies that aim to identify new hybrid host
636 determinants are essential to understand the success of hybrid plant pathogens.
637 Such traits may be identified from comparative genome or transcriptome studies
638 and their functional relevance assessed by reverse genetics and experimental
639 approaches. An intriguing question is whether such traits are inherited from one
640 parental species and only confer a distinct fitness advantage in the genomic
641 background of the hybrid, or if these traits are recombined and represent
642 completely new variants.
643 Finally, a better understanding of the underlying genetics of reproductive
644 barriers and self-versus-non-self recognition between species of fungal plant
645 pathogens could improve predictions of hybridization events in agro-
646 ecosystems. As shown in *N. crassa*, genetic determinants of reproductive barriers
647 can be identified by experimental hybridization assays and QTL mappings
648 (Turner et al. 2011). Cytological analyses of between-species, vegetative hyphal
649 fusions can furthermore be applied to study nuclear behavior during inter-
650 specific fusions and the formation of heteroploid hybrids (Shoji et al. 2015).
651 A number of central questions relating to hybrid evolution can be addressed by
652 experimental evolution approaches. In contrast to retrospective sequence-based
653 analyses, experimental evolution of microbial species provides the opportunity
654 to directly study rates of mutational changes and to identify the nature and order

of these changes. It also allows fitness assays of all generations from progenitor to last evolved population and thereby a comparison of fitness effects of ancestral and derived alleles. Experimental evolution could be applied for example in studies of hybrid pathogens aiming at addressing the fate of genetic variation and genomic incompatibilities in hybrids following multiple generations of asexual or sexual propagation.

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849

Figure legends:

Figure 1: A) Hybridization can occur by asexual fusion or sexual mating between diverged species. B) Recurrent crossing between species and backcrossing of the hybrid with parental species is referred to as introgressive hybridization. In the extreme case of introgressive hybridization, the hybrid is a transient form, while the important outcome of the inter-specific mating is the transfer of genetic material.