Metabolic gene clusters, fungal diversity, and the generation of accessory functions

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Abstract

Ecological interactions are largely determined by adaptive traits deemed "accessory". In plants, fungi, and bacteria, such traits mainly comprise metabolic pathways that produce or transform diverse molecules. While accessory metabolic pathways are pervasive, it is often difficult to identify their genetic bases. Recently, in-depth descriptions of metabolic gene clusters (MGCs), which encode discrete metabolic pathways, have greatly simplified the characterization of genotype-phenotype maps, yet questions of how this genome architecture relates to the evolution of accessory functions remain. Fungi are uniquely positioned to spearhead investigations into these dynamics because they display gradients in clustering across pathways and taxa. This review will focus on the role of MGCs as both agents and consequences of the accessory function evolution that underpins fungal diversification.

MGCs: repositories of accessory metabolic functions

Accessory metabolic functions underpin ecological adaptation and innovation in plants, fungi, and bacteria by determining the outcomes of organismal interactions [1]. Chemical defenses, for example, enable these organisms to overcome competition and resist predation despite their limited mobility. Nutritionally adaptive metabolic pathways can facilitate the breakdown of challenging substrates or protect digested resources from competitors. The accessory metabolites of complex organisms further define the adaptive microbial communities with which they associate [2]. However, there is a general lack of knowledge about the individual genes responsible for these phenotypes. This problem has been partially addressed by investigations at the -omics scale, e.g. inferring and describing networks of accessory genes whose expression or distributions are associated with ecologically important phenotypes [3,4].

Genome structure provides an alternative window into accessory metabolic functions. Specifically, accessory metabolisms are frequently encoded by Metabolic Gene Clusters (MGCs) composed of neighboring, coordinately-expressed genes participating in discrete metabolic pathways, often accompanied by supportive functions like regulation, transport, and self-protection from the encoded phenotype [5, 6]. Because genes cluster in response to natural selection, MGCs are effective proxies of adaptive metabolic functions, and can therefore greatly accelerate the discovery and characterization of accessory pathways contributing to ecological phenotypes [7]. Convergent origins and interspecies exchanges of MGCs can also indicate selection on the accessory functions they encode [8, 9, 10]. Further, the ecology and evolution of MGCs can be inferred independently of the genetic mechanisms and traits they encode, enabling large-scale modeling of chemo-diversification processes [11].

One way to identify accessory function-encoding MGCs is by conducting targeted searches using probabilistic models trained on previously identified clusters. AntiSMASH and the associated ClusterFinder module are general-purpose tools for mining biosynthetic MGCs by similarities in gene sequence, cluster composition, and the spatial distributions of protein family domains in genomes [11, 12]. These tools can be used to identify the genetic basis of real and predicted natural products [13] and further compare potential chemodiversity across fungal populations and lineages [14, 15]. Because such programs use models trained on known pathways/MGCs from a limited set of fungi, bacteria, and plants, “untargeted” cluster prediction methods that use other evolutionary or ecological criteria for identifying gene clusters may be needed to uncover new types of metabolism. Some innovative untargeted approaches include searching for co-location of genes with similar promoter motifs [16], shared evolutionary histories [17], or linkage shared by unexpectedly divergent species [18, 19, 20]. The prediction of biosynthetic MGCs has been the major focus of genome mining initiatives to date, though catabolic MGC discovery is poised to follow suit [18].

MGCs interact with the drivers and constraints of accessory function evolution

Genes for accessory functions can arise through vertical gene duplication (VGD-duplication of a locus within a genome, inherited by offspring), horizontal gene transfer (HGT-duplication from a locus in one genome to a locus in another)(Figure 1), or *de novo* gene evolution [21]. Genes that already perform accessory roles, such as dedicated secondary metabolism structural genes (Nonribosomal Peptide Synthetases, Polyketide Synthetases, etc.) may be the main source of novel accessory genes. Truly novel metabolism also emerges

through duplication of core metabolic genes [22, 23], but it is difficult to distinguish such accessory genes from the core genes from which they are derived purely based on amino acid sequence. MGCs are hotspots for VGD and HGT [24]. These closely-related information copying processes remove the evolutionary constraints that were imposed on the original genes by genetic networks under natural selection; they facilitate adaptive evolution by allowing partitioning of ancestral functions (subfunctionalization) or the emergence of novel functions [25, 26, 27]. HGT of gene modules and MGCs may further impact organismal fitness by copying environmentally adaptive functions into novel genetic backgrounds [9, 28, 29]. A propensity for either type of gene duplication can increase an organism's latent capacity to evolve and elaborate on accessory functions [30]. HGT of MGCs and multigene modules they contain may further accelerate adaptation by bypassing fitness valleys (e.g. toxic transitional states) encountered during stepwise addition of genes to a genome [5, 31].

Linkage, specialization, and the generation of adaptive accessory functions

MGCs provide a framework for testing outstanding hypotheses in evolutionary biology concerning the origins of adaptive phenotypes. For example, enzyme activity by default is promiscuous, which furnishes important variation for evolution of accessory functions, but carries the burden of pathway inefficiency and the potential for toxic interference among pathways [32]. Specialization, in contrast, entails functional optimization and metabolic efficiency, but decreases the overall evolutionary potential of the specialized genes [32]. MGCs may mediate some of these trade-offs by enabling coordination of enzyme function and regulation, reducing their interference with other metabolic processes, while still maintaining promiscuous functions [8]. By decreasing the fitness costs of functionally linked accessory genes, clustering preserves adaptive gene combinations and facilitates their joint incorporation into new phenotypic contexts, accelerating the emergence of accessory function [33]. Differential rates of degeneration and loss among protein functional classes within MGCs lend further insight into the process of adaptive specialization. Recent studies have demonstrated that enzymes are preferentially lost from MGCs while regulators and transporters are selectively retained [34, 35], suggesting specialization could result in enzymatic "dead ends", and that continued innovation requires a regular replenishment of enzymes from conserved or promiscuous functions. The ability to generate and preserve functional linkages in gene clusters would thus impact an organism's ability to generate and incorporate accessory diversity (i.e. its evolvability)[36].

Variation in clustering provides insight into the mechanisms generating accessory functions

Fungi are uniquely positioned to elucidate the mechanisms generating accessory functions and MGC diversity because their known genomic diversity captures variation in clustering across metabolic pathways and across lineages in ways that bacteria, with highly clustered genomes, and plants, with few clusters in their genomes, do not (Figure 1). Specifically, there are significant differences in clustering rates between the two filamentous fungal subphyla, Pezizomycotina and Agaricomycotina. While hundreds of MGCs have been identified in Pezizomycotina, there are only five Agaricomycotina pathways deposited in the MIBiG database v1.4 (mibig.secondarymetabolites.org/repository.html), and only a few more can be found in the literature (Table 1). The MGCs identified in Pezizomycotina fungi further

differ in compositional complexity and the metabolism encoded compared to those identified in Agaricomycotina (Table 1). Known or predicted Agaricomycotina clusters are small, and contain recently duplicated genes, compared to those in Pezizomycotina, which cover a large range of sizes and are compositionally more diverse [37]. Additionally, Agaricomycete biosynthetic pathways employ parallel processes at specific metabolic steps and in the classes of compounds produced [38]. These distinctions suggest accessory metabolism and MGCs in Agaricomycotina have commonalities with those of plants [39], while the genomic structure of accessory metabolism in Pezizomycotina is more similar to bacteria [11]. This overlap complicates assumptions about cluster evolution, where tandem duplications provide a neutral evolutionary mechanism for cluster formation in eukaryotes, and the tendency for genes to be transcribed in operons offers a regulatory mechanism selecting for clustering in bacteria. By providing a range of compositional complexity, but with limited operon-like transcription, the spectrum of fungal gene clustering forces attention to other evolutionary mechanisms driving gene clustering.

To illustrate these trends (Figure 1), certain cluster-encoded metabolisms, such as those based on highly modular nonribosomal peptide synthetase enzymes, along with numerous supporting enzymes, transporters and regulators, are largely restricted to morphologically simple fungi and bacteria [37, 40]. For example, Pezizomycotina have an average of ~10 NRPS containing clusters per genome versus 1.2 in Agaricomycotina. Interestingly, mushroom-forming fungi still produce non-protein peptide metabolites with ribosomally generated oligopeptides encoded in high copy number MSDIN genes, whose products diversify through simple coding mutations [41]. Terpenoid biosynthesis, where biologically meaningful diversity can be readily generated through small modifications and high enzyme promiscuity, so far also seems to be over-represented in morphologically complex fungi and plants [37, 42]. The difference in genome architecture among fungi is especially pronounced in phenolic degradation. While putative phenolic degradation clusters are diverse and complex across Pezizomycotina [18], in Agaricomycotina, most such clustering is the result of tandem duplications and few putative clusters contain more than a single gene family [43]. Disentangling the roles of species-specific metabolism and lineage-based differences in genome evolution mechanisms will become more feasible with greater genome sampling and ecological knowledge of fungi.

Life history traits and genome architecture theory predict a clustering gradient across fungi

It is not entirely clear whether the absolute numbers of clusters identified in the respective lineages reflect differences in propensity to cluster, or if different cluster compositions in Agaricomycotina leave them less likely to be detected using biased models and methods. Nonetheless, the variation we observe is predicted from complex interactions between migration, recombination rates, and selection [44], which differ between fungal subphyla as a result of contrasting reproductive strategies, generation times, and ecological lifestyles. Under strong selection and sufficient gene flow, clustered architectures are favored, because they prevent the influx of maladaptive alleles and preserve the fitness of locally adapted genotypes [44]. For example, when genes participate in the same specialized pathway, clusters reduce recombination between differently optimized enzymes that would be detrimental to their cooperation in metabolic transformations.

The mating strategies of individual organisms may strongly influence genome architecture, the rates of VGD and HGT, and the diversity of accessory functions (Figure 1). For example, many bacteria and fungi participate in large "pan-genomes" containing far greater diversity than can be contained in a single individual. Sharing in pan-genomes is facilitated by mobile elements and unequal recombination mechanisms, and gives access to diverse gene families, gene combinations, and sequence variants. In these types of populations, clusters are more prevalent and they enable the parallel diversification of accessory functions across numerous genomes under diverse environmental selection. In this way, pan-genomes result in varied assortments of discrete pathways in individual genomes. By contrast, fungi and plants that participate in smaller pan-genomes, will have more limited access to gene variants and variable gene combinations, resulting in a smaller number of highly branched and parallel pathways within individual lineages. Pathways in these organisms are constrained to evolve primarily through VGD and intragenomic mutation. Differences in the types of metabolic diversity and in the composition of MGCs found across bacteria, fungi, and plants are consistent with these processes, but specific tests remain to be done.

Ecology is similarly seen as a driver of reproductive strategies that impact selection on genome architecture (Figure 2). Tree-decaying Agaricomycotina often have large, long-lived somatic biomasses with rare, periodic meiotic sexual reproduction. In contrast, the typically small units of rapidly sporulating Pezizomycotina commonly engage in clonal reproduction and self-fertilization, in addition to sexual reproduction. Pervasive, non-meiotic recombination (including parasexuality) in Pezizomycotina [45], similar to bacteria, may explain their substantial pan-genomes and elevated gene flow between populations and across species barriers. The increased proclivity for parasexuality may also favor non-homologous recombination and structural rearrangements of large linkage blocks (even entire chromosomes) that repress recombination. Repression of recombination frees nascent gene clusters to accumulate transposed genes and to drift while becoming selected as a block, effectively acting as a superallele in a population's pan-genome [46, 47]. Indeed, a number of recent studies have confirmed the existence of "idiomorphic" MGC loci in Pezizomycotina fungi, where variably distributed MGCs occupy the same genomic region in different individuals [14, 48, 49, 50, 51]. Supernumerary (dispensible) chromosomes are an extreme example of parasexual/horizontal inheritance of co-adapted blocks of accessory functions. HGT of supernumerary chromosomes in *Fusarium* has been shown to enable infection of a new host by confering multiple adaptive functions [52]. Decreased propensity for genomic rearrangements and constraints on gene flow in Agariciomycotina due to sexual recombination may relax the drive to cluster in this lineage compared with Pezizomycotina, as decreased population-level rates of small-scale genomic rearrangements are also associated with decreased rates of clustering [53]. Intriguingly, ecological selection for self-mating appears to drive formation of mating locus clusters, which relaxes some evolutionary constraints imposed on predominantly sexual populations [54, 55].

One line of evidence supporting the hypothesis that the spectrum of lifestyles and reproductive strategies of fungi impacts patterns of metabolic diversification is the rate of MGC horizontal duplication. It is notable that to date, while large HGTs of MGCs are more commonly inferred in Pezizomycotina (commensurate with higher HGT in general [24]), the only published case of MGC HGT in Agaricomycotina involves dung-decay mushrooms. Dung fungi are driven to

inbreed and reproduce more rapidly due to the patchiness and ephemeral nature of this resource [56]. The case of HGT of the psilocybin cluster among dung-decay fungi [10], contrasts with the other evolutionarily well-characterized Agaricomycotina MGC for luciferin, which evolves through concerted VGD and loss [57]. However, these hypotheses need to be rigorously tested.

Competition among osmotrophs is thought to be the main driver of the diversification of accessory pathways [58]. Interestingly the genomes of fungus-like oomycetes (stramenopiles) are structured more similarly to plants, with extensive tandem gene duplication, but few gene clusters and little accessory metabolism [59, 60]. While oomycetes are similar to fungi in physiology, and their range of ecological roles, they differ by having a dominant diploid stage of their life-cycles, suggesting reproductive strategies as well as nutritional mode are important in genome architecture.

A single gene family illustrates different evolutionary processes among fungal lineages

Different patterns of accessory enzyme diversification in Pezizomycotina and Agaricomycotina exemplify the interplay between accessory functions and gene clustering. For example, phylogenetic analysis supports the emergence of single-domain MT-33 N-methyltransferases by partial duplication of bifunctional ergothioneine synthases in both lineages [61]. These enzymes alternately participate in ergotamine toxin (Pezizomycotina) and methylated tryptophan biosynthesis (Agaricomycotina). Across Agaricomycotina, there have been many recent origins of these enzymes by VGD, and the only observed ancient paralog has itself been subject to ongoing loss. Furthermore, there are no indications that these accessory paralogs are in MGCs or horizontally transferred in Agaricomycotina. In Pezizomycotina, by contrast, while these N-methyltransferases perform a similar function N-methylating indole alkaloids, they have rarely emerged by VGD, and they appear to disperse by HGT as part of complex MGCs [61, 62]. In short, these novel enzymes appear constrained to evolve simple peripheral functions through VGD in isolated lineages of Agaricomycotina, but participate in complex clustered pathways that are shared across species through HGT in Pezizomycotina.

MGC distributions imply ecological functions

The ecological impact of accessory functions in microorganisms can be difficult to discern because species may be opportunistic on the substrate of their isolation, and the genes encoding these functions are often unknown [63]. Equally confounding, sub-populations of a species may be specialized for alternative cryptic niches. MGCs can help disentangle the importance of specific functions for ecological fitness because they enable the rapid identification of genes and gene families of interest contributing to specific phenotypes. For example, the biased distribution and HGT of clustered genes involved in psilocybin production among dung- and insect- associated fungi, coupled with the known effects of psilocybin on serotonin-receptors, led to speculation that this tryptamine alkaloid reduces mycophagy by insects by interfering with neural signaling. Subsequent detection of psilocybin (by a likely a convergent psilocybin pathway) in cicada parasitizing fungi provided additional evidence that this compound is selected for its effects on insects [64]. Similarly, the identification of a MGC found previously in pathogens of woody plants enabled a reverse ecology approach to discovering new hosts for several grass pathogens [7]. And finally, targeted and untargeted

searches for MGCs have further revealed previously unexplored dimensions of accessory function evolution and led to the proposal of their ecological associations [18].

Summary

Accessory functions are the essence of ecological adaptation and innovation, yet they often resist characterization. Genome architecture-focused approaches, such as MGC mining and characterization, promise a relatively straightforward path to cut through genomic noise and decipher ecological relationships in parallel with molecular functions [63, 65]. Although MGCs are observed across all major Kingdoms of life, the diversity of fungal mating strategies and lifestyles is likely responsible for generating fungal-specific variation in clustering rates, positioning fungi and their MGCs as model systems for linking specific ecological, demographic, and genetic processes with the generation of accessory metabolic functions (Figure 2). In particular, elucidating the relative impact of vertical and horizontal gene family expansion (VGD and HGT) on different strategies for metabolic diversification should greatly improve our ability to explain how, when, and why accessory functions evolve.

Figure Legends

Figure 1: *The spectrum of accessory metabolic compartmentalization and diversification.* Bacteria, Ascomycetes, Basidiomycetes, and Plants (shown as silhouettes) capture gradients in accessory metabolic diversification and the storage of accessory metabolic functions within and across individuals. Moving from left to right, pan-genomes become progressively closed (i.e., less variable among individuals), genomic architectures feature fewer metabolic gene clusters encoding accessory functions, and individual genomes transition from encoding many different specialized accessory pathways to few highly diversified pathways. The major candidate mechanisms contributing to the gradients are shown below, and are expected to manifest to different degrees in each of the depicted lineages.

Figure 2: *The eco-evolutionary dynamics of genome architecture*. Ecological selection is the overarching force shaping the evolution of accessory functions, acting through evolutionary processes operating at multiple scales, as discussed in the text. Ecological selection pressures determine the most adaptive population dynamics, including mating/reproduction strategies, optimal effective population size (N_E), and migration rates. In turn, the spectra of these population dynamics determine the mechanisms of genome evolution and metabolic diversification that are ultimately available for generating adaptive variation. Recombination mechanisms and streamlining vary across different life-history strategies and result in the types of evolvability favored and the size of pan-genomes (Figure 1). These drivers simultaneously favor different modes of metabolic diversification such as the combination of gene functions in novel pathways, and their differential optimization/specialization. Contrasting patterns of genome architecture (e.g., gene clustering vs. gene family expansion) are tangible outcomes of interactions among these processes (Figure 1), but they may also feed back on and influence the impact of the main evolutionary drivers. For example, gene clustering favors HGT and pathway specialization, which can mitigate competition and increase access to resources.

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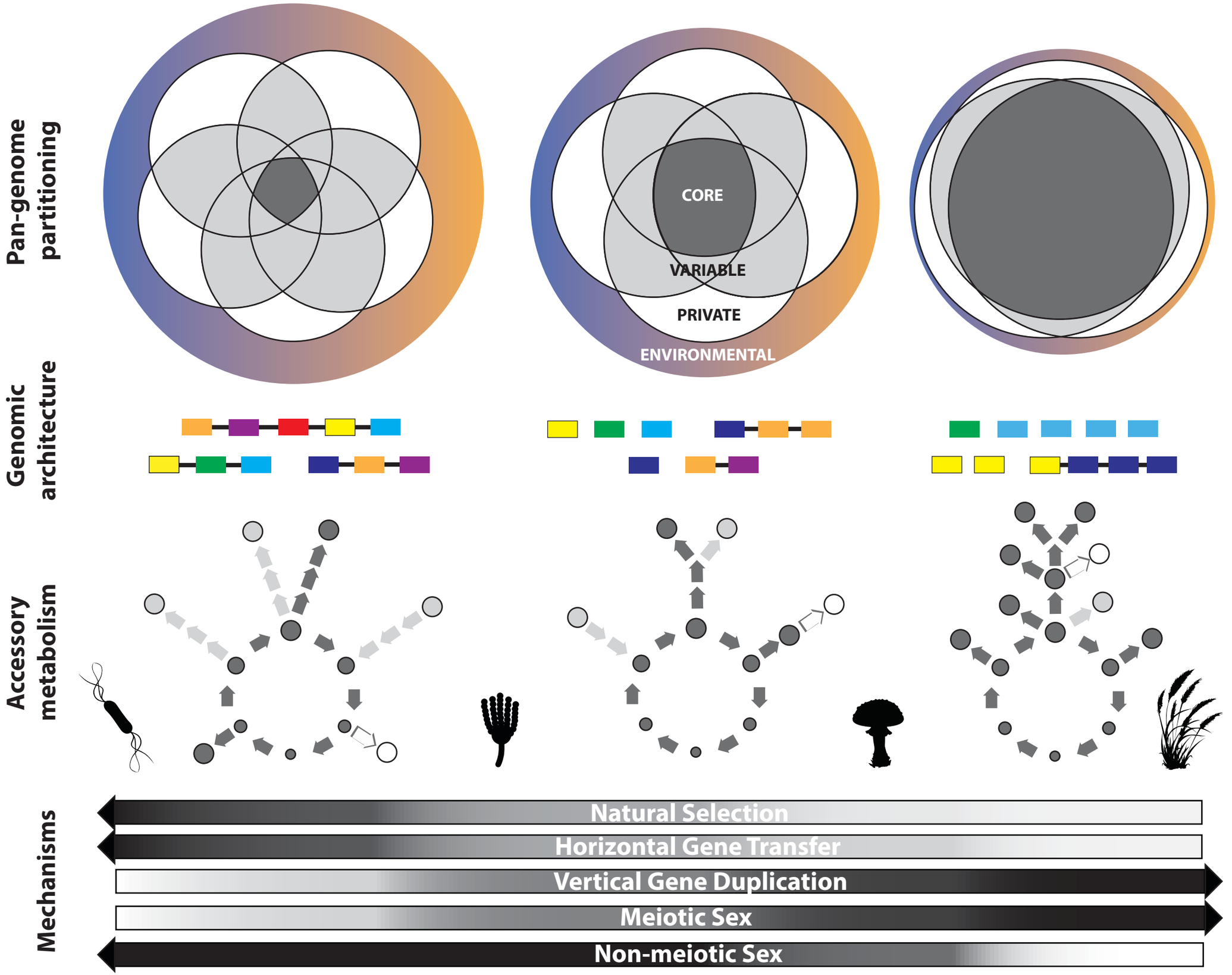
Pan-genome partitioning
Genomic architecture
Accessory metabolism
Mechanisms
CORE
VARIABLE
PRIVATE
ENVIRONMENTAL
Natural Selection
Horizontal Gene Transfer
Vertical Gene Duplication
Meiotic Sex
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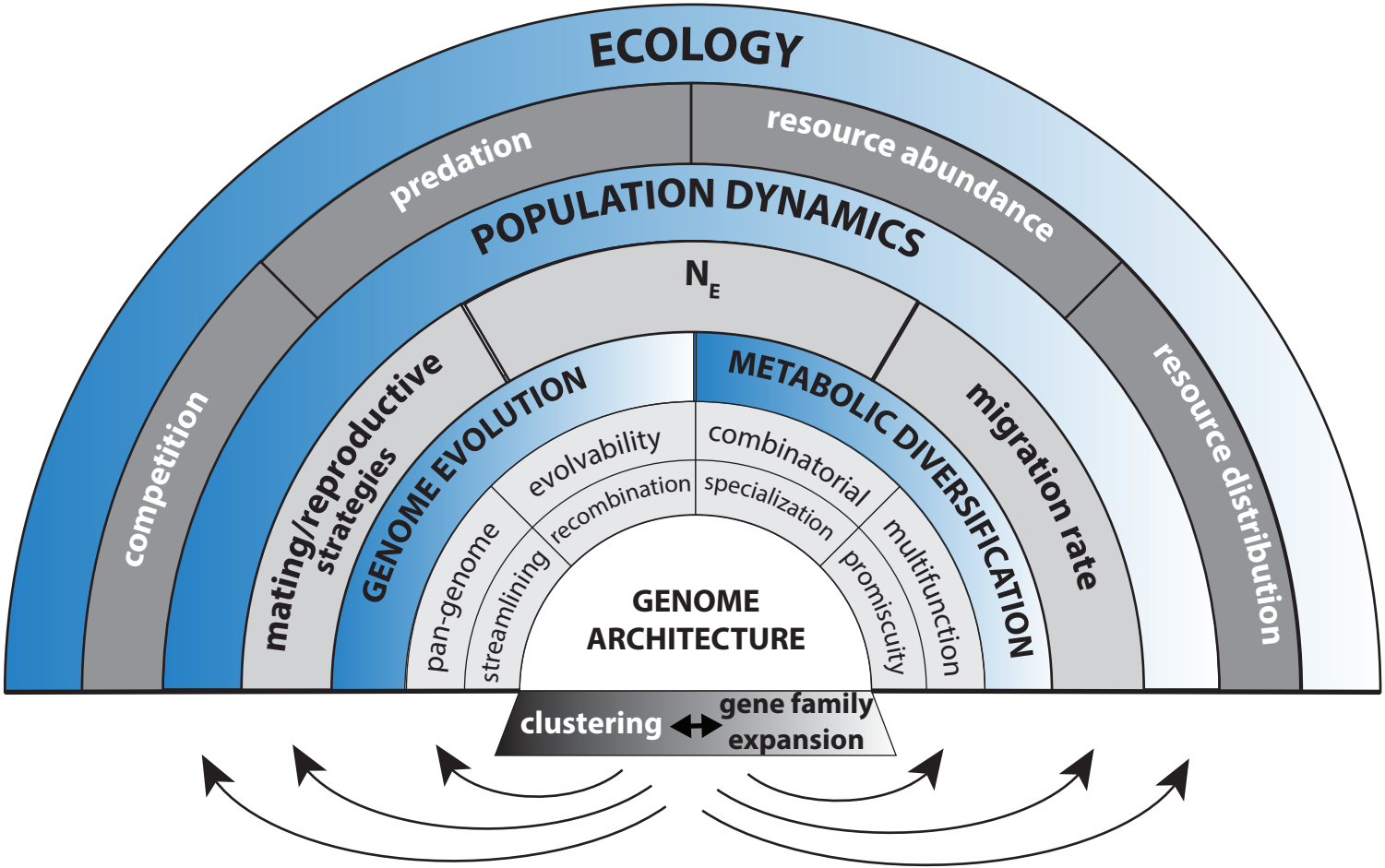
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