

Next Generation Biological Control: the Need for Integrating Genetics and Evolution

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Abstract

Biological control is widely successful for controlling pests, but effective biocontrol agents are now more difficult to obtain due to more restrictive international trade laws. Coupled with increasing demand, the efficacy of existing and new biocontrol agents needs to be improved with genetic and genomic approaches. Although they have been underutilised in the past, applying genetic and

genomic techniques is becoming more feasible from both technological and economic perspectives. We review current methods and provide a framework for using them, incorporating evolutionary and ecological principles. First, it is necessary to identify which biocontrol trait to select and in what direction. Next, the genes or markers linked to these traits need be determined to better target their selection, followed by how to implement this information into a breeding program. Choosing a trait can be assisted by modelling to account for the proper agro-ecological context, and by knowing which traits have sufficiently high heritability values. We provide guidelines for designing genomic strategies in biocontrol programs, which depends on the organism, budget, and desired objective. Genomic approaches start with genome sequencing and assembly. We provide a guide for deciding the most successful sequencing strategy for biocontrol agents. Gene discovery involves quantitative trait loci (QTL) analyses, transcriptomic and proteomic studies, and gene editing. Improving biocontrol practices include marker-assisted selection, genomic selection and microbiome manipulation of biocontrol agents, and monitoring for genetic variation during rearing and post-release. We conclude by identifying the most promising applications of genetic and genomic methods to improve biological control efficacy.

Keywords: artificial selection, biological control, genetics, genome assembly, genomics, insect breeding, microbiome, modelling

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I. Introduction

Biological control, or the use of natural enemies to control a pest, is arguably the best solution for phasing out the large scale use of synthetic pesticides (Thomas & Willis, 1998; Bale, van Lenteren, & Bigler, 2008). It has been broadly applied for hundreds of years and to great success in both greenhouse and open field systems worldwide (van den Bosch, 1971; Stiling & Cornelissen, 2005). Most of the research into the fundamentals of biological control has been from an ecological perspective, focusing on aspects such as optimal foraging and risk monitoring (Wajnberg, Bernstein, & Alphen, 2008; Heimpel & Mills, 2017). Clearly, not all initiated programs resulted in the desired level of pest management, and as such there is considerable room for improvement (Wajnberg, 2004). The reasons for biocontrol programs not always reaching their full potential are manifold and range from releasing the wrong control agents, agents being non-adapted to local environmental conditions, undesired interactions with the native fauna, and evolutionary changes in the pest species upon invasion.

In the past, the default method for improving biocontrol performance was to find a more efficient wild species or strain to be the biocontrol agent (Hassan & Guo, 1991; Hassan, 1994; Nomikou *et al.*, 2001; Hoelmer & Kirk, 2005). However, the Nagoya protocol for Access and Benefit Sharing of Genetics Resources has severely limited international exchange of biological materials, so the practice of successively sourcing more effective biocontrol agents from the field is severely restricted (Cock *et al.*, 2010; Deplazes-Zemp *et al.*, 2018; Mason *et al.*, 2018). Moreover, certain geographical regions have strict regulations on which agents can be used, e.g., only local strains originating from the region itself (Loomans, 2007; Hunt, Loomans, & Kuhlmann, 2011). Concurrently, demand for more effective biocontrol agents is rising, driven by an increased demand for organic products and growth of the organic food production market, valued at 62.9 billion USD as of 2013 (Willer & Lernoud, 2019; Baker, Green, & Loker, 2020). For example, the global biological control market was worth 1.7 billion USD in 2015, with sales growing 3x faster than pesticides (van Lenteren *et al.*, 2018). Additionally, policy developments have aimed to reduce of synthetic pesticide use (van Lenteren *et al.*, 2018) such as a EU-wide neonicotinoid ban (Gross, 2013; Stokstad, 2018) and continuous legal curtailment of organophosphate use in the US and worldwide (Hertz-Picciotto *et al.*, 2018). The rise of the organic market in conjunction with a reduction of pesticide use results in a need for environmentally safer pest control, which is reflected in the rapid growth and increased market value of the biocontrol industry (de Clercq, Mason, & Babendreier, 2011; Dunham, 2015; van Lenteren *et al.*, 2018). It is now more urgent than ever to understand how to effectively and efficiently improve non-native biocontrol agents already in use and to develop novel native biocontrol agents. We review recent developments in the field of biological control that indicate that genetics-based solutions are key (Figure 1).

For decades, it has been advocated to use genetic means to improve the efficacy of biocontrol programs (White, Debach, & Garber, 1970; Hoy, 1986; Hopper *et al.*, 1993; Narang, Bartlett, & Faust, 1993; Nunney, 2003; Routray *et al.*, 2017, Kruitwagen, Beukeboom, & Wertheim, 2018; Lirakis & Magalhaes, 2019). Significant genetic variation has been demonstrated in several key life-history and behavioural traits of potential biocontrol agents (Hoy, 1985; Rousch, 1990; Hopper *et al.*, 1993; Wajnberg, 2004, Ferguson *et al.*, in preparation a). However, despite its proven application in, for example, crop and livestock breeding, there have been few attempts made to improve biocontrol agents through genetic means. We currently witness a revival of the idea to apply genetics to improve biocontrol programs, in terms of exploiting intra- and interspecific variation (Lommen, de Jong, & Pannebakker, 2017; Kruitwagen *et al.*, 2018), performing experimental evolution (Lirakis & Magalhaes, 2019), adapting the microbiome for improving rearing methods (Ras *et al.*, 2017), and population monitoring of released agents (Roderick & Navajas, 2003; Stouthamer & Nunney, 2014; Coelho *et al.*, 2016). Notably, these methods all employ classic genetics principles within a species' existing gene pool, distinguishing them from Genetically Modified Organisms (GMOs) that have DNA from foreign organisms introduced into their genomes. That means that unlike GMOs, the methods discussed here are all compatible with the organic mission common in biocontrol practice. Next to genetic applications, we need an evolutionary perspective on the sustainability and risks of biocontrol programs, for example to enable predictions of future adaptations of pests and agents (Hufbauer & Roderick, 2005; Szűcs *et al.*, 2019). Finally, application of genetics and evolution in biocontrol programs cannot be evaluated without considering the role of the environmental and ecological processes (Thrall *et al.*, 2011). We outline an integrated approach of genetics and evolution in an ecological context as a promising way forward to improve biocontrol practices.

We first identify which organismal traits are important for biological control and should therefore be targeted for improvement (so-called "biocontrol traits"). Next, we present the current state and future prospects of using genetic and genomic methods towards that aim. We consider these methods from evolutionary and ecological contexts, i.e. how these methods can realistically operate in long-term breeding programs and in the field (Figure 2). Because of their prevalence and economic importance (van Lenteren *et al.*, 2018), we focus on programs using arthropod biocontrol agents, although these universal genetics principles overlap with other agents (e.g. nematodes, fungi, bacteria). While the most common form of biological control is augmentative (the recurrent release of a biocontrol agent population not expected to establish permanently, or to supplement an existing population), these methods discussed here can also be applied to classical biological control (release of a new agent with the intention of establishing a self-sustaining population and level of pest control in the area of the pest) and conservation biological control (conserving natural habitat to increase populations of natural enemies). As genomics are key to many of these methods, we provide a key

on how to obtain genome-based data in specific biocontrol contexts (Figure 3). We conclude by reviewing present uses and forecasting applications of the most promising genetic and genomic methods in the future (Figure 4).

II. What are biocontrol traits?

One of the prime reasons preventing the uptake of genetic improvement of biocontrol agents is the difficulty in deciding which traits to optimize. Candidate traits can be roughly subdivided into those related to pest suppression ability, adaptation to abiotic factors, reducing ecological risk, and improving mass production or storage (see Kruitwagen *et al.*, 2018) for a comprehensive overview). For some traits, such as pest kill rate, the direction of improvement may be apparent, as killing more pests is a primary determinant of biocontrol success (Stiling & Cornelissen, 2005). However, for other traits, the direction to take is less obvious. For example, most biocontrol agents attack hosts/prey that are clumped in patches in the environment. From a biocontrol perspective, would it be more effective for the agent to clear patches completely before moving on, or to disperse rapidly to protect a larger total crop area (Wajnberg, Roitberg, & Boivin, 2016; Plouvier & Wajnberg, 2018)? The optimal strategy depends on the specific ecological circumstances faced by the biocontrol agent and the economic harm inflicted by pest species at low density, i.e. the specific agro-ecological situation that the biocontrol practitioner must manage. Optimality models based on behavioural ecology can provide important insight into the critical characteristics of natural enemies for successful biological control (Mills & Kean, 2010; Wajnberg *et al.*, 2016; Lommen *et al.*, 2017). Recently, Plouvier & Wajnberg developed a general modelling framework that identifies the key biocontrol agent life-history traits from an economical perspective of the biocontrol practitioner (Plouvier & Wajnberg, 2018). Under simulated conditions, two different optimized life-history strategies for the agents were found that resulted in higher potential economic returns, differing in plant-leaving decision and host handling time of the biocontrol agent, but also in their respective fecundity, longevity, and dispersal ability. Such a general modelling framework can be parameterised for different biocontrol species and different ecological situations to help identify the key traits to target for genetic improvement.

A key requirement for biocontrol traits to be targeted for selection is the presence of significant heritability (the proportion of the total phenotypic variation between individuals that is due to additive genetic variation), which allows trait values to be shifted towards optimal values by means of (artificial) selection. The amount of standing genetic variation depends on the type of trait and its genetic architecture. Traits closely associated to fitness that are important for biocontrol, such as life-history and behavioural traits (Mousseau & Roff, 1987; Wajnberg, 2004; Lommen *et al.*, 2017; Kruitwagen *et al.*, 2018; Xia *et al.*, 2019), typically have lower heritabilities than physiological

and morphological traits (Mousseau & Roff, 1987). The amount of genetic variation for biocontrol traits is currently poorly investigated and insufficiently known, although progress is being made to measure the heritability of traits related to biocontrol (for a review of the current research, see Ferguson *et al.*, in preparation a). Selection for low heritability traits towards optimal values is possible, but the efficiency of this process depends on a trait's genetic architecture (such as number of genes involved, dominance, epistasis, pleiotropy). It is therefore of key importance to uncover the genetic architecture of biocontrol traits if we aim to efficiently improve them.

III. What genetic information do we need?

1. Genome assembly. Assembling a genome for a biocontrol agent of interest vastly expands the possibilities for generating new knowledge on the genetic architecture of biocontrol traits. A reference genome facilitates studies that focus on gene expression analyses, targeted gene editing, and marker-informed selection. Although producing a high quality genome (high coverage, few gaps) is often portrayed as an essential goal (Bentley, 2006; Faino & Thomma, 2014), a high-level resolved genome may often not be required. Instead, sequences may be collected, assembled, and annotated to the level required for a specific project, and the genome can subsequently be improved further to the level desired by other parties (Papanicolaou *et al.*, 2017). In other words, in more applied circumstances, such as biological control, the aim may be a “good-enough” genome rather than a high quality genome. Also, some applications can already be realised with an incomplete genome, including the quick generation of molecular markers such as microsatellites (Grbić *et al.*, 2011; Abe & Pannebakker, 2017; Kamimura *et al.*, 2019) for low-cost analysis of genetic variation (Baker, Loxdale, & Edwards, 2003; Paspati *et al.*, 2019) and linkage map construction (Niehuis *et al.*, 2010; Beukeboom *et al.*, 2010) in biocontrol agents. Genome assembly goes through various stages: sequencing from an inbred stock or a single individual, aligning the sequences into an assembly, and annotating the assembly with protein-coding information (Ekblom & Wolf, 2014). Although still requiring a considerable amount of labor and funding, recent advances in technology have lowered the cost of sequencing a genome considerably (Wetterstrand, 2019). In the context of biological control, successfully producing a workable genome within one's budget and objectives requires careful strategy. For example, in many cases, the biocontrol agent is too small for DNA extraction from a single individual to be usable for assembling a genome (Richards & Murali, 2015). Pooling many genetically identical individuals is a solution, but how to obtain such a sample varies by species. This is easier and has been done, for instance, with isofemale lines of haplodiploid parasitoids (Werren *et al.*, 2010; Geib *et al.*, 2017) and clonal mites (Hoy *et al.*, 2016), but can be more challenging for species that are more difficult to inbreed (e.g. ladybird beetles (Facon *et al.*, 2011). Figure 3 presents a key for deciding which sequencing strategy to use for various cases in biological

control, accounting for the current state of technologies and the biology of the species (with examples (Ferguson *et al.*, in preparation b, in preparation c; Kraaijeveld *et al.*, 2019; Paspati *et al.*, 2019).

2. Gene discovery. Mapping genes has a long tradition in breeding and research, particularly using quantitative trait loci studies (QTL) (e.g., maize height (Burr *et al.*, 1988); soybean seed morphology (Mansur *et al.*, 1993); pig fatness and growth (Andersson *et al.*, 1994); *Nasonia* parasitoid wasp sex ratio (Pannebakker *et al.*, 2011)). A QTL study uses crosses of individuals with different extreme phenotypes and links their segregation in offspring to molecular marker data to identify the genetic basis of complex traits (Lynch & Walsh, 1998; Beukeboom & Zwaan, 2007). High-throughput sequencing and genome wide association studies (GWAS) have enabled higher resolution mapping screens (Schlötterer *et al.*, 2014) i.e. identification of loci with different allele frequencies between two study populations with different phenotypes of the target trait (Bastide *et al.*, 2013).

For QTL mapping and GWAS, the statistical power to identify causative variants increases with the number of individuals analyzed. In addition, in a QTL approach, power increases with the number of generations invested, but mapping precision is typically lower than GWAS. The genotyping costs can be reduced by relying on sequencing pools of individuals with extreme phenotypes (Pool-GWAS (Schlötterer *et al.*, 2014)). This approach was used to create a genome wide map for body pigmentation in *Drosophila melanogaster* (Bastide *et al.*, 2013) and can theoretically be applied for any target trait in any arthropod. Although individuals for these studies can be sourced from commercial biocontrol populations, these tend to be inbred and represent only a fraction of the genetic variation harbored by natural populations (Rasmussen *et al.*, 2018; Paspati *et al.*, 2019). For exploratory studies, collecting sufficiently variable individuals with clear segregation of phenotype, and thus correspondingly distinct genotypes for candidate loci, may be more easily sampled from wild populations. Alternatively, as it may be legally or logistically difficult to sample multiple natural populations across a large geographic range, accessible commercial strains that are already in use and have contrasting phenotypes may be used instead. For instance, long-established *Trichogramma cacoeciae* strains originating from France and Tunisia have higher fecundity under different temperatures (Pizzol *et al.*, 2010), and would be good candidates for investigating loci linked to climate adaptation.

Another approach to delineating the genomic architecture of biocontrol traits is studying gene expression. Sequencing transcriptomes and proteomes, which are complete RNA and protein expression profiles of an organism respectively, has become increasingly easy and affordable. The advantage of gene expression studies is that they delve into context-dependent phenotypes. For example, gene expression differences of the sexes are highly relevant for parasitoid wasps (Wang,

Werren, & Clark, 2015), because only the female has pest killing ability. It is reasonable to infer that females have sex-specific genotype-phenotype maps in regard to, e.g. chemosensory functions for host interactions, venom production, and egg production. Yet, in these haplodiploid systems, haploid males and diploid females can have the same genome (isogenic diploid state for females and the haploid state for males). Spatiotemporal expression pattern differences of protein and transcript quantity, methylation, RNA splicing, and post-translational modification may be responsible for sex-specific phenotypes, and can be used to find trait-linked loci even when genetic sequences are identical between males and females (Wang *et al.*, 2015). Such studies have been used to delineate the architecture of, for example, sex determination (Verhulst, Beukeboom, & Zande, 2010), oviposition (Pannebakker *et al.*, 2013; Cook *et al.*, 2015), and venom composition (De Graaf *et al.*, 2010) in *N. vitripennis*, and antennal perception of different olfactory cues, i.e. male mate searching versus female host searching in *Cotesia vestalis* (Nishimura *et al.*, 2012) and *Chouioia cunea* (Zhao *et al.*, 2016). Analysis of transcriptomic and proteomic data obtained at different environmental or culturing conditions is also a powerful tool, as it can both identify and quantify patterns of gene expression (Wang *et al.*, 2009). For example, transcriptome analyses of biocontrol agents such as the ladybird *Cryptolaemus montrouzieri* or the parasitic wasps *Cotesia typhae* nov. sp and *Lysiphlebus fabarum* have adapted to alternative prey/hosts by modifying the regulation of genes mainly related to development, digestion, detoxification and virulence (Li *et al.*, 2016; Dennis *et al.*, 2017; Benoist *et al.*, 2017). In addition, the mechanisms underlying resistance to certain pesticides in the predatory mite *Neoseiulus barkeri* and the ladybird beetle *Propylaea japonica* were identified by analysing RNAseq data (Tang *et al.*, 2014; Cong *et al.*, 2016). Also, transcriptome sequencing of an entomopathogenic nematode has revealed multiple expanded gene families that may be involved in parasitism (Dillman *et al.*, 2015). Transcriptomics may also pave the way to understand symbiont-mediated resistance to parasitism (Oliver, Moran, & Hunter, 2005), and help to reverse this effect or to make parasitoids more virulent. Additionally, proteomic analysis of aphid parasitoids *Aphidius colemani* that were either exposed to fluctuating high and low temperatures or to constant cold provided insight on genes and proteins involved in surviving temperature extremes, such as those involved in energy metabolism (Colinet *et al.*, 2007). A final interesting application of transcriptomics for biocontrol is to identify the genetic architecture of memory and learning, as parasitoids can be trained to recognize host species (Huigens *et al.*, 2009). Recently, genes in the Ras and PI3 kinase pathways were found responsible for interspecific differences in *Nasonia* memory retention (Hoedjes *et al.*, 2014, 2015). Gene expression studies can thus contribute to understand adaptation mechanisms of biocontrol agents to a new environment, prey/host defences or novel hosts. Yet careful control of expression data collection, such as consistent life stage or common garden conditions, is important, as phenotypic plasticity can add noise to analyses. Ultimately,

understanding gene expression patterns is essential to allow the preservation of a robust phenotype, or how likely a phenotype is to persist in various agro-ecological environments (Félix & Barkoulas, 2015).

3. Genome editing for exploratory research. Advances in genomics approaches and knowledge have made it possible to modify certain regions in the genome of an organism to study how such modifications reflect in its phenotype. New phenotypic variants can be generated by knocking-down or knocking-out genes. Knocking-down refers to temporary gene expression inhibition through RNA interference (Pratt & MacRae, 2009). Knocking-out refers to permanent alternation through the germ line, and the most advanced of these knock-out approaches is Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) (Hsu, Lander, & Zhang, 2014). Knocking-down or knocking-out candidate life-history trait genes can lead to insight in functions that can be used to optimize selection or breeding of biocontrol agents. For example, it can be used to examine the role of genes in a trait through linkage with the null phenotypes, and those genes can be specifically targeted for selection. At this point in time, gene-editing technology may be exploratory and for fundamental research use, but should not be used in novel biocontrol release programs. Although no external DNA is introduced with knock-down or knock-out, some countries consider gene-editing techniques to be in the same legal category as GMOs (EU; Callaway, 2018) whereas others do not (Australia, Mallapaty, 2019; US, Kim & Kim, 2016; Waltz, 2016). They are therefore subject to much of the same regulations that vary broadly in restrictiveness, e.g. allowable usage in the US versus a complete ban in the EU (reviewed in Alphey & Bonsall, 2018). Moreover, the compatibility of gene-editing with the current “non-GMO” appeal (and therefore marketability) of biological control is questionable.

4. Microbiomes. Recently, there is much interest in the role of the microbiome in organismal functioning. In the context of biological control, it is known that microbes can be responsible for chemical signals that attract parasitoids to their host, and that bacteria can have a defensive role against parasitoids, such as in aphids (Oliver, Moran, & Hunter, 2006; Schmid *et al.*, 2012; Rothacher, Ferrer-Suay, & Vorburger, 2016; Jamin & Vorburger, 2019; Koskinioti *et al.*, 2019; Dicke, Cusumano, & Poelman, 2020). Nowadays, universal DNA markers can be applied to characterize the microbiome, i.e. to identify all bacterial symbionts to at least family or genus level, and their proportionate presence (Ras *et al.*, 2017). This can be used to infer the relative abundance and relative importance of each symbiont in contributing to biocontrol traits. However, despite the enormous attention for the role of microbiomes, we still know very little about whether and how microbes contribute to arthropod life-history traits and biocontrol traits in particular (Janson *et al.*, 2008; Brinker *et al.*, 2019; Gurung, Wertheim, & Falcao Salles, 2019). In addition, the factors that determine the microbiome composition are often not well known. Such information is important to judge how

consistent the microbiome is transgenerationally, and if it can be manipulated through the rearing environment.

IV. How can genetics be used to improve biological control?

1. Artificial Selection. The traditional approach to improving biocontrol agents has been through artificial selection. Artificial selection exploits inter-individual genetic variation of life-history or behavioural traits. For example, agent species have been selected to improve tolerance to climatic conditions to expand their geographic range of use (White *et al.*, 1970), pesticide resistance (allowing their compatibility with pesticide spraying) (Roush & Hoy, 1981a; Spollen & Hoy, 1992), and development time (to speed up production ; Rodriguez-Saona & Miller, 1995). Rather than performing individual crosses with individuals of desired traits, experimental evolution exposes populations to specific environmental conditions for several generations and determines the effect on the trait of interest. Although generally successful (Lirakis & Magalhaes, 2019), selective breeding results have not been used in commercial biocontrol practice to an extent that might be expected, and artificial selection remains underutilised (Wajnberg, 2004; Lommen *et al.*, 2017; Kruitwagen *et al.*, 2018). There are several reasons for this. A first obvious one is lack of sufficient genetic variation because that too few individuals have been collected from nature and used as source population for artificial selection, but little is known about such unsuccessful attempts in the literature. A second possibility is that once selected, the genetic makeup of the selected population will change, e.g., through genetic drift (Roush & Daly, 1990; Stouthamer, Luck, & Werren, 1992; Hopper *et al.*, 1993; Wajnberg, 2004). The conclusion has been that to avoid these issues arising from “selection relaxation,” biocontrol agents must be continuously re-selected, which can be economically prohibitive. There is, however, little empirical evidence for this, and a recent study on *Drosophila* indicates that laboratory populations may not change that much in life-history parameters compared to their natural counterparts (Michalak *et al.*, 2019).

Trade-offs between life-history traits are also a factor in biocontrol evolution. Life-history theory poses that an organism has limited resources to allocate to each trait. To select for the enhancement of one or more traits, as is the goal in biocontrol breeding, pleiotropic and disadvantageous changes can occur in other traits, and the overall effect on the biocontrol function of the organism can be unpredictable (Stearns, 1989; Roff, 2007). For example, it has been noted that traits corresponding to high yield and ease of use under laboratory and industrial conditions are favourable, but adaptation to captivity may come at the expense of an agent’s efficacy in the field (Mackauer, 1976; Hopper *et al.*, 1993; Sørensen, Addison, & Terblanche, 2012; Sánchez-Rosario *et al.*, 2017). It is also logistically difficult to phenotype complex traits for small organisms as is needed for traditional breeding.

However, solutions to many of these problems are at hand. There is evidence, for example, that selection relaxation may not be as problematic as previously believed. Theoretically, if the selection regime is strong enough, the trait goes to fixation in a population, and is no longer subject to drift (Falconer & Mackay, 1996). This may be reflected in selection persistence being documented in insect lines even after many generations of no active maintenance on the trait (e.g. (White *et al.*, 1970; Croft & Meyer, 1973; Roush & Hoy, 1980; see Lirakis & Magalhaes, 2019). It is also standard industrial practice to conduct quality control of biocontrol agent products (van Lenteren, 2003), which effectively screens against lines that have lost desirable traits.

Key to these solutions is understanding the genetic architecture of the trait under selection, which theoretically allows the combination of individual phenotype selection with molecular genetics through applying marker-assisted selection (Lande & Thompson, 1990). When, for instance, we know the loci and alleles associated with a desired or undesired target trait, we can breed a more efficient biocontrol agent by selecting for the former and avoiding the latter. Not all trade-offs may be detrimental for biological control. For example early reproduction may come at a cost of longevity (Williams, 1996), but a long life may not be important to a captive population's net productivity if it is frequently supplemented with new, fecund individuals. Knowing the genetic underpinning of trade-offs between traits would further assist in understanding and preventing unwanted trade-offs.

An unfavorable life-history trade-off of a trait may take several forms, for instance at a detriment to another trait or another stage in the biocontrol program (e.g. a trait selected for mass-rearing improvement leads to reduced field performance). In such cases, genetics can again potentially provide the solution if the genomic architecture underlying these trade-offs is known (Figure 4B). For example, antagonistic pleiotropy (genes operating on multiple traits but in opposing directions) is known for fecundity versus longevity in the melon fly, *Zeugodacus cucurbitae* (Miyatake, 1997) and higher larval survival versus lower adult body weight in *D. melanogaster* (Bochdanovits & de Jong, 2004); additional studies can uncover these in biocontrol agent species. If there are variable pathways corresponding to different trade-offs, it should be possible to choose to select through one with the fewest unfavorable trade-offs, exploiting the ubiquitous presence of genetic redundancy.

Learning behaviour is another biocontrol trait that responds to selection. Parasitoids can be directly selected to better recognize pest species (Dukas, 2000; Rahmani *et al.*, 2009; Schausberger *et al.*, 2010; van den Berg *et al.*, 2011; Hoedjes *et al.*, 2014, 2015; Kraaijeveld *et al.*, 2018; Kruidhof *et al.*, 2019), or indirectly to more efficiently recognise recruitment signals from plants following herbivore attack (tritrophic interactions) (van der Putten *et al.*, 2001; Turlings & Wäckers, 2004). Juvenile predatory *Phytoseiulus persimilis* can be trained to accept specific prey species and retain this habit throughout its lifetime (Rahmani *et al.*, 2009), as can numerous parasitoid wasps (reviewed

in Kruidhof *et al.*, 2019). Delineating the genetic architecture of memory and learning can thus assist in developing applications in breeding.

Knowledge of the genetic architecture of traits can assist with artificial selection through deliberate targeting of linked genes. This is particularly helpful for traits that are laborious or challenging to phenotype repeatedly. For example, for many arthropods, assaying lifetime reproductive output would require counting thousands of offspring and would require waiting until the animal dies. In the meantime, work has already been invested in caring for the next generation whether or not their progenitors prove to have high lifetime fecundity. Instead, rather than the trait itself, selection can target a linked molecular marker, which is called marker-assisted selection (MAS) (Lande & Thompson, 1990). Identification of candidate genes and QTL through the aforementioned gene discovery methods are key to developing direct markers for genes that control the trait of interest, or markers that are in linkage disequilibrium with the trait and are proximate to the coding gene. Although much work remains to uncover the genetic bases of traits, there are already good results documented for arthropods linking genes and QTL to foraging (Page *et al.*, 2000), grooming (Oxley, Spivak, & Oldroyd, 2010), and *Varroa* mite resistance in honey bees (Behrens *et al.*, 2011), fertility in the parasitoid *Leptopilina clavipes* (Pannebakker *et al.*, 2004), and sex ratio (Pannebakker *et al.*, 2011), memory retention and olfaction (Hoedjes *et al.*, 2014, 2015), host specificity (Desjardins *et al.*, 2010), and pupal diapause (Paolucci *et al.*, 2016) in *N. vitripennis*.

2. Genomic selection. For complex traits with highly polygenic bases or genes with complicated epigenetic effects, direct and linkage equilibrium MAS may not be possible. In such cases, it is possible to use markers that are linked to a total breeding value instead of any specific phenotype (reviewed in (Dekkers, 2004)). Genomic selection employs this concept to potentially circumvent the need for proving marker causality. The statistical method of genomic selection uses information from genome-wide DNA-markers such as single nucleotide polymorphisms to select for complex traits (Meuwissen, Hayes, & Goddard, 2001) (Figure 4C). It is particularly helpful when artificial selection (marker assisted or not) is hampered by low heritability either through strong environmental noise and/or low levels of additive genetic variance. The genomic selection method considers markers distributed throughout the whole genome and estimates an effect of each marker, irrespective of the statistical significance of this effect. The total estimated genetic effect of an individual is the sum of the effects of all its markers as the genomic estimated breeding value (GEBV). By including effects of all markers, this method avoids missing a substantial portion of the genetic variance contributed by loci of minor effects, in contrast to methods that aim to identify the causal genes underlying traits. Although genomic selection still requires the collection of both genotypes and phenotypes, this work

only needs to be done for the initial reference population (and then at infrequent iterations as the predictive power of the reference population is gradually reduced over generations).

An advantage of genomic selection over traditional selection methods is that a higher accuracy of GEBVs can be achieved for traits of low heritability, and for traits that cannot be recorded on the selected candidate itself, but can be predicted through its genotype (Meuwissen *et al.*, 2001). Over the last decade, genomic selection has proven its potential in animal breeding, i.e., dairy cattle (Hayes *et al.*, 2009; Luan *et al.*, 2009; VanRaden *et al.*, 2009) and pigs (Lopes, 2016), but it has not yet been applied to biocontrol agents. Genomic selection methods may be particularly useful because GEBV can be estimated directly from the genotype, without the need for accurate pedigrees that are lacking for most biocontrol agents. One current challenge to genomic selection is the cost of large-scale SNP panels, but these are already undergoing a rapid drop in difficulty and expense. Also, collection of a sufficient amount of DNA may require sacrificing the selected candidate, but breeding of close relatives, such as offspring or full siblings, may offer a solution.

3. Population genetics. The availability of genetic markers allows for population genetic analyses, either at a coarse scale such as in the case of microsatellite panels, or at fine scale such as dense SNP panels based on assembled genomes. A powerful application of population genetics in biocontrol is monitoring of agents released into the field (Figure 4A), allowing for the assessment of their impact on existing natural enemies and monitoring their performance. Traditional neutral markers have been successfully used for performance monitoring of released strains (Hufbauer, 2004; Kazmer, Luck, & Mar, 2007; Guzmán-Larralde *et al.*, 2014). However, high density population genomic methods, such as GBS, allow for more detailed tracking of the introgression of the genetic material into previously released populations (Stouthamer & Nunney, 2014). Despite the importance of tracking the fate of released strains and their associated alleles, there has been little effort invested in genotype-based post-release monitoring in the field, possibly because of its logistic difficulty (Blossey & Skinner, 1999; Coombs & McEvoy, 1999). Typically, this approach involves bringing preserved individuals back to the laboratory for DNA extraction, PCR amplification, and sequencing. A recent novel approach allows real-time identification of a biocontrol agent in the field by loop-mediated isothermal amplification (LAMP) (Lee, 2017). This is a low-cost alternative to PCR that can be conducted in a single test tube and at a single temperature. In a biocontrol context, this method has been tested with the Asian chestnut gall wasp parasitoid *Tormyus sinensis* (Colombari & Battisti, 2016). It can efficiently be employed for measuring parasitisation rate in the field, especially as it can be used for juvenile stages when species identification is most difficult. The measurement of parasitisation rates in the field is not only useful for measuring the performance of the biocontrol agent, it also can help to assess non-target effects of released biocontrol agents (Garipey *et al.*, 2015;

Stahl *et al.*, 2019d). Genetic markers for post-release assessment is one of the most straightforward means for studying an agent's ecology in the field, but more advanced ecological interactions can also be examined for ways to optimize success.

Another important use of population genetics in biocontrol is the assessment of genetic variation. Loss of genetic variation is expected to occur in all captive populations, through inbreeding, selection, and random genetic drift, even when mass-reared at high numbers (Mackauer, 1976). Although this does not always result in fitness or performance loss, experiments have shown severe effects of inbreeding depression and domestication on the reproductive performance of large captive populations of *Drosophila* (Woodworth *et al.*, 2002) and a biocontrol population of a Chinese strain of the parasitoid *Bindoxys communis* (Garipey, Boivin, & Brodeur, 2014). Specific care should be taken when culturing parasitoid wasps. As haplodiploid species, parasitoids are generally expected to suffer less from inbreeding depression for fitness traits, but in some species a loss of genetic variation for sex-determination loci results in the loss of reproductively competent males, and can lead to extinction of captive populations (Stouthamer *et al.*, 1992; Zayed & Packer, 2005; Hein, Poethke, & Dorn, 2009; Retamal *et al.*, 2016; Zaviezo *et al.*, 2018; Leung, van de Zande, & Beukeboom, 2019). These potentially large effects on the fitness and performance make the monitoring of genetic variation a key part of mass-culturing biocontrol agents. For those species with an assembled genome, whole-genome Genotype-By-Sequencing (GBS) techniques (Baird *et al.*, 2008) allow for fine-scale population analyses by providing accurate allele frequency estimates to track evolution at a genomic scale and identify genomic regions under selection in contrasting ecological situations (Davey & Blaxter, 2010). This can also lead the way to unravelling the genetic architecture of relevant biocontrol traits.

4. Microbiome manipulation. Microbiomes may constitute an important target for modifying biocontrol agent performance. The composition of the microbial community of an organism can be altered through rearing conditions (e.g. a probiotic diet), via a breeding regime or by genetic manipulation (Grau, Vilcinskis, & Joop, 2017; Ras *et al.*, 2017). For example, *D. melanogaster* fed a probiotic bacterium were less susceptible to infections of pathogenic bacteria (Blum *et al.*, 2013). Sterile male performance was enhanced and *Pseudomonas* pathogen levels were reduced when *Ceratitis capitata* (the Mediterranean fruit fly) was fed bacterial supplements (Ami, Yuval, & Jurkevitch, 2010). Although these studies focused on flies used in sterile insect technique (mass releasing sterile males to outcompete wild individuals), the same principles can be applied to biocontrol agents. Probiotic diets can potentially improve the performance of mass-reared parasitoids directly, as can feeding on factitious hosts with a specific microbiome indirectly (Ras *et al.*, 2017; Koskinioti *et al.*, submitted). It is also possible for host specimens to transmit their

microbes to released biocontrol agents and influence their physiology and ecology (Schuler *et al.*, 2013). In such cases, microbial screening with genetic markers would be useful for investigating microbiome shifts that are responsible for phenotype alterations. It is also known that microbes can be responsible for chemical signals that attract parasitoids to their host. This implies that biocontrol agents can potentially be trained using these host microbiomes to be more efficient at finding hosts. In the case of defensive microbes in pest species, exposure to such microbes during development may confer immunity in the next generation (Ras *et al.*, 2017). These applications are, however, still theoretical and will require microbiome determination of specific biocontrol agent-pest pairs, identification of relevant symbionts, and development of methods to optimize their use.

A specific class of potentially useful symbionts are those that manipulate their hosts' reproduction, such as *Wolbachia* (Dedeine *et al.*, 2001; Vavre, Fouillet, & Fluery, 2003; Werren, Baldo, & Clark, 2008). *Wolbachia* can cause cytoplasmic incompatibility, which acts as a reproductive barrier among species or strains (Bourtzis *et al.*, 1996; Fouillet *et al.*, 2000; Gotoh, Noda, & Hong, 2003; Werren *et al.*, 2008). However, it is possible to cure arthropods of *Wolbachia* with antibiotics, permitting interspecies hybridisation or inter-strain reproduction (Breeuwer & Werren, 1995) and perhaps more radically, intentional re-infection with chosen *Wolbachia* strains (Grenier *et al.*, 1998; Watanabe, Kageyama, & Miura, 2013). This can, for example, be exploited to create strains that cannot interbreed with native congeners, reducing ecological risk. *Wolbachia* is also implicated in thelytokous reproduction (female parthenogenesis) in numerous insect species, such as the *Drosophila* parasitoids *Asobara* and *Leptopilina* (Breeuwer & Werren, 1995; Dedeine *et al.*, 2001; Schidlo *et al.*, 2002; Kremer *et al.*, 2009), aphid parasitoids (Starý, 1999), and *Trichogramma* species (Stouthamer, Luck, & Hamilton, 1990; Stouthamer & Kazmer, 1994). Thelytoky is particularly significant for parasitoids in biocontrol because only females have host-killing ability. In addition, parthenogenesis induction by *Wolbachia* can be used as a tool for advanced genotypic selection, which exploits the gamete duplication mechanism that underlies the parthenogenesis induction of *Wolbachia* and allows for fast selection of beneficial gene combinations in parasitoids for biocontrol (Russell & Stouthamer, 2011). In species that do not carry *Wolbachia*, intentional infection (Yamashita & Takahashi, 2018) can potentially be used to alter reproduction and life-history traits. Such transfection applications require careful testing, as *Wolbachia* phenotypes are not always the same between species (Veneti *et al.*, 2012). Also, *Wolbachia* can reduce the relative number of other potentially beneficial symbiotic bacteria (Audsley, Ye, & McGraw, 2017; Ye *et al.*, 2017) and conversely, other microbiota can outcompete *Wolbachia* (Kondo, Shimada, & Fukatsu, 2005; Goto, Anbutsu, & Fukatsu, 2006; Hughes, Rivero, & Rasgon, 2014; Rossi *et al.*, 2015). These competition dynamics within microbiomes (Brinker *et al.*, 2019; Gurung *et al.*, 2019) are an important

consideration when releasing manipulated strains into the field, as is the fact that new microbes introduced via hosts may become permanent fixtures in their ecosystem.

V. Conclusion

(1) It is a misconception that genetic solutions to biocontrol problems have been too complex to attempt, explaining the perceived “lack of progress” in the past several decades (Poppy & Powell, 2004; Lommen *et al.*, 2017; Kruitwagen *et al.*, 2018). Rather, the simpler approach of sourcing superior strains from nature has been more common.

(2) Improvement of biological control is highly needed, both in terms of better performance of existing agents as well as for expansion of the number and targets of new agents. Despite their general applicability, animal breeding techniques have not been exploited to their full potential in the biocontrol field. There are a number of reasons for this that, in contrast to e.g. livestock breeding, can be attributed to how biocontrol agents need to perform in a complex ecological environment. This makes it hard to decide which traits to optimize, but we are progressively gaining more knowledge on this, for instance by applying modelling frameworks (Plouvier & Wajnberg, 2018).

(3) In the new era of stricter trade laws and higher commercial demand, a new horizon of genetics in biocontrol practice is emerging (Figure 1). Several novel approaches are at hand, yet, each application requires proper contextualisation for a realistic projection of success.

(4) Areas where research and development are still needed can be staged (Figure 4). Marker-based methods (such as field-tracking and strain identification) are already being implemented (Figure 4A). Others are not yet in use but are imminently possible, such as integrating knowledge of genetic architecture to develop more effective breeding programs (Figure 4B). Still, others, such as genomic selection are currently largely in the theoretical realm. This may be due to novel technologies being too prohibitively labor intensive or expensive, or still requiring troubleshooting.

(5) The rapid development of genomic sequencing techniques and the resulting cost reductions (Wetterstrand, 2019) will find its way to biocontrol as it did to other biological disciplines such as microbiology, medicine, ecology and conservation (Handelsman, 2004; Hudson, 2008; Tautz, Ellegren, & Weigel, 2010; Ashley, 2016; Hohenlohe *et al.*, 2018; Supple & Shapiro, 2018).

Furthermore, even in the more advanced applications, it is likely that a first success will lead to rapid embracement by the scientific community and industry, parallel to e.g. the development of the PCR technique and the human genome project. For example, a proof-of-principle study on genomic selection for a biocontrol agent would prove its feasibility even if cost and efficiency still need further optimisation (Xia *et al.*, 2019) (Figure 4C).

(6) Novel methodology to uncover the genetic architecture of life-history traits, in combination with increasing investment in research and development by biocontrol companies, will rapidly expand the

knowledge base of the biocontrol field. Gene-editing techniques are a useful research tool for delineating this genetic architecture, but as the current biocontrol market depends on a reputation of using more traditional genetics methods, now is not the time to use gene-edited organisms in the field.

(7) Genetic variation lies at the basis of the potential of any organismal trait to evolve and hence our ability to improve traits. We have described the current genetic methodologies to uncover the genetic basis of important biocontrol traits. This knowledge can be used to design artificial selection programs, either by traditional selective breeding (White *et al.*, 1970; Ram & Sharma, 1977; Voroshilov, 1979; Roush & Hoy, 1981b; Hoy, 1986; Rosenheim & Hoy, 1988; Spollen & Hoy, 1992; Zhang *et al.*, 2018), or more sophisticated genomic selection (Xia *et al.*, 2019) or experimental evolution approaches (Lirakis & Magalhaes, 2019). Notably, fundamental insight has been obtained from laboratory investigations of the genetic model species *D. melanogaster* and *N. vitripennis*, and there are good reasons to believe that similar approaches can efficiently be applied to biocontrol agents. In fact, some species, notably haplodiploid parasitoid wasps, may confer additional advantages for efficient mass rearing, such as the possibility to alter progeny sex ratios and select for host searching learning behaviour.

(8) Another important requirement is the further integration of fundamental and applied research in biological control. The authors of this article have been collaborating within Breeding Invertebrates for Next Generation BioControl (BINGO)(BINGO-ITN, 2019). This is a 4-year EU-funded Marie Skłodowska-Curie Innovative Training Network (ITN) (BINGO-ITN, 2019) to “advance the current state of knowledge on the use of genetic variation in biocontrol practice with the simultaneous development of a new breed of young researchers that have an extensive suite of interdisciplinary skills that allows them to rise to the challenges of improving the efficiency of biological pest control through selective breeding of natural enemies in a broad range of agricultural systems and environmental conditions”. This has led to measurable scientific progress (Ras *et al.*, 2017; Lirakis, Dolezal, & Schlötterer, 2018; Stahl *et al.*, 2019c, 2019d; Stahl, Babendreier, & Haye, 2019b; Stahl *et al.*, 2019a; Stahl, Babendreier, & Haye, 2018; Plouvier & Wajnberg, 2018; Balanza, Mendoza, & Bielza, 2019; Koskinioti *et al.*, 2019; Xia *et al.*, 2019; Leung *et al.*, 2019; Lirakis & Magalhaes, 2019; Paspatis *et al.*, 2019; Le Hesran *et al.*, 2019; Ferguson *et al.*, in preparation b, in preparation c) as well as to an increasing awareness of the potential of biocontrol agent breeding among scientists, the biocontrol industry, growers, and the general public. We hope that this review will further stimulate the application of genetic and evolutionary methodology in next generation biological control. [7802 words]

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Figures

Figure 1. Overview of potential of genetic methods to address biocontrol challenges.

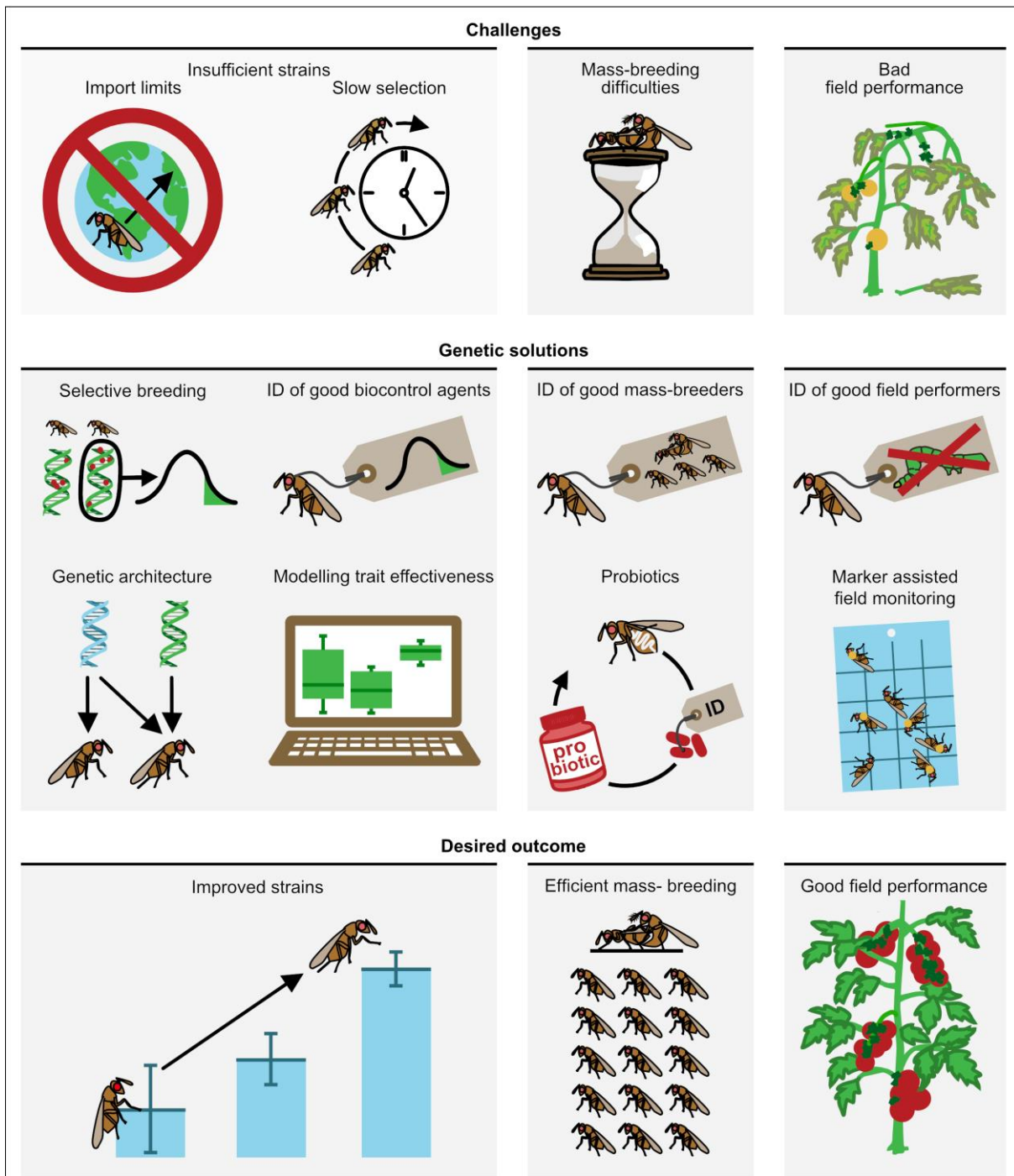


Figure 2. Guide to the use of genetic methods in research and development, sorted according to research question.

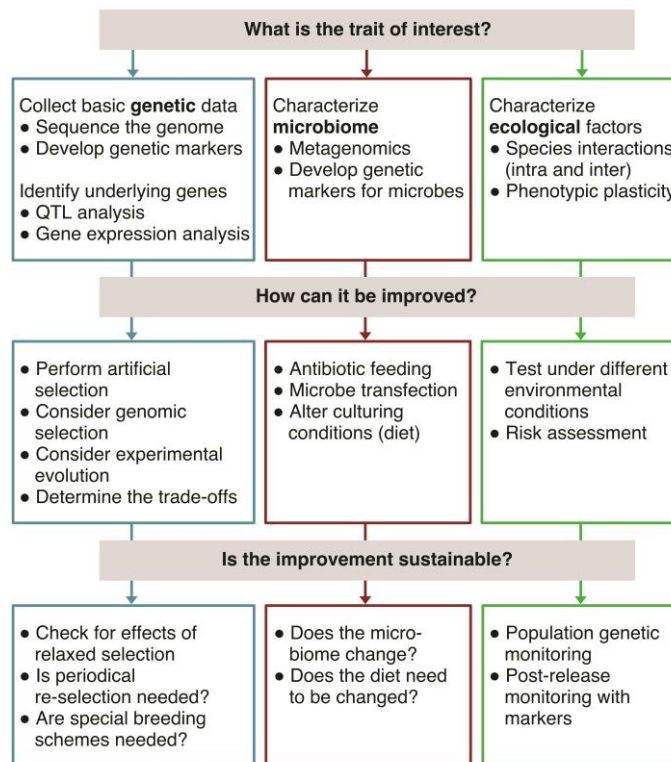


Figure 3. Sequencing strategy key for obtaining genomes of biocontrol agents.

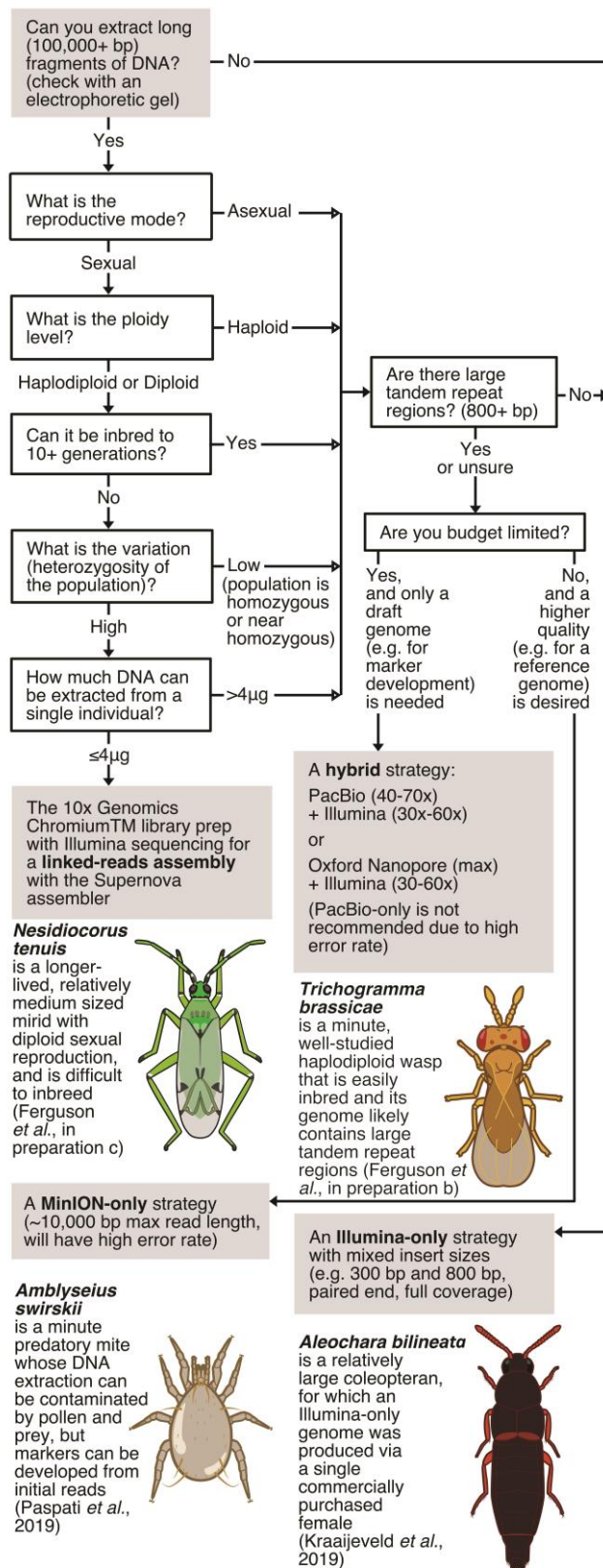


Figure 4. Examples of the application of genetic techniques in biocontrol, in increasing order of complexity. A) Genotyping for field monitoring of released biocontrol agents. B) Design optimal rearing strategy based on genetic architecture of traits of interest. C) Genomic selection to improve polygenic or hard-to-phenotype traits.

