

# Molecular Identification and Characterization of Biological Control Agents of Red Palm Weevil

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## About the Article

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## ABSTRACT

The red palm weevil is a date palm pest that is very destructive with a devastating economic and ecological impact on the global population. The traditional methods of management such as the use of chemical insecticides and pheromone traps do not always suffice because of the pesticide resistance, environmental risk and because larvae hide in the shadows and has a feeding habit. Biological control agents such as fungi, bacteria, nematodes, parasitoids as well as predators have been offered as effective and eco-friendly alternatives to the control of pests. The identification, characterization and application of biological control agents have been altered by molecular tools (DNA barcoding, PCR-based, multilocus phylogenetics, next-generation sequencing) providing a means to select the precise strain of the agent, identify cryptic diversity and predict efficacy and ecological, allowing adaptation. Molecular characterization of native biological control agents that are used to treat a variety of regions show that they are continuously more effective in increasing the efficiency of the pest control. Nonetheless, the bottlenecks such as the dearth of reference databases and lack of accessibility to sophisticated technologies in the underdeveloped nations notwithstanding, in the developing nations, incorporating molecular methods with biological control agents gives a viable way forward towards successful, area specific control of the red palm weevil. It is not only a method of reducing the usage of chemical pesticides but also a method of providing ecological and agricultural system with long-term sustainability.

## INTRODUCTION

Red palm weevil (*Rhynchophorus ferrugineus* Olivier) is widely regarded as one of the most destructive invasive pests of palm species worldwide, including date palm (*Phoenix dactylifera*), coconut (*Cocos nucifera*) and ornamental palms<sup>1</sup>. Native to South and Southeast Asia, it has rapidly spread to the Middle East, North Africa and parts of Europe, becoming a major global biosecurity threat<sup>2</sup>. The larval stages cause severe internal damage by tunneling within palm trunks, weakening the vascular system and ultimately leading to death of the tree<sup>3</sup>. The economic impact of RPW infestations is estimated to reach billions of dollars annually due to yield losses, tree mortality and eradication costs<sup>3</sup>.

Conventional control strategies have relied primarily on chemical insecticides applied through trunk injections, crown drenching and pheromone traps<sup>5</sup>. Although, these approaches may provide short-term suppression, they suffer from significant limitations, including environmental contamination, the development of pesticide resistance and inadequate effectiveness against concealed larval stages<sup>6</sup>. Moreover, the prolonged use of chemical control disrupts beneficial arthropod populations and poses risks to both ecosystem integrity and human health<sup>7</sup>. Consequently, there is an urgent need to incorporate environmentally sustainable alternatives into integrated pest management programs<sup>8</sup>.

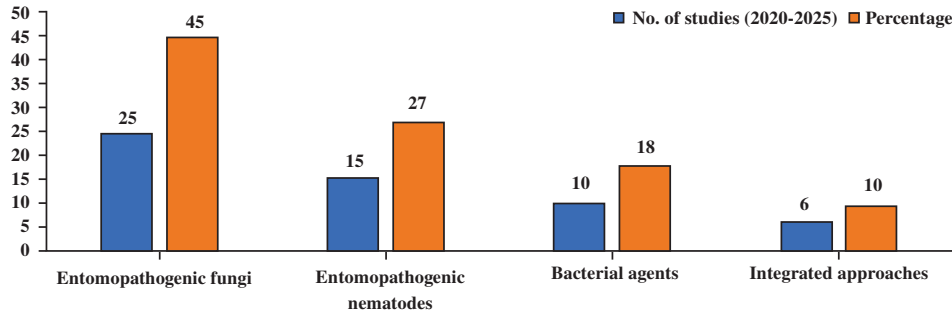


Fig. 1: Distribution of research studies (2020-2025) on different biological control agents against RPW

Table 1: Comparison of conventional and biological control approaches for the red palm weevil (RPW)

Approach	Examples/Agents	Mode of Action	Advantages	Limitations	Citations
Conventional (Chemical)	Insecticides (trunk injection, crown drenching), pheromone traps	Direct toxicity to larvae/adults; pheromone-based mass trapping	Rapid action; widely available; initially effective	Environmental contamination; pesticide resistance; poor penetration into concealed larvae; harmful to beneficial organisms; health risks	Khan <i>et al.</i> , <sup>9</sup>
Biological (Microbial and Natural Enemies)	Entomopathogenic fungi ( <i>Beauveria</i> , <i>Metarhizium</i> ); Entomopathogenic nematodes ( <i>Steinernema</i> , <i>Heterorhabditis</i> ); Bacteria ( <i>Bacillus</i> , <i>Serratia</i> )	Infection through cuticle or natural openings; symbiotic bacteria release; toxin-mediated mortality	Eco-friendly; target-specific; compatible with IPM; potential for long-term suppression	Variable field performance; sensitive to environmental factors; slower kill rate; requires molecular identification and formulation optimization	Yang <i>et al.</i> , <sup>15</sup>

Table 1 presents a comparative analysis of conventional and biological control methods against RPW. While chemical insecticides and pheromone traps can offer rapid population reduction, their effectiveness is constrained by resistance development, environmental pollution and limited efficacy against hidden larvae<sup>9</sup>. In contrast, biological control agents such as entomopathogenic fungi, nematodes and bacteria act through natural infection mechanisms, are environmentally benign and can be readily integrated into IPM strategies. Despite challenges related to formulation and field stability, biological control represents a more sustainable and long-term solution for RPW management<sup>10</sup>.

Biological Control Agents (BCAs) are increasingly recognized as effective and environmentally sustainable tools for suppressing RPW populations. Both larvae and adults of RPW are highly susceptible to entomopathogenic fungi (EPF), particularly *Beauveria bassiana* and *Metarhizium anisopliae*<sup>11</sup>. Several isolates have been evaluated under laboratory and semi-field conditions, with reported mortality rates exceeding 80%<sup>12</sup>. Entomopathogenic nematodes (EPNs), including *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, are also highly infective, entering the host through natural openings and releasing symbiotic bacteria that cause death of the host<sup>13</sup>. These bacteria, such as *Serratia nematodiphila* and *Bacillus thuringiensis*, have likewise demonstrated antagonistic activity against RPW under experimental conditions<sup>14</sup>.

Collectively, these BCAs form the foundation of sustainable RPW management programs when integrated with cultural and pheromone-based strategies<sup>15</sup>.

Figure 1 illustrates the distribution of research efforts on biological control agents targeting RPW between 2020 and 2025. The majority of studies have focused on entomopathogenic fungi, owing to their proven pathogenicity and adaptability across diverse field conditions<sup>16</sup>. Entomopathogenic nematodes represent the second most extensively investigated group, largely due to their capacity to target concealed life stages. Although, bacterial agents remain comparatively underexplored, laboratory studies have produced promising results and integrated approaches combining multiple BCAs with pheromone-based systems are increasingly advocated for sustainable RPW management<sup>17</sup>. This trend reflects growing emphasis on environmentally friendly alternatives to chemical control.

Despite their promising efficacy, large-scale application of BCAs requires precise identification and molecular characterization<sup>18</sup>. Morphological traits alone are insufficient due to the presence of cryptic species, strain-level variation and environmentally induced phenotypic plasticity<sup>19</sup>. Entomopathogenic fungi and nematodes have undergone substantial taxonomic and systematic revisions through the use of molecular tools such as DNA barcoding (ITS, COI), multilocus sequence typing (EF1- $\alpha$ , RPB1, RPB2) and phylogenomic analyses<sup>20</sup>. For example, ITS sequencing has

Table 2: Major biological control agents evaluated against the red palm weevil (RPW) and their key characteristics

Biological control agent	Representative examples	Mode of action	Key findings	Citations
Entomopathogenic fungi (EPF)	<i>Beauveria bassiana</i> , produce toxins <i>Metarhizium anisopliae</i>	<i>Penetrate cuticle</i> , lab and and enzymes causing host death	>80% adult mortality in field trials; oil-based and nano- formulations improve persistence under arid conditions; effective with pheromone traps	Irshaid <i>et al.</i> <sup>76</sup>
Bacterial agents	<i>Bacillus thuringiensis</i> (Bt), <i>Serratia marcescens</i>	Bt Cry toxins disrupt midgut cells; <i>Serratia</i> produces enzymes and metabolites (proteases, prodigiosin)	Bt: larval mortality within 72 hrs; <i>Serratia</i> : strong insecticidal effects; recombinant Bt strains under development	Guo <i>et al.</i> <sup>124</sup>
Entomopathogenic nematodes (EPNs)	<i>Steinernema carpocapsae</i> , <i>Heterorhabditis</i> <i>bacteriophora</i>	Release symbiotic bacteria ( <i>Xenorhabdus</i> , <i>Photorhabdus</i> ) inside host	Infect concealed larvae/pupae; gel and chitosan carriers enhance field survival; integrated with IPM	Nurashikin-Khairuddin <i>et al.</i> <sup>10</sup>
Parasitoids and predators	<i>Chelonus</i> spp. (egg parasitoid), <i>scolia erratica</i> (larval/pupal predator), ants, beetles	Parasitism and predation reduce RPW populations	Egg parasitism reduces early infestations; wasps and predators attack larvae/pupae; under- documented but promising for organic systems	Sarwar <sup>125</sup>

been used to identify virulent *Beauveria* and *Metarhizium* isolates recovered from RPW cadavers<sup>21</sup>, while multilocus phylogenetic approaches have resolved species complexes within *Heterorhabditis* and *Steinernema*<sup>22</sup>.

Bacterial BCAs have similarly been characterized using molecular techniques, leading to the identification of *Serratia* and *Bacillus* isolates associated with RPW that exhibit high insecticidal activity<sup>23</sup>. Moreover, molecular studies have elucidated toxin-host interactions, such as the binding of *B. thuringiensis* Cry toxins to midgut receptors in RPW<sup>24</sup>. These insights facilitate the selection of highly potent strains for bioformulation development and field application. Recent advances in genomics and metagenomics have further strengthened RPW biocontrol research<sup>25</sup>. Sequencing of the RPW genome has provided valuable information on host-pathogen interaction mechanisms and molecular targets<sup>26</sup>, while metagenomic profiling of the RPW gut microbiota has revealed bacterial communities capable of antagonizing or synergizing with applied BCAs<sup>27</sup>. Integration of such molecular data enhances the predictability and stability of BCA performance under diverse ecological conditions<sup>2</sup>.

Nevertheless, several challenges remain. The lack of comprehensive barcode databases for most entomopathogens hampers their accurate identification, particularly in under-studied regions<sup>28</sup>. Inconsistencies in marker resolution, limited access to sequencing facilities and regulatory constraints on the registration of microbial agents further restrict the development and deployment of molecularly characterized BCAs<sup>29</sup>. Despite these limitations, the efficacy and reliability of RPW biocontrol agents continue to improve due to the increasing integration of molecular tools in biological control research<sup>23</sup>. This review

synthesizes literature published between 2020 and 2025 on the molecular identification and characterization of entomopathogenic fungi, nematodes and bacteria for the management of *Rhynchophorus ferrugineus*<sup>11</sup>. It also highlights the critical role of molecular approaches in the selection, standardization and implementation of BCAs for the sustainable management of red palm weevil<sup>12</sup>.

**Biological control agents of RPW:** Biological control agents (BCAs) represent a promising, environmentally friendly alternative to chemical insecticides for the management of red palm weevil (RPW), *Rhynchophorus ferrugineus*. Entomopathogenic fungi, particularly *Beauveria bassiana* and *Metarhizium anisopliae*, are widely utilized due to their ability to infect both adult and larval stages and induce high mortality under laboratory and field conditions<sup>30</sup>. Similarly, entomopathogenic nematodes (EPNs) such as *Steinernema* and *Heterorhabditis* have demonstrated considerable potential in penetrating palm tissues to infect larvae and pupae, thereby suppressing RPW populations<sup>31</sup>. In addition to microbial agents, natural predators (e.g., ants, earwigs, and certain beetles) and parasitoids (e.g., *Scleroderma guani* and *Chelisoche morio*) contribute to secondary reductions in RPW infestations<sup>32</sup>. Furthermore, recent advances integrating BCAs with semiochemical-based trapping and cultural control practices have enhanced their effectiveness within integrated pest management (IPM) frameworks<sup>33</sup>. Overall, biological control agents constitute an essential, eco-friendly, and long-term strategy for mitigating the economic and ecological impacts of RPW. Table 2 summarizes the principal BCAs evaluated against RPW, including representative taxa, modes of action, and key findings.

According to this summary, entomopathogenic fungi and nematodes are consistently effective against concealed developmental stages of RPW, bacterial agents can induce rapid larval mortality, and parasitoids and predators play an emerging yet underdocumented role in pest management programs<sup>34</sup>.

**Entomopathogenic fungi:** Among the most extensively studied biological control options against *Rhynchophorus ferrugineus* are entomopathogenic fungi (EPF), particularly *Beauveria bassiana* and *Metarhizium anisopliae*<sup>35</sup>. These fungi infect weevils through cuticular penetration, followed by the production of toxins and enzymes that result in host death within a few days<sup>36</sup>. Recent studies have emphasized strain variability, with highly virulent isolates causing more than 80% adult mortality in both laboratory and field trials<sup>4</sup>. Novel formulations, including oil-based and nanoemulsified conidia, have substantially improved persistence and efficacy under high-temperature and arid conditions typical of date palm orchards<sup>36</sup>. Large-scale applications of *M. anisopliae* in Saudi Arabia and the United Arab Emirates have demonstrated that combining fungal treatments with pheromone-based trapping systems significantly reduces weevil infestation levels<sup>37</sup>.

**Bacterial agents:** RPW has been evaluated against bacterial antagonists such as *Bacillus thuringiensis* (Bt) and *Serratia marcescens*, both of which exhibit insecticidal properties<sup>38</sup>. Bt-based biopesticides containing Cry toxins have been shown to be orally toxic to RPW larvae, causing midgut epithelial disruption and resulting in death within 72 hs<sup>39</sup>. Similarly, *Serratia marcescens* has demonstrated strong entomopathogenic activity, particularly through the production of proteins and the prodigiosin pigment, which possess antimicrobial and insecticidal properties<sup>40</sup>. Moreover, lineage-based bioinsecticides are under development, including genetically engineered Bt strains and recombinant plasmids with enhanced efficacy<sup>39</sup>.

**Entomopathogenic nematodes:** Entomopathogenic nematodes (EPNs), particularly *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, are obligate parasites that harbor symbiotic bacteria (*Xenorhabdus* and *Photorhabdus*, respectively), which are responsible for host mortality<sup>41</sup>. EPNs have been demonstrated to infect concealed RPW larvae and pupae within palm trunks, making them particularly suitable for managing this cryptic pest<sup>13</sup>. The use of gel and chitosan carriers has been shown to enhance nematode survival and infectivity under field conditions<sup>10</sup>. Increasingly, IPM programs integrating pheromone trapping and cultural practices are incorporating EPNs as a core biological control component<sup>42</sup>.

**Parasitoids and predators:** The application of natural enemies remains less developed compared with microbial agents; however, parasitoids and predators are increasingly recognized as potential tools for RPW suppression. RPW eggs have been reported to be parasitized by braconid parasitoids such as *Chelonus* spp., which can reduce initial infestations in palm plantations<sup>23</sup>. Natural suppression also involves ant species and predatory beetles, although evidence for their effectiveness is largely ecological and remains poorly documented<sup>43</sup>. These natural enemies may nonetheless strengthen sustainable control strategies, particularly in organic and low-pesticide production systems<sup>10</sup>.

**Molecular identification and characterization approaches:** Molecular technologies have substantially advanced the identification, characterization and deployment of Biological Control Agents (BCAs) against Red Palm Weevil (RPW), *Rhynchophorus ferrugineus*<sup>44</sup>. Conventional morphological methods often fail to detect cryptic species or explain strain-level variation that contributes to inconsistent field performance<sup>45</sup>. By integrating molecular approaches, researchers can achieve precise taxonomic resolution, assess genetic diversity and identify virulence-associated traits of entomopathogenic fungi, bacteria and nematodes<sup>46</sup>. This section provides an overview of key molecular methods, including DNA barcoding, PCR-based techniques, next-generation sequencing (NGS) and phylogenetic analyses, which together underpin modern, sustainable RPW management strategies<sup>47</sup>. Table 3 presents a comparative overview of molecular tools used for the identification and characterization of RPW biological control agents<sup>48</sup>, detailing the genetic markers, target organisms and applications. It demonstrates how combining DNA barcoding, PCR-based methods, NGS and phylogenetics enhances strain resolution, virulence assessment and biosafety evaluation in RPW biocontrol programs<sup>49</sup>.

**DNA barcoding:** Molecular diagnostics in pest and Biological Control Agent (BCA) research have increasingly relied on DNA barcoding. In insects, the universal barcode is the mitochondrial cytochrome oxidase subunit I (COI) gene, whereas the internal transcribed spacer (ITS) region is widely used for fungi<sup>50</sup>. For nematodes, the 18S rRNA gene and the D2-D3 expansion segments of the 28S rRNA gene are commonly employed<sup>51</sup>. These markers have proven effective in resolving ambiguities arising from morphological plasticity. For example, COI sequencing has successfully distinguished *Steinernema carpocapsae* from the morphologically similar *S. feltiae*, ensuring that only the most virulent nematode strains are incorporated into RPW biocontrol programs<sup>52</sup>. Similarly, ITS barcoding of

Table 3: Summary of molecular approaches used for the identification and characterization of biological control agents (BCAs) against the red palm weevil.

Approach	Common Markers/Genes	Target Organisms (BCAs)	Applications	Key Advantages	References
DNA Barcoding	COI (insects, nematodes), ITS (fungi), 18S and 28S rRNA (nematodes)	Fungi, nematodes, RPW	Species-level identification, resolving cryptic species, strain confirmation	High accuracy, global databases (BOLD, GenBank)	Singh <i>et al.</i> <sup>52</sup>
PCR-Based methods	ITS, COI, 16S rRNA, RAPD, ISSR, AFLP	Fungi, bacteria, nematodes	Rapid diagnostics, genetic diversity analysis, field-ready detection (LAMP, portable PCR)	Fast, cost-effective, strain differentiation	Antil <i>et al.</i> <sup>126</sup>
Next-Generation Sequencing (NGS)	Whole-genome, metagenome, transcriptome	Fungi, nematodes, symbiotic bacteria	Virulence gene discovery, microbiome studies, QC of commercial products	High-throughput, functional insights	Zhang <i>et al.</i> <sup>65</sup>
Phylogenetic analysis	EF1- $\alpha$ , RPB1, RPB2, $\beta$ -tubulin, WGS data	Fungi, nematodes	Taxonomy, strain selection, biosafety assessment	Clarifies evolutionary relationships, ensures ecological safety	Sun <i>et al.</i> <sup>71</sup>

*Beauveria* and *Metarhizium* isolates recovered from RPW cadavers has enabled rapid identification of entomopathogens, facilitating mass production and field formulation<sup>53</sup>.

DNA barcoding and reference libraries have become essential tools for fungal identification, enabling rapid, accurate species discrimination and enhancing traceability across different geographic regions<sup>54</sup>. DNA barcoding data are also integrated into international databases, including BOLD and GenBank, providing comparative frameworks for RPW biocontrol research<sup>55</sup>. Nevertheless, this approach has limitations, as single-locus barcoding is often insufficient to discriminate closely related strains; therefore, multilocus or genomic methods are required to complement it<sup>56</sup>.

**PCR-based methods:** PCR-based techniques are cost-effective, sensitive and rapid tools for BCA identification and gene analysis. Species-specific primers targeting conserved regions of the ITS, COI and 16S rRNA genes have been extensively used to detect microbial agents within RPW-infected tissues<sup>57</sup>. Multiplex PCR further enables the simultaneous detection of multiple pathogens, including *Beauveria*, *Metarhizium* and *Bacillus thuringiensis*, in a single reaction, thereby streamlining diagnostics in integrated management programs<sup>58</sup>.

Molecular marker systems such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR) and Amplified Fragment Length Polymorphism (AFLP) have been applied to assess intra- and inter-population genetic diversity. RAPD markers, for instance, have revealed substantial genetic variation among *Beauveria bassiana* strains, some of which are associated with enhanced thermotolerance and pathogenicity toward RPW<sup>59</sup>. Similarly, ISSR markers applied to *Metarhizium anisopliae* isolates have shown that strains from arid environments possess genetic adaptations linked to desiccation tolerance<sup>60</sup>. AFLP analysis has been particularly valuable in nematodes, revealing cryptic divergence among *Heterorhabditis bacteriophora* populations across diverse agroecological regions<sup>52</sup>.

PCR-based diagnostics are also being adapted for field use through portable PCR platforms and Loop-mediated Isothermal Amplification (LAMP), enabling rapid identification of entomopathogens in RPW-infested plantations<sup>61</sup>. Collectively, these technologies bridge laboratory research and field application, improving diagnostic turnaround and enhancing the responsiveness of Integrated Pest Management (IPM) programs<sup>62</sup>.

**Next-generation sequencing (NGS):** Next-generation sequencing (NGS) has revolutionized molecular entomology and microbial ecology by enabling high-throughput, high-resolution characterization of genetic material<sup>2</sup>. Metagenomic analyses of RPW-associated microbiomes have revealed diverse bacterial and fungal communities, some of which may act antagonistically or synergistically with applied BCAs<sup>63</sup>. Such information is essential for predicting the stability and performance of microbial formulations under field conditions<sup>64</sup>.

Whole-genome sequencing of entomopathogenic fungi has identified key virulence-associated genes, including those encoding cuticle-degrading enzymes, secondary metabolite biosynthetic clusters and stress-response pathways<sup>65</sup>. Similarly, genomic studies of entomopathogenic nematodes and their symbiotic bacteria (*Xenorhabdus* and *Photorhabdus*) have uncovered toxin secretion systems and antimicrobial peptide biosynthesis pathways that are critical for overcoming insect immune defenses<sup>66</sup>. Population genomic analyses of *Beauveria* and *Metarhizium* using NGS have further demonstrated their capacity for divergence and adaptation to specific agroecological niches<sup>67</sup>.

These genomic investigations are complemented by transcriptomic analyses that identify differentially expressed genes during RPW larval infection, providing molecular markers for strain improvement<sup>1</sup>. Moreover, NGS technologies are increasingly applied in the quality control of commercial BCA products, as entire microbial formulations can be sequenced to verify strain identity, detect contaminants and ensure functional reliability in the

field<sup>68</sup>. This is particularly relevant in regions such as the Middle East and South Asia, where counterfeit or substandard microbial products may undermine farmer confidence in biological control strategies<sup>69</sup>.

**Phylogenetic analysis:** Phylogenetic approaches are essential for elucidating evolutionary relationships, species boundaries and ecological adaptation of Biological Control Agents (BCAs). The application of Multilocus Sequence Typing (MLST) using EF1- $\alpha$ , RPB1, RPB2 and tubulin genes has become widespread in defining the taxonomy of *Beauveria*, *Metarhizium* and *Isaria* species. These analyses have revealed well-supported clades that are often associated with differences in virulence, thermal tolerance and host specificity. Whole-genome phylogenomic approaches have further uncovered cryptic lineages within entomopathogenic nematodes such as *Heterorhabditis* and *Steinernema*, enabling the identification of strains with high reproductive capacity and enhanced virulence against concealed RPW larval stages<sup>69</sup>.

Phylogenetic trees constructed using Bayesian and maximum-likelihood methods also demonstrate geographic structuring, highlighting the importance of selecting locally adapted strains for effective biocontrol deployment<sup>71</sup>. Importantly, phylogenetic analyses support biosafety assessments by enabling estimation of non-target risks and potential ecological disturbances associated with candidate strains placed within specific evolutionary lineages<sup>72</sup>. Consequently, such analyses have become a regulatory requirement in many countries prior to the large-scale release of microbial biocontrol agents.

**Case studies and global research progress:** Over the past decade, the application of molecular tools to identify and characterize BCAs against the red palm weevil (*Rhynchophorus ferrugineus*) has advanced substantially. Case studies from diverse geographic regions demonstrate that entomopathogenic fungi, nematodes and bacteria are most effective when molecular diagnostics guide their selection and deployment<sup>73</sup>. Collectively, these studies reflect a global shift toward molecularly informed biocontrol programs and provide comparative insights into relative effectiveness across ecological contexts. Graph 2 illustrates the performance of molecularly characterized BCAs in managing RPW in different regions, showing that locally adapted fungal and nematode isolates are generally the most effective<sup>74</sup>.

**Case studies from the middle east:** The Middle East, particularly the Gulf region, has experienced some of the most severe RPW infestations and has consequently become a major center for biocontrol research<sup>75</sup>. In Saudi Arabia,

molecular identification of *Beauveria bassiana* isolates using ITS and TEF1- $\alpha$  markers demonstrated that specific genetic clusters were strongly associated with virulence against RPW, with local isolates causing larval mortality rates exceeding 85%<sup>76</sup>. Similarly, multilocus phylogenetic analyses of *Metarhizium anisopliae* strains isolated from infested date palms in Oman confirmed their adaptation to arid environments and their expression of cuticle-degrading enzymes<sup>77</sup>.

Entomopathogenic nematodes (EPNs) have also received considerable attention in the region. COI and ITS sequencing revealed the presence of *Steinernema abbasi* and *Heterorhabditis indica* in UAE soils, both of which exhibited rapid pathogenicity against concealed RPW larvae<sup>78</sup>. Field isolates achieved mortality rates exceeding 75%, outperforming non-local strains. Additionally, metagenomic analyses of RPW cadavers in Kuwait identified fungal endophytes, including *Cladosporium* and *Aspergillus* spp., which may exert antagonistic interactions with RPW<sup>17</sup>.

**Advances from India:** India, a leading producer of coconuts and date palms, has reported several successful case studies involving molecularly identified BCAs. RAPD and ISSR markers revealed substantial genetic diversity among *Beauveria bassiana* isolates from Kerala and Tamil Nadu, with certain strains exhibiting enhanced heat tolerance and pathogenicity under tropical conditions<sup>80</sup>. The virulence of *Metarhizium anisopliae* isolates against RPW was confirmed through ITS-based characterization of strains collected in Andhra Pradesh, with larval mortality reaching up to 90% within 10 days<sup>81</sup>. Nematode diversity has also been elucidated using molecular tools; *Steinernema siamkayai* and *Heterorhabditis indica* were verified using COI and 28S rRNA sequences and native strains were found to outperform imported isolates under field conditions<sup>10</sup>. Rapid on-site detection of EPNs has further been enabled by portable PCR assays, reducing diagnostic delays during field implementation<sup>41</sup>. Overall, Indian case studies emphasize the application of molecular markers to select heat-tolerant and highly virulent strains for deployment in tropical agroecosystems<sup>82</sup>.

**Research progress in Pakistan:** Pakistan has emerged as a key contributor to RPW molecular biocontrol research, particularly in date palm-producing regions of Sindh and Balochistan<sup>83</sup>. ITS and COI sequencing confirmed the presence of diverse entomopathogenic fungi, including *Beauveria*, *Metarhizium* and *Lecanicillium* spp., many of which possess haplotypes adapted to local desert environments<sup>84</sup>. Comparative bioassays demonstrated that native isolates exhibited higher virulence and spore viability under arid conditions than commercial strains<sup>85</sup>. Molecular

barcoding of nematodes further revealed that several *Heterorhabditis* and *Steinernema* species are prevalent in Punjabi and Sindhi soils, expanding the known diversity of Pakistani EPNs<sup>86</sup>. Field trials showed that native isolates of *H. indica* achieved over 75% mortality of RPW larvae, supporting their suitability for incorporation into local IPM programs<sup>87</sup>. Recent studies employing qPCR monitoring have also assessed EPN persistence in palm soils, providing critical insights into their survival dynamics under harsh climatic conditions<sup>88</sup>.

**European research contributions:** Europe, particularly Mediterranean countries such as Spain and Italy, has made substantial contributions to molecularly guided RPW biocontrol research<sup>89</sup>. Multilocus phylogenetic analyses of *Metarhizium anisopliae* and *Isaria fumosorosea* in Spain identified specific clades associated with increased virulence and thermal stability<sup>90</sup>. Simple sequence repeats (SSRs) and AFLP markers have been applied to monitor strain identity and genetic stability in commercial *Beauveria bassiana* formulations, ensuring product quality and consistency<sup>91</sup>. In Italy, metagenomic profiling of RPW microbiota has been used to identify bacterial symbionts that influence susceptibility to entomopathogens, providing new insights into combined microbial biocontrol strategies<sup>2</sup>. Field trials conducted in Europe and elsewhere consistently demonstrate that locally adapted, molecularly verified isolates are more effective in controlling RPW infestations than exotic strains, underscoring the importance of region-specific biocontrol solutions<sup>92</sup>.

**Comparative effectiveness of molecularly characterized BCAs:** Interregional comparisons consistently demonstrate that molecular characterization is essential for the effective selection of BCAs. For fungal agents, ITS and multilocus sequencing have been successfully applied to distinguish highly virulent strains of *Beauveria bassiana* and *Metarhizium anisopliae*, enabling their targeted deployment across desert, tropical and Mediterranean climatic conditions<sup>93</sup>. Similarly, COI-based barcoding has revealed cryptic taxonomic diversity within *Steinernema* and *Heterorhabditis*, ensuring that only the most virulent lineages are released<sup>13</sup>.

Molecularly validated bacterial agents, including *Bacillus thuringiensis* and *Serratia marcescens*, have likewise been shown to exert significant insecticidal effects against RPW<sup>38</sup>. Comparative analyses consistently indicate that molecularly verified native isolates exhibit greater adaptability, enhanced survival and superior field performance compared with imported or uncharacterized strains<sup>94</sup>.

**Challenges and limitations:** Despite rapid advances in the molecular diagnostics of Biological Control Agents (BCAs) against *Rhynchophorus ferrugineus* (RPW) (Fig. 2), significant constraints continue to limit their widespread integration into integrated pest management (IPM) programs<sup>72</sup>. These challenges include incomplete reference databases, variability in molecular marker performance and persistent technical and financial barriers in developing countries. Addressing these limitations is critical for developing sustainable and globally applicable management strategies<sup>95</sup>. Table 4 summarizes the key issues restricting the molecular characterization and utilization of BCAs against RPW, identifying three major constraints: The lack of consistent reference sequences, variability among molecular markers and technical and financial limitations in developing nations. These factors collectively affect accurate identification, strain selection and the broader adoption of molecular tools in IPM programs<sup>96</sup>.

The application of molecular tools for the identification and characterization of BCAs against RPW is associated with several critical shortcomings. One of the primary challenges is the scarcity of high-quality reference sequences. Reliable detection of entomopathogenic fungi and nematodes depends on curated databases such as GenBank, UNITE and BOLD; however, most RPW-associated BCAs remain underrepresented. In particular, although *Beauveria bassiana* and *Metarhizium anisopliae* are widely used, strains from arid and tropical regions are inadequately represented, limiting the accurate characterization of isolates from the Middle East and South Asia. Even when sequences are available, annotation errors remain common, with fungal ITS entries in GenBank reportedly comprising approximately 25% misidentified and 10% poorly annotated sequences<sup>99</sup>. Such inaccuracies can lead to false positives in barcoding analyses and the incorrect selection of strains for RPW control<sup>100</sup>.

Similar challenges occur in nematode identification, as COI and 28S rRNA reference sequences for *Steinernema* and *Heterorhabditis* are limited for RPW-endemic regions, complicating the confirmation of cryptic lineages<sup>101</sup>. Native isolates from countries such as Pakistan and Oman often do not match existing reference sequences, resulting in uncertainty regarding their phylogenetic placement<sup>102</sup>.

Another major limitation is the variability of molecular markers. Although, the Internal Transcribed Spacer (ITS) region serves as the universal fungal barcode, it provides insufficient resolution within the *Beauveria* and *Metarhizium* species complexes relevant to RPW<sup>102</sup>. Multilocus sequencing approaches using TEF1- $\alpha$ ,  $\beta$ -tubulin and RPB1/2 have been proposed to improve species delimitation; however, their inconsistent application across laboratories hampers cross-regional comparisons<sup>103</sup>. In nematodes, mitochondrial COI is widely used, yet intraspecific variation often overlaps with interspecific

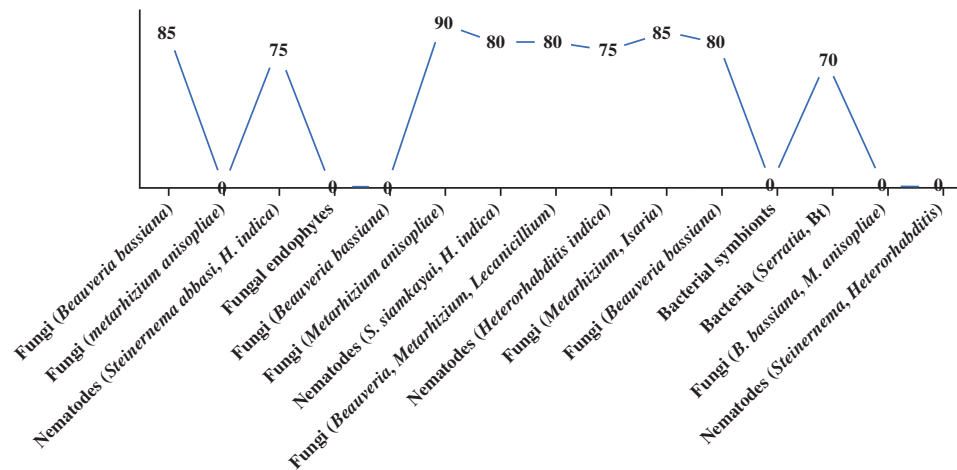


Fig. 2: Effectiveness of molecularly characterized BCAs against RPW mortality (%)

Table 4: Key challenges and limitations in molecular diagnostics of biological control agents (BCAs) against the red palm weevil (RPW)

Category	Specific Issues	Consequences for RPW BCA Research	Citations
Reference databases	Limited representation of <i>Beauveria</i> , <i>Metarhizium</i> , <i>Steinernema</i> , <i>Heterorhabditis</i> in GenBank, UNITE, BOLD	Reduced accuracy in isolate identification; cryptic lineages remain unresolved	Nilsson <i>et al.</i> , <sup>97</sup>
	Annotation errors (up to 25% of fungal ITS entries misidentified)	Risk of false positives in barcoding; misguides strain selection	Pham <i>et al.</i> , <sup>99</sup>
Molecular Markers	ITS: universal barcode but poor resolution in species complexes	Misidentification within <i>Beauveria</i> and <i>Metarhizium</i>	Stengel <i>et al.</i> , <sup>123</sup>
	Inconsistent adoption of TEF1- $\alpha$ , $\beta$ -tubulin, RPB1/2 across labs	Difficult to compare regional studies	Knot <i>et al.</i> , <sup>86</sup>
Technical and Financial Constraints	Limited access to sequencing platforms, bioinformatics pipelines, culture banks	Dependence on foreign institutions; delays in outputs	Singh-Moodley <i>et al.</i> , <sup>105</sup>
	High costs of DNA kits, PCR reagents, sequencing services	Fewer isolates processed; incomplete population studies	Mfuh <i>et al.</i> , <sup>106</sup>

divergence, complicating taxonomic resolution<sup>86</sup>. Furthermore, the use of multiple markers (COI, ITS, 28S rRNA) across different studies complicates data integration<sup>18</sup>.

Marker-based analyses are also affected by technical biases. RAPD and ISSR markers are highly sensitive to PCR conditions and generally lack reproducibility, whereas AFLP and SSR markers are more robust but require specialized expertise and equipment, limiting their use in resource-constrained laboratories<sup>104</sup>. Although, advanced technologies such as SNP arrays, RAD-seq and whole-genome sequencing (WGS) offer higher resolution and trait association, they remain impractical and cost-prohibitive in many RPW-affected regions<sup>48</sup>. Global initiatives, including UNITE+INSDC and regional barcoding consortia, have sought to address these gaps, yet progress has been limited, particularly in representing isolates from under-sampled agroecosystems<sup>97</sup>.

Technical and financial constraints in developing nations further exacerbate these challenges, as these regions bear the greatest burden of RPW infestations. Laboratories in South Asia, the Gulf and North Africa often lack access to sequencing platforms, bioinformatics pipelines and long-term culture facilities<sup>105</sup>. This limitation restricts the

feasibility of multilocus or genomic studies and necessitates reliance on international collaborations<sup>106</sup>. In addition, the cost of imported DNA extraction kits, PCR reagents and sequencing services is frequently two to three times higher than in developed countries, limiting the number of isolates that can be processed and preventing large-scale population studies<sup>107</sup>.

Bioinformatics expertise is similarly scarce, even where molecular data are generated. Many laboratories lack trained personnel in sequence alignment, phylogenetic analysis and genome assembly, increasing dependence on foreign collaborators and delaying research outputs<sup>108</sup>. Insufficient funding also precludes the establishment of national repositories containing reference isolates and sequences. As a result, indigenous BCAs remain underrepresented in global databases, contributing to persistent knowledge gaps<sup>109</sup>. This situation is further compounded by policy constraints, as many RPW-affected countries prioritize short-term chemical control measures over long-term investment in molecular biocontrol research<sup>110</sup>. Collectively, the lack of reference sequences, marker variability, technological and financial limitations and weak institutional support undermine the reliability and scalability of molecular approaches for developing and deploying BCAs

against RPW. Addressing these bottlenecks requires strengthened international collaboration, sustained investment in local research capacity and coordinated efforts to establish inclusive genomic repositories that represent agroecosystems most affected by RPW.

**Future prospects:** Molecular tools are entering a transformative phase in the biological control of *Rhynchophorus ferrugineus* (RPW). Beyond conventional barcoding, emerging approaches now enable high-throughput screening, real-time diagnostics and predictive deployment of biological control agents (BCAs)<sup>111</sup>. The following sections outline key innovations likely to shape the future of RPW biocontrol.

**Multi-omics integration for strain selection:** Multi-omics pipelines linking genotype to phenotype represent the next frontier in BCA development. Genomic and transcriptomic sequencing reveal genes associated with cuticle penetration, toxin production, sporulation and stress tolerance, while metabolomics and proteomics identify biomarkers of virulence and environmental resilience<sup>112</sup>. Such integrated strategies enable the pre-selection of elite isolates prior to resource-intensive field evaluations, thereby streamlining strain development and deployment<sup>113</sup>. Recent studies emphasize the value of whole-genome sequencing of *Beauveria* and *Metarhizium*, which has uncovered virulence factors and stress-response traits essential for performance in desert and tropical environments<sup>111</sup>. Multi-omics-based screening is expected to reduce trial-and-error approaches and accelerate commercialization.

**Portable sequencing and rapid diagnostics:** Field-ready sequencing technologies are emerging as powerful tools for RPW monitoring and biocontrol implementation. On-site identification of entomopathogenic fungi, nematodes and bacteria can now be achieved using nanopore sequencers and qPCR platforms<sup>114</sup>. These systems enable real-time verification of strain identity during mass production, ensure formulation quality and minimize diagnostic delays in the field. Furthermore, nanopore sequencing facilitates tracking of strain persistence and microbial dynamics within palm orchards<sup>115</sup>. Recent reviews indicate that portable sequencing is increasingly applied in agricultural pest diagnostics and is feasible even in low-resource settings<sup>116</sup>. Incorporating these platforms into routine RPW biocontrol monitoring is expected to enhance both efficacy and timeliness of interventions.

**Microbiome and metagenomics in biocontrol:** The microbiome is increasingly recognized as a central factor in determining BCA efficacy. The gut microbiota of *Rhynchophorus ferrugineus* (RPW) and palm-associated microbial communities strongly influence pathogen

virulence and the survival of entomopathogens<sup>117</sup>. Metagenomic profiling has identified both antagonistic microbes that reduce infection efficacy and synergistic endophytes that enhance biocontrol outcomes. Future strategies may involve co-engineering compatible microbial consortia or manipulating the microbiome to suppress antagonistic species. The integration of insect microbiome metagenomics into pest management strategies represents a promising approach to applying microbial ecology in RPW biocontrol<sup>118</sup>. Incorporating microbiome data into BCA selection and formulation pipelines is an emerging opportunity for sustainable management of RPW.

**Advances in formulation and mass production:** Even highly virulent strains fail to achieve efficacy if formulations cannot withstand field conditions. The survival and performance of fungal and nematode BCAs are being enhanced through encapsulation technologies, including nano- and microcapsules, oil-based carriers and alginate gels, which improve shelf life, UV resistance and heat tolerance<sup>52</sup>. Large-scale production is also becoming more cost-effective through submerged liquid fermentation and optimized downstream processing<sup>112</sup>. Studies conducted between 2022 and 2024 indicate that these innovations increase survival rates under arid conditions and prolong product longevity, enabling broader application in date palm regions<sup>112</sup>. Such advances ensure more reliable, regionally adapted formulations capable of consistent field performance.

**Genomic evidence in regulation and policy:** Regulatory approval of microbial BCAs has historically been a bottleneck. Molecular tools can facilitate this process by providing precise genomic identification of strains, confirming genetic stability and verifying the absence of undesirable traits, such as toxin production or antibiotic resistance. International policy reports recommend incorporating DNA barcoding, multilocus sequence typing (MLST) and whole-genome sequencing into BCA registration protocols<sup>112</sup>. This genomics-based regulatory framework not only accelerates approvals but also fosters global trust and acceptance of microbial biocontrol products<sup>120</sup>.

**Regional hubs and capacity building:** Developing countries face substantial technical and financial challenges in implementing molecular tools for RPW management. Establishing regional sequencing centers and mobile laboratory networks could provide shared access to sequencing, bioinformatics pipelines and culture collections<sup>121</sup>. Lessons from genomic surveillance in public health indicate that such hubs can serve multiple countries cost-effectively<sup>87</sup>. For RPW, these hubs could maintain reference libraries of fungal, nematode and bacterial isolates, standardize molecular protocols and strengthen international collaboration.

**Artificial intelligence and predictive models:** Digital technologies and artificial intelligence (AI) are emerging as complementary tools to molecular data. By integrating omics profiles, phenotypic assays and environmental information, machine learning models can predict which BCA strains will perform optimally under specific climatic conditions. Molecular surveillance combined with AI-based decision-support systems can guide strain selection, timing of application and formulation combinations in the field<sup>112</sup>. Recent research demonstrates the potential of AI in pest biocontrol and microbial ecology, providing a foundation for RPW-specific predictive frameworks<sup>122</sup>. The future of precision biocontrol lies in the integration of data science and molecular biology to optimize sustainable pest management.

## CONCLUSION

*Rhynchophorus ferrugineus* (red palm weevil, RPW) is a highly destructive pest of date palms and related crops, with cryptic feeding habits, high reproduction and adaptability that render conventional control largely ineffective. Over the past two decades, biological control agents (BCAs) have emerged as a sustainable alternative but their success depends on precise identification, strain characterization and adaptation to local ecological conditions, an area where molecular tools have revolutionized RPW management. Techniques such as DNA barcoding, PCR, next-generation sequencing and phylogenetic analysis have improved species identification, uncovered hidden genetic diversity and resolved strain-level ambiguities. This has enhanced the predictability and effectiveness of BCAs, including *Beauveria bassiana*, *Metarhizium anisopliae*, *Steinernema* and *Heterorhabditis*, while enabling discovery of novel microbial agents and ensuring quality in commercial formulations. Global case studies highlight the impact of molecular approaches: Arid-adapted isolates in the Middle East, heat-tolerant virulent strains in South Asia and quality-assured commercial products in Europe demonstrate the adaptability and broad applicability of molecularly guided biocontrol strategies. Challenges remain, particularly in developing countries, including incomplete reference databases, variable molecular markers, limited infrastructure, high costs and regulatory barriers. Addressing these is crucial to fully exploit molecular methods within Integrated Pest Management (IPM). Future prospects involve integrating molecular biology with practical pest management using portable sequencing, metagenomics and AI-based predictive models. These innovations allow rapid diagnostics, informed strain selection, field monitoring and development of region-specific, climate-resilient BCAs, reducing dependence on chemical pesticides. In conclusion, molecular identification and characterization have reshaped

RPW biocontrol, bridging taxonomy, genetics and applied management. Their continued development and integration into IPM programs hold promise for sustainable RPW control and provide a framework for managing other invasive pests globally, contingent on long-term research, international collaboration and translation into field-ready solutions.

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