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Research Article

Conferring the Midas Touch on Integrative Taxonomy: A Nanogold-Oligonucleotide Conjugate-Based Quick Species Identification Tool

Rahul Kumar [1,2] **and Ajay Kumar Sharma** [1]

[1] *University Department of Zoology, Vinoba Bhave University, Hazaribagh 825301, India*
[2] *Department of Zoology, Sheodeni Sao College, Kaler 824127, India*

Correspondence should be addressed to Rahul Kumar; rahuldayanand33@gmail.com

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Nanogold or functionalized gold nanoparticles (GNPs) have myriad applications in medical sciences. GNPs are widely used in the area of nanodiagnostics and nanotherapeutics. Applications of GNPs in taxonomic studies have not been studied vis-à-vis its extensive medical applications. GNPs have great potential in the area of integrative taxonomy. We have realized that GNPs can be used to visually detect animal species based on molecular signatures. In this regard, we have synthesized gold nanoparticles (<20 nm) and have developed a method based on interactions between thiolated DNA oligonucleotides and small-sized GNPs, interactions between DNA oligonucleotides and target DNA molecules, and self-aggregating properties of small-sized GNPs under high salt concentrations leading to a visible change in colour. Exploiting these intermolecular and interparticle interactions under aqueous conditions, in the present work, we have demonstrated the application of our procedure by using a DNA oligonucleotide probe designed against a portion of the mitochondrial genome of the codling moth *Cydia pomonella*. This method is accurate, quick, and easy to use once devised and can be used as an additional tool along with DNA barcoding. This tool can be used for distinguishing cryptic species, identification of morphovariants of known species, diet analysis, and identification of pest species in quarantine facilities without any need of performing repetitive DNA sequencing. We suggest that designing and selecting a highly specific DNA probe is crucial in increasing the specificity of the procedure. Present work may be considered as an effort to introduce nanotechnology as a new discipline to the extensive field of integrative taxonomy with which disciplines like palaeontology, embryology, anatomy, ethology, ecology, biochemistry, and molecular biology are already associated for a long time.

1. Introduction

Species identification is central to the area of taxonomy. Nowadays, it has become a trend to identify and study a species using both morphological as well as molecular data. Especially when describing an insect species, mitochondrial DNA-based approaches are quite popular. Mitochondrial DNA-based DNA barcoding is one of the most preferred molecular tools among modern insect taxonomists. The design of the pair of universal primers against the mitochondrial cytochrome oxidase-I (mtCO-I) gene has revolutionized the field of taxonomy [1, 2]. Phylogenetic analyses based on the sequences of both mitochondrial as well as nuclear genes provide a better resolution in tracing inter and intraspecific similarities and differences [3]. As a common practice in DNA barcoding, a stretch of mtCO-I is amplified using universal primers followed by sequencing of the amplicon and sequence analysis postsequencing [2]. Amplifying and sequencing the DNA of every specimen is not possible. Morphologically similar-looking specimens may not always belong to the same species, but it sounds like a redundant, nonfeasible, and time-consuming task to amplify and sequence the DNA of all specimens (when the number of specimens is very high) belonging to the same species. To

tackle such situations, we have developed a methodology that can quickly detect a species based on its molecular signature. This tool would help to reduce the need for repetitive sequencing and can be employed to authenticate barcodes in resource-limited setups. Our method utilizes functionalized gold nanoparticles (GNPs) and their unique properties. There is a tsunami of literature dealing with the application of gold nanoparticles in different areas of biological sciences, but we could not find even a single study dealing with the application of GNPs in taxonomic studies of higher animals or even higher plants. GNPs have huge applications in both nanodiagnostics and nanotherapeutics [4, 5]. Nanodiagnostic tools based on GNPs include plasmon resonance biosensors, dot-immunoassay, immune chromatography, and different homophase methods [4]. For the present work, we have used, with some modifications, one of the homophase methods which involves interaction between thiolated ssDNA (small single-stranded DNA molecules) and small-sized functionalized GNPs, the interaction between thiolated ssDNA-GNP complexes and target DNA molecules, and colour change in the solution as a result of aggregation of the particles under conditions of high ionic strength [6–8]. Since the publication of the genomic sequence of *Drosophila melanogaster* in 2000, which was the first insect genome to be sequenced, a large number of different insect genomes have been sequenced and studied in detail [9, 10]. Instead of the availability of a huge amount of insect genomic data in the public domain, being the most diverse taxa with the largest number of species in the entire animal kingdom, the genomic information of a large number of insect species is still not available. The size of the nuclear genome is far greater than the size of the mitochondrial genome. It makes the mitochondrial genome be sequenced in less time with fewer budgets and easier to analyze. In recent years, more insect mitochondrial genomes have been sequenced and studied in comparison to their nuclear genomes. The mitochondrial genome is the most extensively studied genomic system in insects [11]. Therefore, in the present study, we have selected a short stretch of the mitochondrial genome of an insect for designing a unique oligonucleotide to be used as a part of our probe. This method was found to be accurate, quick, and easy to use once devised and can be used as an additional tool along with DNA barcoding.

2. Materials and Methods

2.1. Materials. Gold (III) chloride trihydrate ($HAuCl_4.3H_2O$), trisodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$), sodium borohydride ($NaBH_4$), magnesium sulphate ($MgSO_4$), ethidium bromide, and agarose were purchased from Sigma Aldrich. All buffers were manually prepared using chemicals of analytical grade. The GeneRular 1 kb DNA ladder was purchased from Thermo Scientific. Micropipette tips, centrifuge tubes, and PCR tubes were purchased from Tarsons.

2.2. Species Selection. The codling moth *Cydia pomonella* was used for our studies. The oligonucleotide probe and primers were designed against a stretch of its mitochondrial DNA. The cotton bollworm moth *Helicoverpa armigera* has been used as the control. Complete mitochondrial genome sequences of both of these economically important moths are publicly available and are well characterized too. Therefore, we preferred these species for our studies.

2.3. Analysis of Mitochondrial Genome. Complete mitochondrial genome sequences of *Cydia pomonella* were retrieved from the NCBI (National Center for Biotechnology Information) Genome database in FASTA (stands for "FAST-All") format. Graphical circular and linear maps of the mitochondrial genome were prepared from this sequence using OGDRAW (Organellar Genome DRAW) and MITOS (MITOchondrial genome annotation Server) to demarcate the position of genes and direction of open reading frames (Figure 1(a)) [12, 13]. The same strategy was followed for the control, *Helicoverpa armigera* (Supplementary Figure 1).

2.4. Mitochondrial DNA Extraction. First, the mitochondria were isolated from larval tissue of *Cydia pomonella* and *Helicoverpa armigera* using a previously described organelle isolation protocol [14]. In each case, only one caterpillar was used for mitochondrial isolation. Isolated mitochondria of each species were then used to isolate mitochondrial DNA [15]. It was isolated using the DNeasy 96 Blood and Tissue Kit by Qiagen. For each species, mitochondrial DNA isolation was performed multiple times, and all samples were pooled together and vacuum dried to remove excess water for better concentration. Isolated mitochondrial DNA was quantified using a NanoDrop 2000 Spectrophotometer by Thermo Scientific.

2.5. Designing of Oligonucleotide Probe. Multiple primer pairs were generated from the complete mitochondrial genome sequence of *Cydia pomonella* using NCBI Primer-BLAST (Basic Local Alignment Search Tool). Out of these primers, the most unique oligonucletide sequence was selected by running NCBI BLAST using each primer sequence. The sequence which was considered the "most unique" sequence exhibits the least cross-species sequence similarity within the order Lepidoptera in BLAST results. This sequence was found to be absent in the mitochondrial genome of *Helicoverpa armigera,* which was used as the control. This oligonucleotide was labeled with a thiol group (-SH) at the 5′ end to enable its conjugation with GNPs to be used as a probe (Supplementary Figure 2). The 5′ thiol-modified oligonucleotide was synthesized on a 25 nM scale according to standard procedure and supplied in lyophilized form by Eurofins.

2.6. Characterization of Oligonucleotide Probe. For characterization of oligonucleotide probe, PCR was performed. The oligonucleotide probe itself was used as a forward primer along with a reverse primer to amplify a stretch of 1332 bases of the mitochondrial genome sequence of *Cydia pomonella* (Figure 1(b)). The reverse primer was selected after analyzing its properties using the IDT OligoAnalyzer with respect to the

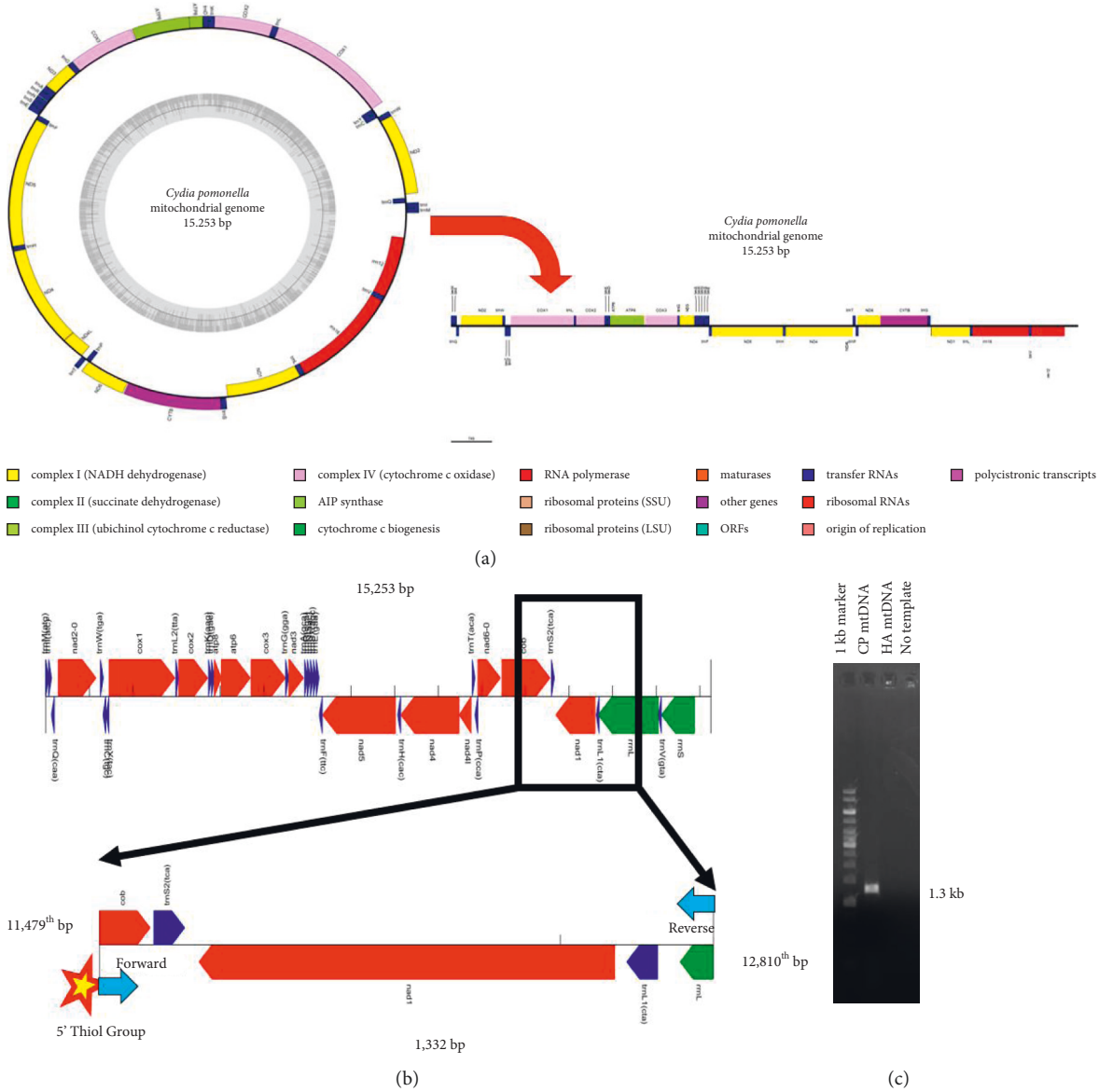

Figure 1: Designing and characterization of oligonucleotide probe. (a) Circular and linearized maps of mitochondrial genome of codling moth *Cydia pomonella*. (b) Designing of oligonucleotide probe and PCR primers. (c) Gel showing PCR product at 1.3 kb in case of mitochondrial DNA of *Cydia pomonella* (CA mtDNA). *Helicoverpa armigera* mitochondrial DNA (HA mtDNA) was used as negative control.

forward primer [16] (Supplementary Figure 2). Primers were synthesized at a 25 nM scale according to standard procedure and were supplied in lyophilized form by Eurofins. PCR was performed using the mitochondrial DNA of *Cydia pomonella* as a template. Mitochondrial DNA of *Helicoverpa armigera* was used as the control. PCR products were run on a 0.8% agarose gel containing ethidium bromide for visualization under UV light using the gel documentation system.

2.7. Synthesis of GNPs. Gold nanoparticles were synthesized using a two-step chemical reduction method (reduction followed by stabilization) [17]. Briefly, 10 ml of 1 mM of gold (III) chloride trihydrate ($HAuCl_4.3H_2O$) solution was taken in a conical flask wrapped with silver foil and kept for stirring on a magnetic stirrer. To this solution, 400 μl of a 500 μg/ml solution of ice-chilled sodium borohydride ($NaBH_4$) was added drop-wise and left for 30 seconds. Then, 200 μl of a 5% solution of trisodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$) was added and left for another 30 seconds. Citrate-capped GNPs were formed. In this two-step method, the reduction was achieved by the addition of $NaBH_4$, and stabilization was carried out by $Na_3C_6H_5O_7.2H_2O$ (Figure 2(a)).

2.8. Characterization of GNPs. Size distribution analysis of a tenfold diluted freshly prepared sample of GNPs was done by dynamic light scattering (DLS) using a Zetasizer (Malvern Instruments, Malvern, UK) equipped with the 5 mW

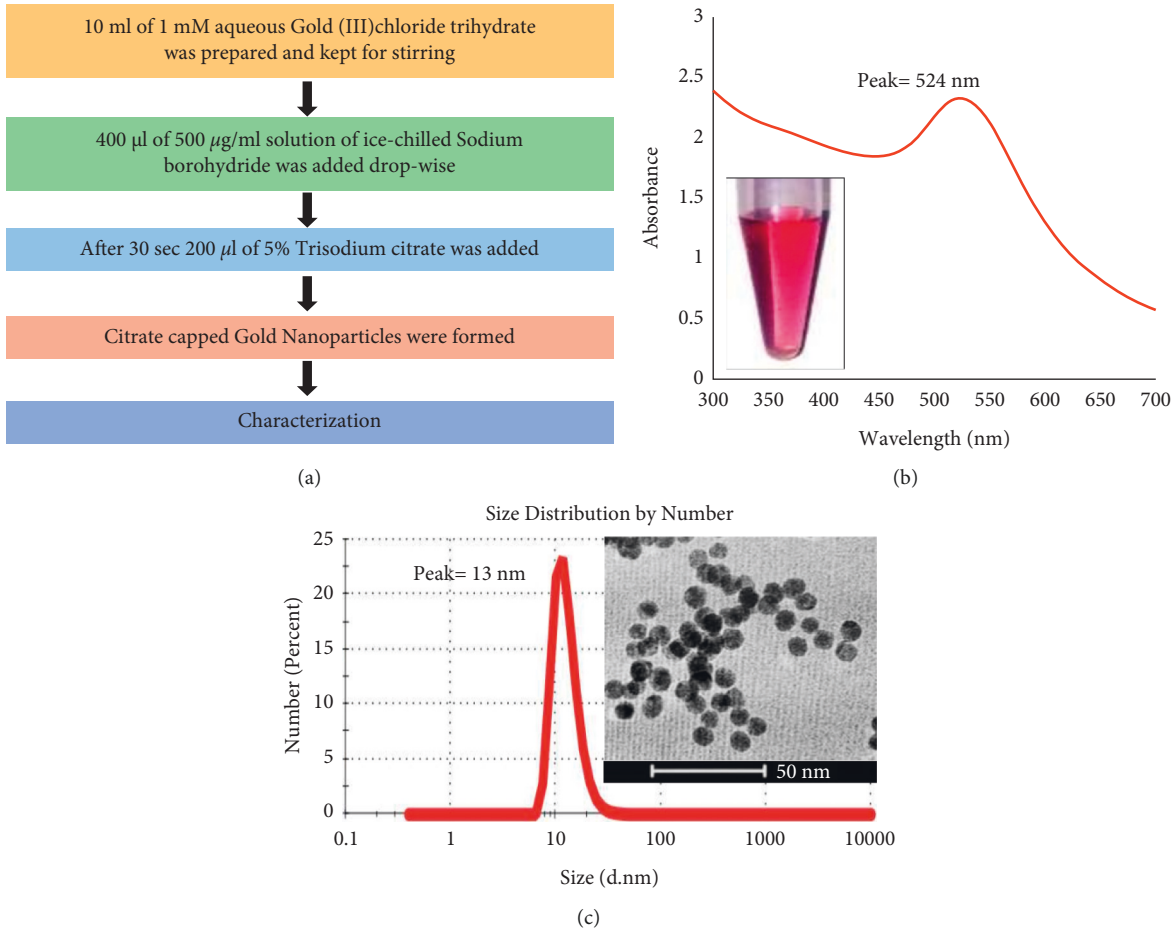

Figure 2: Synthesis and characterization of GNPs. (a) Two-step chemical reduction method of GNP synthesis. In this two-step method, reduction was achieved by addition of sodium borohydride and stabilization was carried out by trisodium citrate dehydrate. (b) Characterization of GNPs for its colour using visual observations and absorbance using the UV-visible spectrophotometer. (c) Characterization of GNPs for its size distribution using Zetasizer and shape using the transmission electron microscope (TEM).

helium/neon laser. For morphological characterization, one drop of the same sample was poured on 300-mesh carbon-coated copper grids and dried at room temperature before loading into the transmission electron microscope (TEM) for imaging, which was done using a high-resolution TEM (TECNAI, T20G2, TEM, FEI, Inc., Hillsborough, OR, USA) operated at 200 kV. The absorbance of the same sample was determined using an Evolution 220 UV-visible spectrophotometer (Thermo Scientific). The colour of the sample was also recorded. The molar concentration of GNPs was also calculated using the absorbance of the sample at 450 nm, determined as above and the value of the extinction coefficient of GNPs at 450 nm, for specific particle size, as previously reported [18]. This calculation provides an average estimate of the molar concentration of GNPs.

2.9. Preparation of GNP-Oligonucleotide Conjugate. Conjugation of GNP-oligonucleotide was performed using a method modified from previous studies [8, 19, 20] (Figure 3(a)). Two sets of conjugation reaction mixtures were prepared. One set of reaction mixtures had a 1 μM oligonucleotide probe in 1 ml of GNPs solution and the other set had a 0.5 μM oligonucleotide probe in 1 ml of GNPs solution. Two reaction mixtures were prepared for the comparison of sensitivity in detection among different concentrations of conjugates. Both reaction mixtures were kept inside the orbital shaker and incubated overnight at 50°C. To each reaction mixture, phosphate buffer, SDS, and NaCl solution were added to obtain a final concentration of 10 mM (pH 7.4), 0.01% (weight/volume), and 0.1 M, respectively, and they were kept in an orbital shaker for incubation at 50°C for 48 hours. After incubation, both reaction mixtures were centrifuged at 15,000 rpm for 30 min at 4°C followed by washing with washing buffer twice. The washing buffer is 100 mM phosphate buffer saline (PBS) (with 0.01% SDS and 100 mM NaCl). The GNP-oligonucleotide conjugate is finally resuspended in the same washing buffer and stored at 4°C in dark.

2.10. Characterization of GNP-Oligonucleotide Conjugate. The absorbance of the GNP-oligonucleotide conjugate sample was determined using an Evolution 220 UV-visible spectrophotometer (Thermo Scientific) and compared with the absorbance of unconjugated GNPs. A similar approach for characterization has also been used by other workers [20, 21].

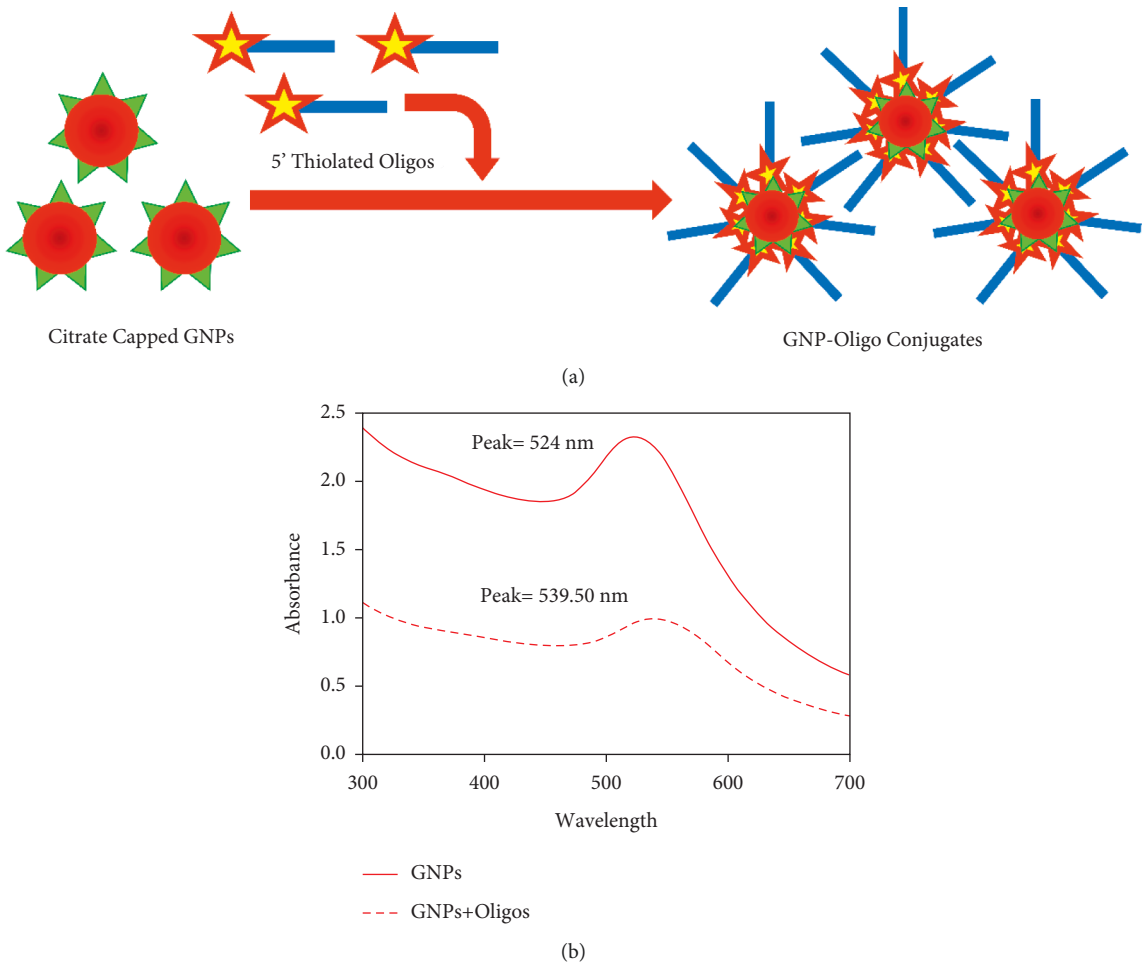

Figure 3: Preparation and characterization of GNP-oligonucleotide conjugate. (a) Schematic representation of conjugation of GNPs and thiolated oligonucleotide probe. (b) Characterization of GNP-oligonucleotide conjugate using the UV-visible spectrophotometer. Red shift in absorbance peak indicates conjugation success.

2.11. Hybridization of GNP-Oligonucleotide Conjugate with Mitochondrial DNA. Hybridization and optimization of biomolecules were performed based on previous studies with some modifications [8, 22]. A hybridization reaction mixture was prepared by mixing 20 μl of 50 mM of *Cydia pomonella* mitochondrial DNA and 20 μl of GNP-oligonucleotide conjugate in a PCR tube. The hybridization reaction mixture was incubated for 5 minutes by placing it on a thermoshaker preheated at 95°C, followed by incubation for further 5 minutes at 63°C for hybridization. After hybridization, 6 μl of the above mixture is aliquoted into 6 different PCR tubes. Afterwards, for optimization of salt concentration, 6 different concentrations of magnesium sulphate ($MgSO_4$), namely, 3 mM, 15 mM, 30 mM, 60 mM, 80 mM, and 100 mM, were added to each of the abovementioned reaction mixtures, respectively. Milli-Q water was used as a negative control in place of *Cydia pomonella* mitochondrial DNA in the hybridization mixture. A change in red or pink indicates a positive result, while a blue colour change indicates a negative result, which can be observed visually. Assessing the sensitivity and specificity of the GNP-oligonucleotide conjugates is also a part of optimization. For assessing the sensitivity of GNP-oligonucleotide conjugate, 6 different concentrations of *Cydia pomonella* mitochondrial DNA were prepared using serial dilution, namely, 5 ng/μl, 10 ng/μl, 20 ng/μl, 30 ng/μl, 40 ng/μl, and 50 ng/μl. Each DNA concentration was used for hybridization with GNP-oligonucleotide conjugate in separate PCR tubes. After hybridization, an optimized concentration of $MgSO_4$ was added and the colour of the solution was recorded. In the negative control, Milli-Q water was added in place of DNA. Similarly, to assess the specificity of GNP-oligonucleotide conjugate, 50 ng/μl of mitochondrial DNA of *Cydia pomonella* and *Helicoverpa armigera* was hybridized with GNP-oligonucleotide conjugate in separate PCR tubes. After hybridization, an optimized concentration of $MgSO_4$ was added and the colour of the solution was recorded.

3. Results

3.1. Quantification of Mitochondrial DNA. Nanodrop measurements provide a value of 21 ng/μl for *Cydia pomonella* mitochondrial DNA and 18 ng/μl for *Helicoverpa armigera* mitochondrial DNA.

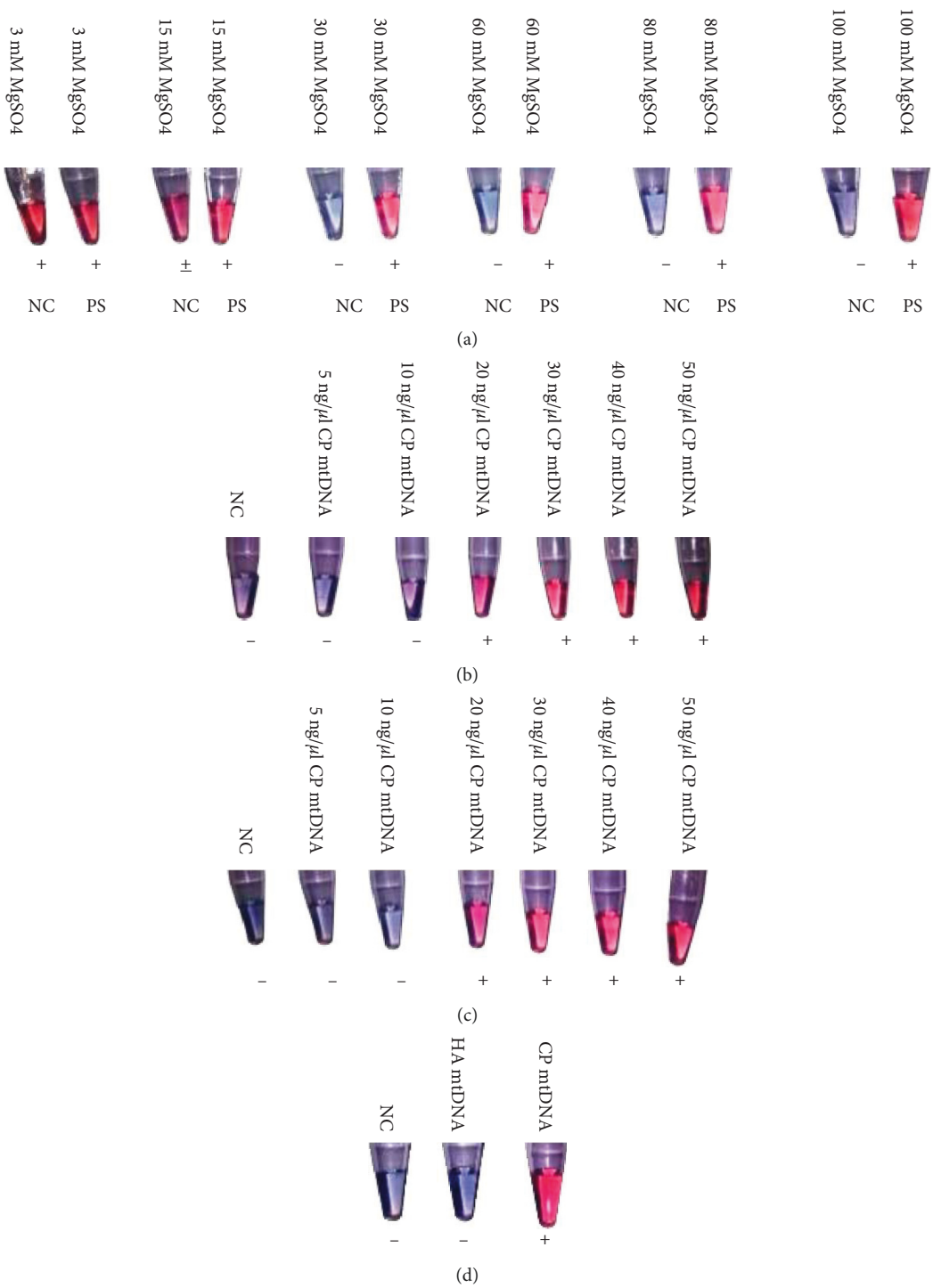

Figure 4: Continued.

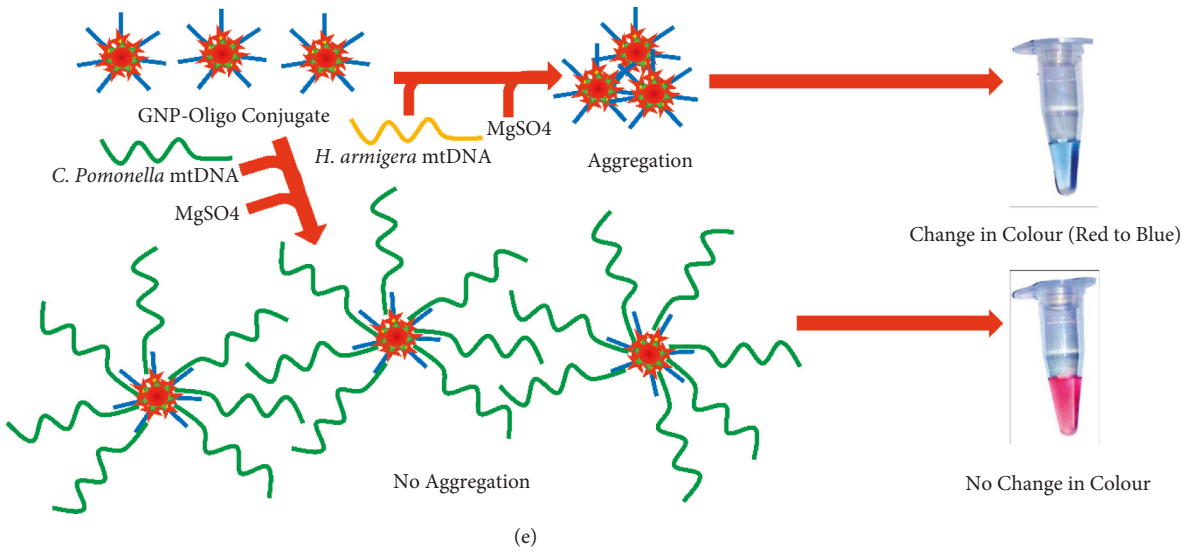

Figure 4: Hybridization of GNP-oligonucleotide conjugate with mitochondrial DNA. (a) Optimization of $MgSO_4$ concentration. (b) Assessment of GNP-oligonucleotide conjugate sensitivity of GNP-oligonucleotide conjugate prepared using 1 μM oligonucleotide probe. (c) Assessment of GNP-oligonucleotide conjugate sensitivity of GNP-oligonucleotide conjugate prepared using 0.5 μM oligonucleotide probe. (d) Assessment of GNP-oligonucleotide conjugate specificity. (e) Graphical representation of the process of hybridization between GNP-oligonucleotide conjugate and mitochondrial DNA, and its outcome. NC, negative control without mtDNA; PS, sample containing *Cydia pomonella* mtDNA; CP, *Cydia pomonella*; HA, *Helicoverpa armigera*; (+), positive result; (−), negative result.

3.2. Characterization of Oligonucleotide Probe. The gel image shows successful amplification of the targeted stretch of DNA after PCR. A bright DNA band is visible in the case of the PCR product where the *Cydia pomonella* mitochondrial DNA template was used. There is no amplification in the case of the *Helicoverpa armigera* mitochondrial DNA template, which was used as a negative control (Figure 1(c)).

3.3. Characterization of GNPs. The size distribution of GNPs was found to be around 13 nm as revealed by Zetasizer measurements (Figure 2(c)). TEM imaging shows that these particles are spherical in shape (Figure 2(c)). The UV-visible spectrophotometric data show the peak of the curve at 524 nm, which is the value of maximum absorption by the particles (Figure 2(b)). The colour of GNPs was found to be red in solution (Figure 2(b)). Furthermore, the absorbance of the sample at 450 nm was found to be 1.85, and the extinction coefficient of spherical GNPs at 450 nm for 13 nm particle size was noted as $1.39 \times 10^8\ M^{-1}\ cm^{-1}$ from a previously published report [18]. Using these values, the molar concentration of GNPs was calculated as 13.3 nM. This value is an average estimate, not an absolute quantity. Therefore, for conjugation experiments, GNPs were taken in volume (ml) in place of molar concentration.

3.4. Characterization of GNP-Oligonucleotide Conjugate. UV-visible spectrophotometric measurements show a shift in peak from 524 nm in the case of unconjugated GNPs to 539.50 nm in the case of GNP-oligonucleotide conjugate. This red shift confirms the process of conjugation (Figure 3(b)).

3.5. Optimization of $MgSO_4$ Concentration. Hybridization of the GNP-oligonucleotide conjugate (prepared using 0.5 μM oligonucleotide probe) with *Cydia pomonella* mitochondrial DNA was followed by the addition of 6 different concentrations of $MgSO_4$ as mentioned above. In the negative control, Milli-Q water was used in place of *Cydia pomonella* mitochondrial DNA. Using 30 mM, 60 mM, 80 mM, and 100 mM $MgSO_4$, *Cydia pomonella* can be clearly distinguished from negative control (Figure 4(a)). 100 mM was selected as the optimal concentration of $MgSO_4$.

3.6. Assessment of GNP-Oligonucleotide Conjugate Sensitivity. Among different concentrations of *Cydia pomonella* mitochondrial DNA used for hybridization with the GNP-oligonucleotide conjugate, 20 ng/μl, 30 ng/μl, 40 ng/μl, and 50 ng/μl remained red after addition of $MgSO_4$, whereas other solutions with lower concentrations of DNA and the negative control having Milli-Q water in place of DNA turned blue. Therefore, the detection limit was found to be 20 ng/μl for *Cydia pomonella* mitochondrial DNA. Also, both GNP-oligonucleotide conjugates, the one prepared using a 1 μM oligonucleotide probe and the other using a 0.5 μM oligonucleotide probe, show similar results with different concentrations of target DNA. Both of these conjugates are equally sensitive to target detection

(Figures 4(b)and 4(c)). Therefore, we selected GNP-oligonucleotide conjugate prepared using a 0.5 μM oligonucleotide probe for assessment of the specificity of this procedure.

3.7. Assessment of GNP-Oligonucleotide Conjugate Specificity. The specificity of the GNP-oligonucleotide conjugate was evaluated by hybridizing conjugates with mitochondrial DNA of *Cydia pomonella* and *Helicoverpa armigera* individually. Mitochondrial DNA of *Cydia pomonella* displays successful hybridization after addition of $MgSO_4$ as the solution remains red. The solution containing mitochondrial DNA of *Helicoverpa armigera* turns blue after addition of $MgSO_4$, indicating no hybridization (Figure 4(d)).

4. Discussion

Every method has some advantages and disadvantages. Being time-consuming and expensive, though considered the gold standard, the use of DNA barcoding for every similar-looking specimen of an already known species sounds like a redundant exercise. Simple to use and an inexpensive tool like the one developed by us can be used for quick detection of species just by observing colour of the solution visually in place of repeated DNA sequencing procedures and without the use of expensive and difficult-to-handle instruments (Figure 4(e)). Once prepared, the GNP-oligonucleotide conjugate is stable at room temperature for almost a month and can be stored at 4°C for a longer duration for multiple usages, which makes this method cost-effective as shown by other thiolated ssDNA-GNP complex-based methods previously published [23]. This method can further be used to authenticate DNA barcodes by providing additional evidence of a species' molecular identity in less time. The present method has great potential in a variety of applications associated with species identification. This method can be used to distinguish cryptic species. Such species appear morphologically similar but are genetically different. Contrary to this, some species exhibit polymorphism. Morphovariants of a species are morphologically different but are the same in terms of genetic identity. Such individuals can be identified using this method. This method can be employed for the rapid identification of pest species in quarantine facilities. Using this method, animal and plant species being consumed by other animals as food can also be identified (diet analysis). The method is sensitive and specific. The present method is a simple demonstration of a procedure in its preliminary stage, which could be made more robust and error-proof by adopting a few modifications, which are discussed in the subsequent paragraph.

The specificity of this method is highly dependent upon the uniqueness of the oligonucleotide probe sequence. We have used mitochondrial DNA for designing oligonucleotide probes as the nuclear genome sequences of most insects are not available and the DNA barcoding of most insects also relies on the sequence of mtCO-I. But designing an exclusively unique oligonucleotide probe based on the mitochondrial genome is not always possible because of its small size, absence of introns, and conservation of mitochondrial genes across taxa. Also, the chances of cross-binding of the oligonucleotide probe designed against mitochondrial DNA with nuclear DNA cannot be ruled out (when nuclear genome sequence is not available). This is the reason why we selectively extracted mitochondrial DNA (excluding nuclear DNA). The nuclear genomes of eukaryotes are huge in size and differ considerably across as well as within species. Using bioinformatics tools, it is possible to design such an oligonucleotide probe whose sequence is exclusive to a particular eukaryotic species by scanning its whole genome (both nuclear and mitochondrial). This unique probe can then be conjugated with GNPs and be further used as described in our method. The use of such a probe would make this method highly specific with negligible chances of detection failure or false detection. Similarly, the sensitivity of this method can be further enhanced by using an ultrapure GNP- oligonucleotide conjugate. The conjugate solution prepared by us was purified (washed to remove unbound particles) using multiple rounds of centrifugation at high speed, but it cannot be ruled out as it may have negligible amounts of unbound GNPs or free oligonucleotides or both. To get an ultrapure conjugate solution with no unbound GNPs and free oligonucleotides, advanced chromatographic methods can be used after the centrifugation step. These methods can also provide estimates of the respective percentages of GNP-oligonucleotide conjugate, unbound GNPs, and free oligonucleotides in solution [8].

Taxonomy is at the core of understanding biodiversity. Like other scientific disciplines, taxonomy has also progressed significantly from being a traditional morphology-based approach to being a modern multisource approach. The modern approach does not lessen the importance of the traditional morphology-based approach but rather strengthens it. The modern multisource approach involves information from various sources like morphology, behaviour, mitochondrial DNA, nuclear DNA, ecology, enzymes, chemistry, reproductive compatibility, cytogenetics, life history, and whole genome scans. Such a multisource approach is the backbone of integrative taxonomy, a synthesis of different traditional and modern approaches. Integrative taxonomy reduces the chances of misidentification and other taxonomic errors and has made the process of identification easier, more efficient, and more reliable. Palaeontology, embryology, anatomy, ethology, ecology, biochemistry, and molecular biology are the major fields of studies with significant applications in integrative taxonomy [24, 25]. Instead of huge development in the field of integrative taxonomy, the application of nanotechnology in this area has not been realized yet, unlike in other popular disciplines. In biological sciences, nanotechnology is emerging as a great tool with huge potential. Applications of nanotechnology in different fields of biology are already being explored. Nanodiagnostics (including nanodetection, nanoimaging, and nanoanalytics) and nanotherapeutics, which are the subareas of nanomedicine, are the most preferred areas of biological sciences where the application of nanotechnology is being explored today [26]. The focus is on regenerative medicine, cancer diagnosis and treatment,

neuromorphic engineering, tissue engineering, development of biosurfectants, biomedical nanosensors, enhancing bioavailability and bioactivity of drugs, pathogen detection, stem cell biology, and molecular imaging [27–32]. Some of the nonmedical applications of nanotechnology include the development of pesticide, herbicide, and fertilizer nanoformulations; designing of pest and agrochemical nanosensors; development of nanodevices for genetic engineering, crop improvement, and animal breeding; increasing shelf life of harvested crops; and creation of biomimetic materials [33–36]. Instead of the huge applications of nanotechnology, its application in integrative taxonomy has not been realized yet. We believe that nanotechnology also has great potential in the field of integrative taxonomy. This new area of discourse may be called "nanotaxonomy." It can add new dimensions to modern taxonomic studies if explored systematically.

5. Conclusion

In the present study, we have reported a novel method for the detection of insect species based on their molecular signature by using a GNP-oligonucleotide conjugate which can distinguish one species from another by a simple change in the colour of the solution that can be observed by the naked eye. Use of mitochondrial genome sequence for probe designing is the unique strategy. This method can help in saving time and money spent on repetitive barcoding experiments on apparently similar looking specimens. This method has high sensitivity (detection limit = 20 ng/μl) and specificity. The specificity of this method can further be enhanced by designing a species exclusive probe with negligible cross-species similarity employing whole genome scanning assisted by advanced bioinformatic tools. The present work may be considered as a small step towards bridging the existing gap between integrative taxonomy and nanotechnology.

Data Availability

The data used to support the findings of this study are included within the article and the supplementary material.

Disclosure

This work was presented as a poster at the Ento'21 Conference organized by the Royal Entomological Society (RES), London, from 23rd August, 2021 to 27th August, 2021.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1. Linearized mitochondrial genome map of *Helicoverpa armigera*. Supplementary Figure 2. Design of an oligonucleotide probe against a portion of the mitochondrial genome of *Cydia pomonella* and PCR primers for its characterization. The sequence of the DNA segment to be amplified is shown in the 5′ to 3′ direction. The sequence of the probe, which is also the forward primer, is shown in red letters, and the reverse primer is shown in green. (*Supplementary Materials*)

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