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ORCID® iDs	Rahul Kumar - https://orcid.org/0000-0002-8320-7861

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

*Email ID: rahuldayanand33@gmail.com (corresponding author)

Abstract

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 ultra-small 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 visible change in colour. Exploiting these intermolecular and interparticle interactions under aqueous conditions, in present work, we have demonstrated the application of our procedure by using 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. We suggest that designing and selection of a highly specific DNA probe is crucial in increasing 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.

Keywords

DNA barcoding; gold nanoparticles; integrative taxonomy; nanotechnology.

Introduction

Species identification is central to the area of taxonomy. Now a days it has become a trend to identify and study a species using both morphological as well as molecular data. Especially while describing an insect species, mitochondrial DNA based approach is quite popular. Mitochondrial DNA based DNA barcoding is one of the most preferred molecular tool among modern insect taxonomists. Designing 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 post-sequencing [2]. Amplifying and sequencing DNA of every specimen isn't possible. Morphologically similar looking specimens may not always belong to the same species but it sounds redundant, non-feasible and time consuming task to amplify and sequence DNA of all specimens (when number of specimens are very high) belonging to the same species. To tackle such situations, we have developed a methodology which can quickly detect a species based on its molecular signature. This tool would help to reduce the need of repetitive sequencing and can be employed to authenticate barcodes in resource limited setups. Our method utilizes functionalized gold nanoparticles (GNPs) and its unique properties. There is a tsunami of literature dealing with application of gold nanoparticles in different areas of biological sciences but we could not find even a single study dealing with 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 present work we have repurposed with some modifications one of the homophase methods which involves interaction between thiolated ssDNA (small single stranded DNA molecules) and ultrasmall functionalized GNPs, 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 publication of genomic sequence of *Drosophila melanogaster* in 2000, which is 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 public domain, being the most diverse taxa with largest number

of species in entire animal kingdom, genomic information of a large number of insect species are still not available. Size of nuclear genome is far greater than the size of mitochondrial genome. It makes mitochondrial genome to be sequenced in less time with less budget and easier to analyze. In recent years, more insect mitochondrial genomes have been sequenced and studied in comparison to the nuclear genomes. Mitochondrial genome is the most extensively studied genomic system in insects [11]. Therefore, in present study we have selected a short stretch of 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.

Materials and methods

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. GeneRular 1 kb DNA ladder was purchased from Thermo Scientific. Micropipette tips, centrifuge tubes and PCR tubes were purchased from Tarsons.

Species selection

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

Analysis of mitochondrial genome

Complete mitochondrial genome sequences of *C. pomonella* was retrieved from NCBI Genome data base in FASTA format. Graphic circular and linear maps of the mitochondrial genome was prepared from this sequence using OGDRAW and MITOS to demarcate position of genes and direction of open reading frames (Figure 1a) [29,30]. Same strategy was followed for the control, *H. armigera* (Supplementary figure 1).

Mitochondrial DNA extraction

First, the mitochondria was isolated from larval tissue of *C. pomonella* and *H. armigera* using previously described organelle isolation protocol [31]. Isolated mitochondria of each species was then used to isolate mitochondrial DNA [32]. It was isolated using 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 NanoDrop 2000 Spectrophotometer by Thermo Scientific.

Designing of oligonucleotide probe

Multiple primer pairs were generated from the complete mitochondrial genome sequence of *C. pomonella* using NCBI Primer-BLAST. Out of these primers, the most unique oligonucletide sequence was selected by running NCBI BLAST using each primer sequence. The sequence which was assigned to be the most unique sequence exhibits least cross species sequence similarity within its order in BLAST result. This sequence was found to be absent in the mitochondrial genome of *H. armigera* which was used as control. This oligonucleotide was labeled with thiol group (-SH) at 5' end to enable its conjugation with GNPs to be used as probe (Supplementary figure 2). The 5' thiol modified oligonucleotide was synthesized at 25 nM scale according to standard procedure and supplied in lyophilized form by Eurofins.

Characterization of oligonucleotide probe

For characterization of oligonucleotide probe, PCR was performed. The oligonucleotide probe itself was used as forward primer along with a reverse primer to amplify a stretch of 1332 bases of the mitochondrial genome sequence of *C. pomonella* (Figure 1b). Reverse primer was selected after analyzing its properties using IDT Oligoanalyzer with respect to the forward primer [33] (Supplementary figure 2). Primers were synthesized at 25 nM scale according to standard procedure and supplied in lyophilized form by Eurofins. PCR was performed using mitochondrial DNA of *C. pomonella* as template. Mitochondrial DNA of *H. armigera* was used as control. PCR products were run on 0.8% agarose gel containing ethidium bromide for visualization under UV light using gel documentation system.

Synthesis of GNPs

GNPs were synthesized using two-step chemical reduction method (reduction followed by stabilization) [34]. 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 magnetic stirrer. To this solution, 400 µl of 500 µg/ml solution of ice-chilled Sodium borohydride ($NaBH_4$) was added drop wise and left for 30 seconds. Then, 200 µl of 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, reduction was achieved by addition of $NaBH_4$ and stabilization was carried out by $Na_3C_6H_5O_7.2H_2O$ (Figure 2a).

Characterization of GNPs

Size distribution analysis of tenfold diluted freshly prepared sample of GNPs was done by dynamic light scattering (DLS) using a Zetasizer (Malvern Instruments, Malvern, UK) equipped with 5 mW 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 high-resolution TEM (TECNAI, T20G2, TEM, FEI, Inc. Hillsborough, OR, USA) operated at 200 kV. Absorbance of same sample was determined using Evolution 220 UV-visible spectrophotometer (Thermo Scientific). Colour of the sample was also recorded. Molar concentration of GNPs was also calculated using absorbance of the sample at 450 nm determined as above and value of extinction coefficient of GNPs at 450 nm for specific particle size as previously reported [12]. This calculation provides an average estimate of the molar concentration of GNPs.

Preparation of GNP-oligonucleotide conjugate

Conjugation of GNP-oligonucleotide was performed using a method modified from previous studies [8,35,36] (Figure 3a). Two set of conjugation reaction mixture was prepared. One set of reaction mixture had 1 µM oligonucleotide probe in 1 ml of GNPs solution and the other set had 0.5 µM oligonucleotide probe in 1 ml of GNPs solution. Two reaction mixtures were prepared for comparison of sensitivity in detection among different concentrations of conjugates. Both reaction mixtures were kept inside orbital shaker and incubated overnight at 50^o C. To each reaction mixture, phosphate buffer, SDS and NaCl solution was added to obtain final concentration of 10 mM (pH 7.4), 0.01 % (weight/volume), and 0.1 M respectively, and was kept in orbital shaker for incubation at 50^o 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. Washing buffer is 100 mM 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.

Characterization of GNP-oligonucleotide conjugate

Absorbance of GNP-oligonucleotide conjugate sample was determined using Evolution 220 UV-visible spectrophotometer (Thermo Scientific) and compared with the absorbance of unconjugated GNPs. Similar approach for characterization has also been used by other workers [36,37].

Hybridization of GNP-oligonucleotide conjugate with mitochondrial DNA

Hybridization and optimization of biomolecules were performed based on previous studies with some modifications [8,38]. Hybridization reaction mixture was prepared by mixing 20 µl of 50 mM of *C. 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 pre-heated at 95° C, followed by incubation for further 5 minutes at 63° C for hybridization. After hybridization, 6 µl of above mixture is aliquot into 6 different PCR tubes. Afterwards, for optimization of salt concentration 6 different concentrations of Magnesium sulphate ($MgSO_4$), *viz.* 3 mM, 15 mM, 30 mM, 60 mM, 80 mM and 100 mM, was added to each of above mentioned reaction mixtures respectively. Milli-Q water was used as negative control in place of *C. pomonella* mitochondrial DNA in the hybridization mixture. Red or pink colour indicates positive result while blue colour change indicates negative result which can be observed visually. Assessing sensitivity and specificity of GNP-oligonucleotide conjugate is also a part of optimization. For assessing the sensitivity of GNP-oligonucleotide conjugate, 6 different concentrations of *C. pomonella* mitochondrial DNA was prepared using serial dilution, viz. 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, optimized concentration of $MgSO_4$ was added and colour of the solution was recorded. In negative control, Milli-Q water was added in place of DNA. Similarly, for assessing the specificity of GNP-oligonucleotide conjugate, 50 ng/µl of mitochondrial DNA of *C. pomonella* and *H. armigera* were hybridized with GNP-oligonucleotide conjugate in separate PCR tubes. After hybridization, optimized concentration of $MgSO_4$ was added and colour of the solution was recorded.

Results

Quantification of Mitochondrial DNA

Nanodrop measurements provide a value of 21 ng/µl for *C. pomonella* mitochondrial DNA and 18 ng/µl for *H. armigera* mitochondrial DNA.

Characterization of oligonucleotide probe

Gel image shows successful amplification of the targeted stretch of DNA after PCR. Bright DNA band is visible in case of the PCR product where *C. pomonella* mitochondrial DNA template was used. There is no amplification in case of *H. armigera* mitochondrial DNA template which was used as negative control (Figure 1c).

Characterization of GNPs

The size distribution of GNPs was found to be around 13 nm as revealed by Zetasizer measurements (Figure 2c). TEM imaging shows that these particles are spherical in shape (Figure 2c). UV-visible spectrophotometric data shows peak of the curve at 524 nm which is the value of maximum absorption by the particles (Figure 2a). The colour of GNPs was found to be red in solution (Figure 2a). Further, absorbance of the sample at 450 nm was found to be 1.85 and extinction coefficient of spherical GNPs at 450 nm for 13 nm particle size was noted as 1.39×10^{8} $M^{-1}cm^{-1}$ from a previously published report [12]. Using these values molar concentration of GNPs was calculated as 13.3 nM.

Characterization of GNP-oligonucleotide conjugate

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

Optimization of $MgSO_4$ concentration

Hybridization of GNP-oligonucleotide conjugate (prepared using 0.5 µM oligonucleotide probe) with *C. pomonella* mitochondrial DNA was followed by addition of 6 different concentrations of $MgSO_4$ against as mentioned above. In negative control, Milli-Q water was there in place of *C. pomonella* mitochondrial DNA. Using 30 mM, 60 mM, 80 mM and 100 mM $MgSO_4$ *C. pomonella*

202 can be clearly distinguished from negative control (Figure 4a). 100 mM was selected as the optimal
203 concentration of $MgSO_4$.

204 **Assessment of GNP-oligonucleotide conjugate sensitivity**

205 Among different concentrations of *C. pomonella* mitochondrial DNA used for hybridization with
206 the GNP-oligonucleotide conjugate, 20 ng/µl, 30 ng/µl, 40 ng/µl and 50 ng/µl remained red after
207 addition of $MgSO_4$ whereas other solutions with lower concentrations of DNA and the negative
208 control having Milli-Q water in place of DNA turned blue. Therefore, the detection limit was found
209 to be 20 ng/µl for *C. pomonella* mitochondrial DNA. Also, both GNP-oligonucleotide conjugates,
210 the one prepared using 1 µM oligonucleotide probe and the other using 0.5 µM oligonucleotide
211 probe, show similar results with different concentrations of target DNA. Both of these conjugates
212 are equally sensitive in target detection (Figure 4b-c). Therefore, we selected GNP-oligonucleotide
213 conjugate prepared using 0.5 µM oligonucleotide probe for assessment of specificity of this
214 procedure.

215 **Assessment of GNP-oligonucleotide conjugate specificity**

216 Specificity of GNP-oligonucleotide conjugate was evaluated by hybridizing conjugates with
217 mitochondrial DNA of *C. pomonella* and *H. armigera* individually. Mitochondrial DNA of *C.*
218 *pomonella* displays successful hybridization after addition of $MgSO_4$ as the solution remains red.
219 The solution containing mitochondrial DNA of *H. armigera* turns blue after addition of $MgSO_4$
220 indicating no hybridization (Figure 4d).

221 **Discussion**

222 Every method has some advantages and disadvantages. Being time consuming and expensive,
223 though considered gold standard, use of DNA barcoding for every similar looking specimen of
224 already known species sounds to be a redundant exercise. A simple to use and inexpensive tool
225 like the one developed by us can be used for quick detection of species just by observing colour
226 of the solution visually in place of repeated DNA sequencing procedure and without use of
227 expensive and difficult-to-handle instruments. Once prepared, GNP-oligonucleotide conjugate is
228 stable at room temperature for almost a month and can be stored at 4^{o} C for a longer duration for
229 multiple usages which makes this method cost effective as shown by other thiolated ssDNA-GNP
230 complex based methods previously published [13]. The method has high sensitivity and specificity.

Specificity of the method is highly dependent upon uniqueness of the oligonucleotide probe sequence. We have used mitochondrial DNA for designing of oligonucleotide probe as nuclear genome sequence of most of the insects are not available and DNA barcoding of most insects also rely on sequence of mtCO-I. But designing an exclusively unique oligonucleotide probe based on mitochondrial genome is not always possible because of its small size, absence of introns and conservation of mitochondrial genes across taxa. Nuclear genomes of eukaryotes are huge in size and differ considerably across as well as within species. Using bioinformatic 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. Use of such a probe would make this method highly specific with negligible chances of detection failure or false detection. This method can further be used to authenticate DNA barcode by providing additional evidence of species molecular identity in small time.

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 modern multisource approach. Modern approach doesn't lessen the importance of traditional morphology based approach rather strengthens it. 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 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 reliable. Palaeontology, embryology, anatomy, ethology, ecology, biochemistry and molecular biology are the major field of studies with significant applications in integrative taxonomy [14,15]. Instead of huge development in the field of integrative taxonomy, application of nanotechnology in this area has not been realized yet unlike other popular disciplines. Nanotechnology is emerging as a great tool with a huge potential in biological sciences. Applications of nanotechnology in different fields of biology are already being explored. Nanodiagnostics (include nanodetection, nanoimaging and nanoanalytics) and nanotherapeutics, which are the sub-areas of nanomedicine, are the most preferred areas of biological sciences where application of nanotechnology is being explored today (Bayda *et al.*, 2019). 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 [19–24]. Some of the non-medical applications of nanotechnology include 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; creation of biomimetic materials, etc. [25–28]. Instead of 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. It can add new dimensions to the modern taxonomic studies if explored systematically.

Conclusion

In present study, we have reported a novel method for detection of insect species based on its molecular signature by using a GNP-oligonucleotide conjugate which can distinguish one species from another by simple change in the colour of solution which can be observed by 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. 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. Present work may be considered as a small step towards bridging the existing gap between integrative taxonomy and nanotechnology.

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Conflict of interest

None

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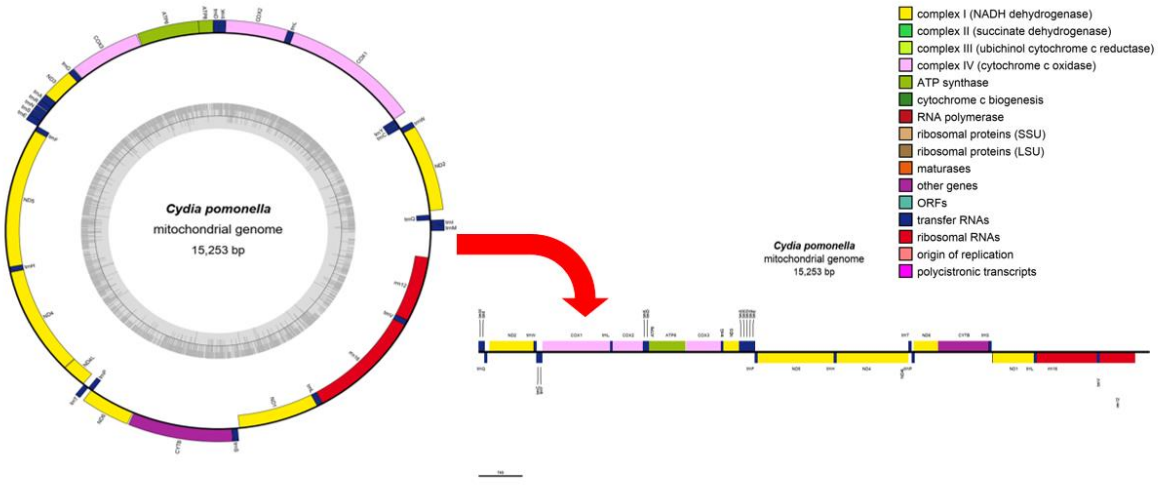
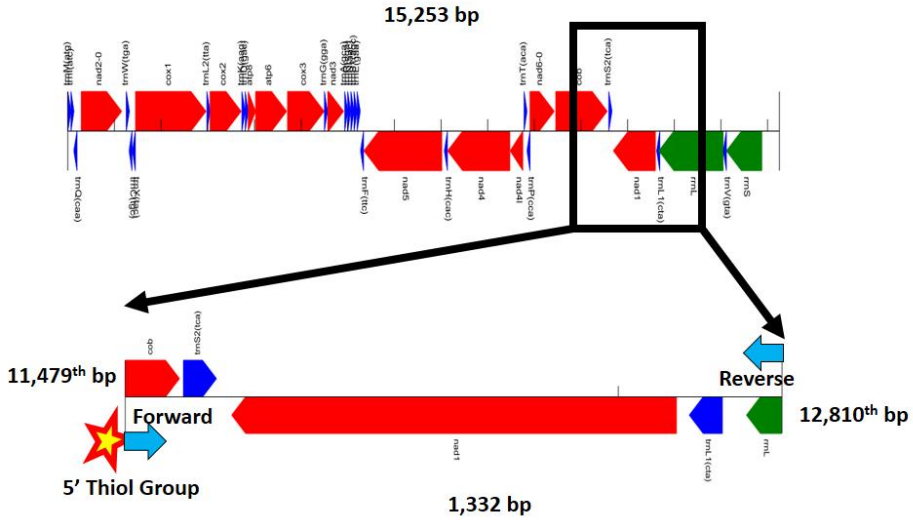
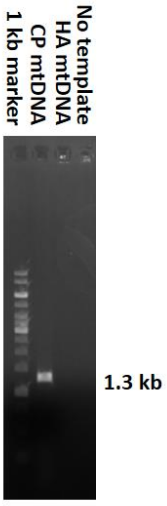

Cydia pomonella
mitochondrial genome
15,253 bp
Cydia pomonella
mitochondrial genome
15,253 bp
complex I (NADH dehydrogenase)
complex II (succinate dehydrogenase)
complex III (ubichinol cytochrome c reductase)
complex IV (cytochrome c oxidase)
ATP synthase
cytochrome c biogenesis
RNA polymerase
ribosomal proteins (SSU)
ribosomal proteins (LSU)
maturases
other genes
ORFs
transfer RNAs
ribosomal RNAs
origin of replication
polycistronic transcripts

(a)


15,253 bp
11,479th bp
Forward
5' Thiol Group
Reverse
12,810th bp
1,332 bp

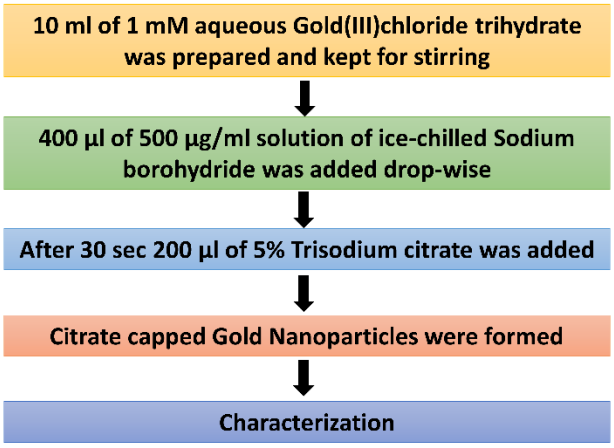
(b)


No template
HA mtDNA
CP mtDNA
1 kb marker
1.3 kb

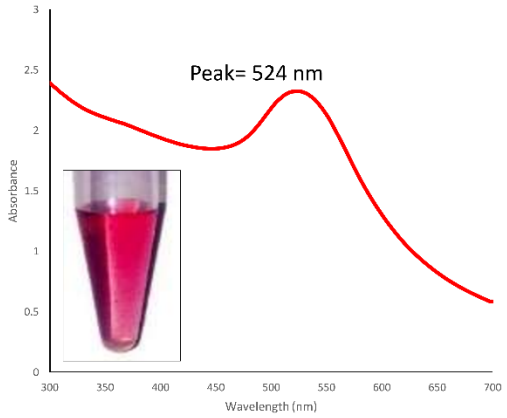
(c)

455

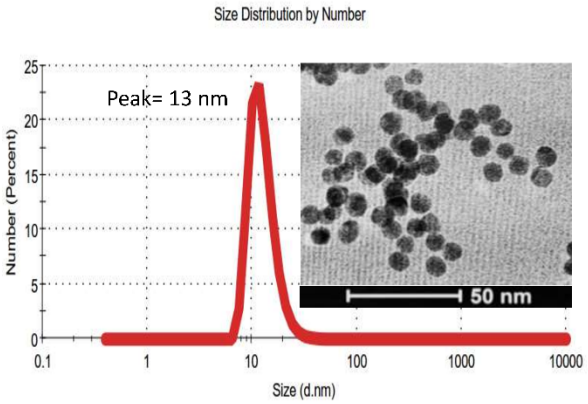
Figure 1. Designing and characterization of oligonucleotide probe. (a) Circular and linearized maps of mitochondrial genome of codling moth *C. pomonella*. (b) Designing of oligonucleotide probe and PCR primers. (c) Gel showing PCR product at 1.3 kb in case of mitochondrial DNA of *C. pomonella* (CA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


10 ml of 1 mM aqueous Gold(III)chloride trihydrate was prepared and kept for stirring
400 µl of 500 µg/ml solution of ice-chilled Sodium borohydride was added drop-wise
After 30 sec 200 µl of 5% Trisodium citrate was added
Citrate capped Gold Nanoparticles were formed
Characterization

(a)


Peak= 524 nm
Absorbance
Wavelength (nm)

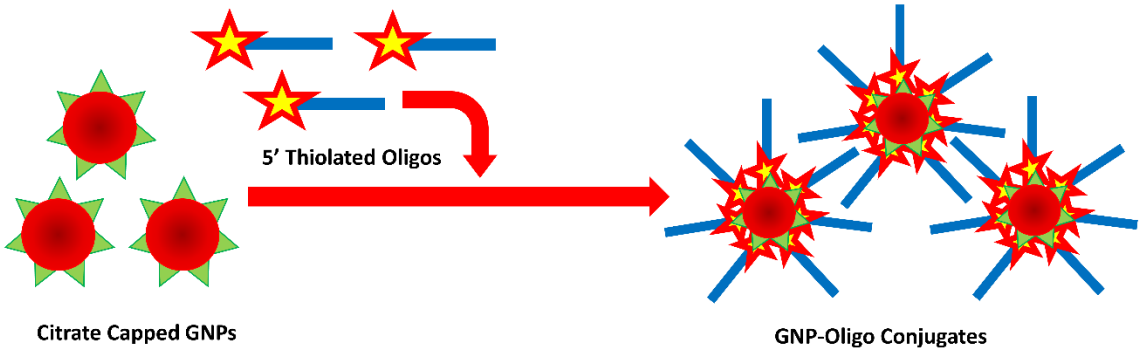
(b)


Size Distribution by Number
Peak= 13 nm
Number (Percent)
Size (d.nm)
50 nm

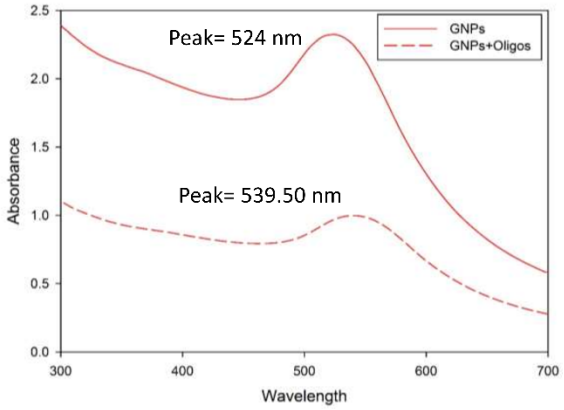
(c)

461

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


5′ Thiolated Oligos
Citrate Capped GNPs
GNP-Oligo Conjugates

(a)


Peak= 524 nm
Peak= 539.50 nm
GNPs
GNPs+Oligos
Absorbance
Wavelength
2.5
2.0
1.5
1.0
0.5
0.0
300
400
500
600
700

(b)

468

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 UV-visible spectrophotometer. Red shift in absorbance peak indicates conjugation success.

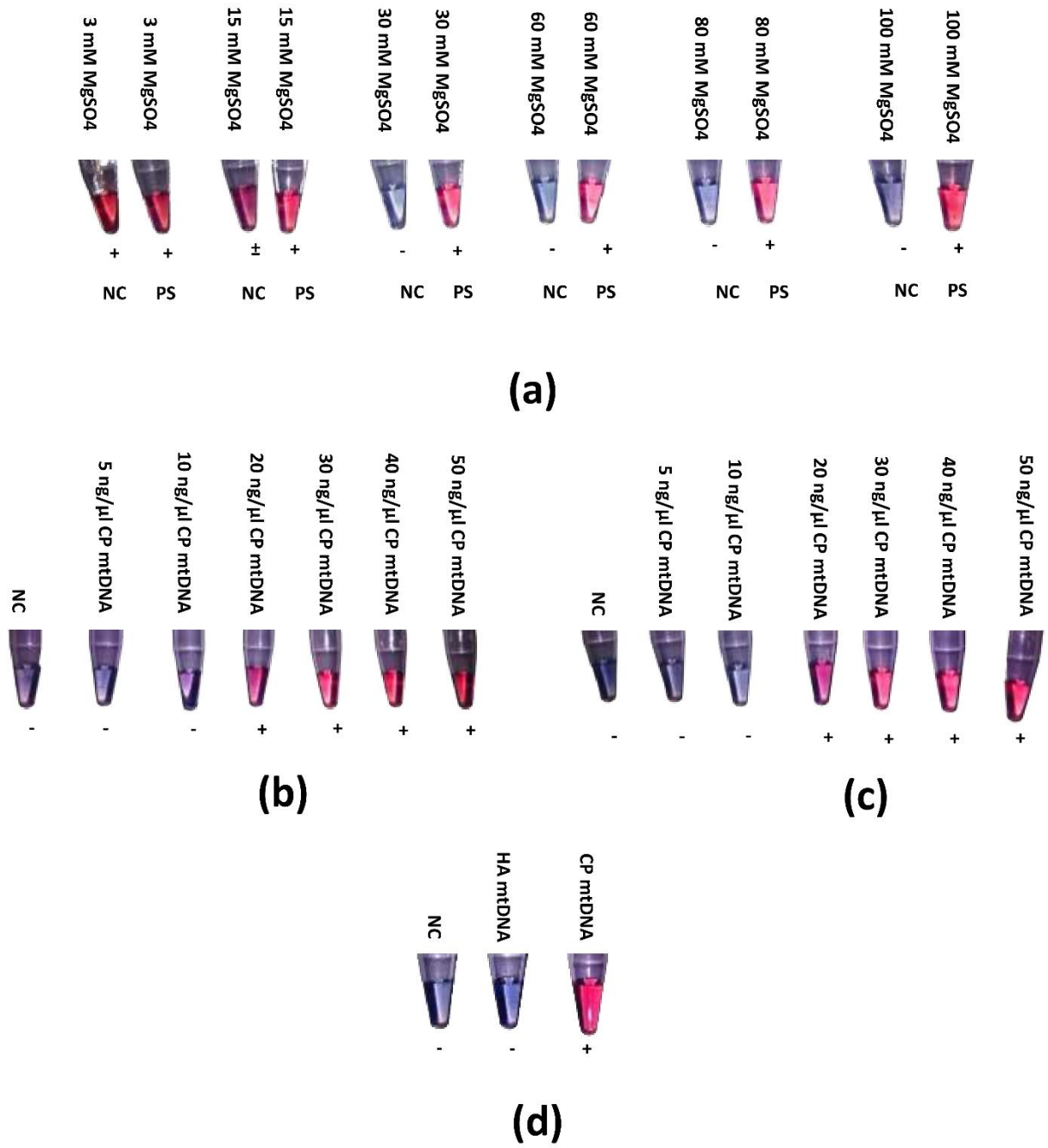

3 mM MgSO4
3 mM MgSO4
15 mM MgSO4
15 mM MgSO4
30 mM MgSO4
30 mM MgSO4
60 mM MgSO4
60 mM MgSO4
80 mM MgSO4
80 mM MgSO4
100 mM MgSO4
100 mM MgSO4
+
+
+
+
-
+
-
+
-
+
-
+
NC
PS
NC
PS
NC
PS
NC
PS
NC
PS
(a)
NC
5 ng/µl CP mtDNA
10 ng/µl CP mtDNA
20 ng/µl CP mtDNA
30 ng/µl CP mtDNA
40 ng/µl CP mtDNA
50 ng/µl CP mtDNA
-
-
-
+
+
+
+
(b)
NC
5 ng/µl CP mtDNA
10 ng/µl CP mtDNA
20 ng/µl CP mtDNA
30 ng/µl CP mtDNA
40 ng/µl CP mtDNA
50 ng/µl CP mtDNA
-
-
-
+
+
+
+
(c)
NC
HA mtDNA
CP mtDNA
-
-
+
(d)

Figure 4. Hybridization of GNP-oligonucleotide conjugate with mitochondrial DNA. (a) Optimization of MgSO4 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. NC= negative control without mtDNA; PS = sample containing *C. pomonella* mtDNA; CP = *C. pomonella*; HA = *H. armigera*; (+) = positive result, (-) = negative result.