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DNA Finger printing techniques in identification of drugs of natural origin

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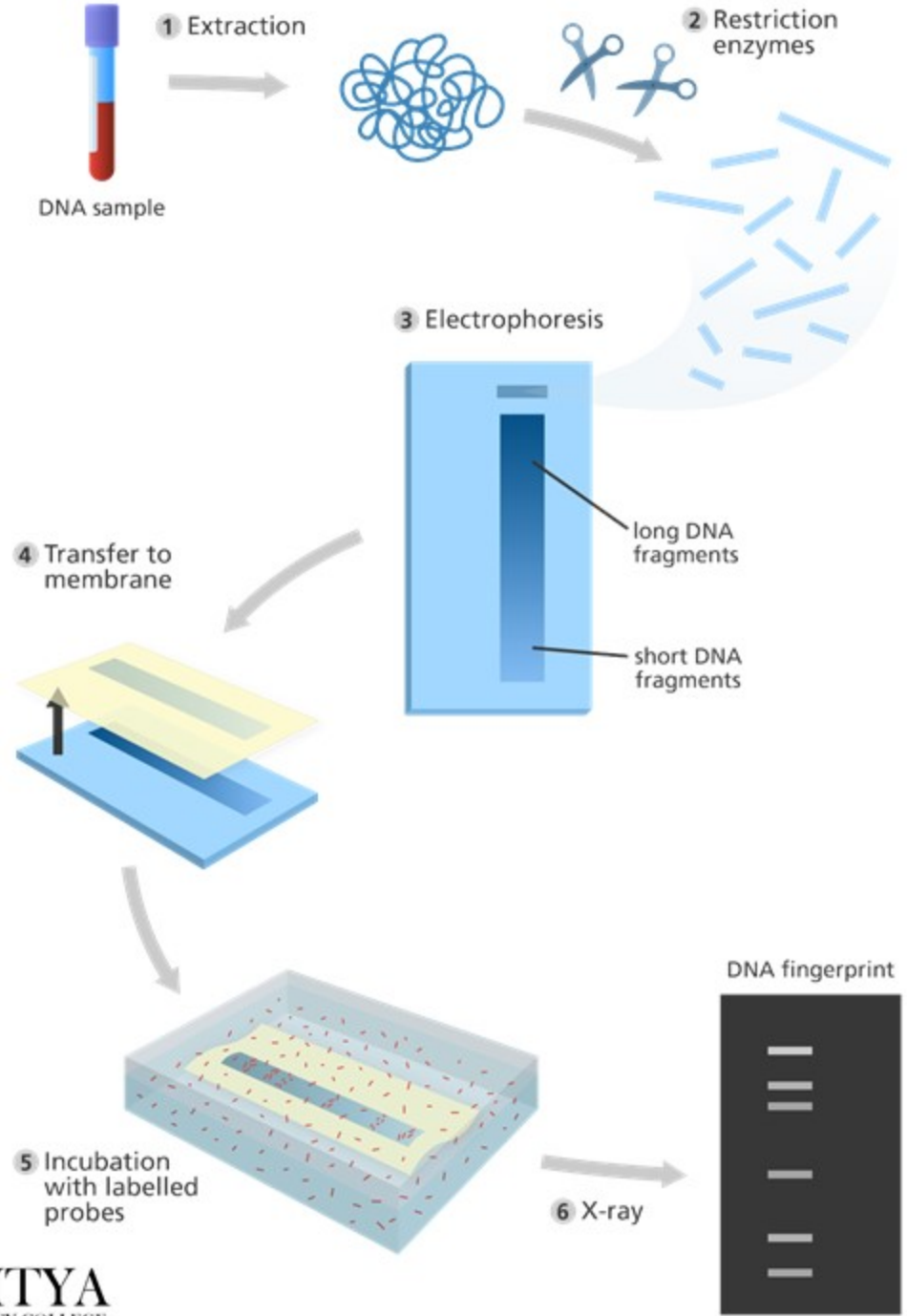
Substitution

- Substitution could potentially endanger the health and safety of the consumers.
- Substitution of samples could arise due to more than one co-occurring species sharing the same vernacular name and hence leading to confusion for the collectors.
- Alternatively, it could occur due to the inability of the collector to distinguish two or more co-occuring species because of their close morphological similarity.
- A number of techniques have been developed to identify medicinal plants in trade including the use of morpho-taxonomical keys, histological techniques, chemical fingerprinting and DNA-based approach, each having their own advantages and disadvantages.

DNA fingerprinting

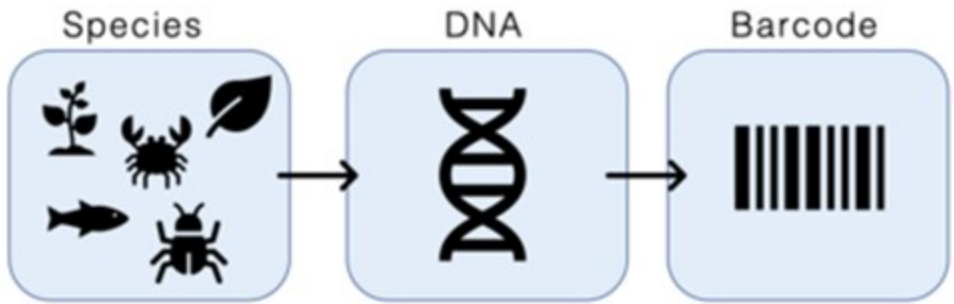
- DNA fingerprinting was invented in 1984 by Professor Sir Alec Jeffreys after he realised you could detect variations in human DNA, in the form of these minisatellites.
- DNA fingerprinting is a technique that simultaneously detects lots of minisatellites in the genome to produce a pattern unique to an individual. This is a DNA fingerprint.
- The probability of having two people with the same DNA fingerprint that are not identical twins is very small.

DNS finger printing



DNA barcode

- DNA barcoding has been extensively used as an accurate, cost-effective, and reliable tool to identify medicinal plant material used in trade.
- DNA barcoding to detect adulterations, including product substitution, contamination, use of endangered species for medicinal purposes and the use of unlabeled fillers that pose considerable health concerns.
- Biological reference material (BRM) consisting of taxonomically authenticated samples of each of the species along with other co-occurring and congeneric allied species that are likely to be used in adulteration was established.
- Using the DNA barcode sequences of the BRM as reference, the analysis indicated that the substitution ranged from 20 to 100%



DNA barcoding

- DNA barcoding is a method of species identification using a short section of DNA from a specific gene or genes.
- The premise of DNA barcoding is that, by comparison with a reference library of such DNA sections (also called "sequences"), an individual sequence can be used to uniquely identify an organism to species, in the same way that a supermarket scanner uses the familiar black stripes of the UPC barcode to identify an item in its stock against its reference database.
- These "barcodes" are sometimes used in an effort to identify unknown species, parts of an organism, or simply to catalog as many taxa as possible, or to compare with traditional taxonomy in an effort to determine species boundaries.

Collection of sample

- Using the identified trade names and vernacular names, raw drug samples of the different species were collected.
- Region-specific vernacular names and trade names were obtained from the literature or from “Ayurvedic Pharmacopoeia of India”.
- The trade samples included different plant parts such as fruits, leaf, stem, bark and roots were collected.
- All the market samples were collected for a single species, and multiple individuals (at least three) were sampled for DNA analysis.
- Most of the market samples were difficult to identify morphologically as they were in dry form and had not retained the characteristic features of the plant.
- Each market sample was given a unique sample number so as to ensure a chain of custody protocol.
- Each market sample also contained details about the date and location of collection, shop name, and collection number.

Table 1 Percentage of species adulteration in the raw herbal trade of medicinal plants in South India

Sl. no.	Trade name	Corresponding scientific name	Major source supply	Parts used	Percentage of adulteration
1.	Bilva/bael	*Aegle marmelos* (L.) Corrêa	W	Leaf, root, fruit	0
2.	Kalmegh/nilavembu	*Andrographis paniculata* (Burm.f.) Nees	C/W	Leaf	0
3.	Shatavari	*Asparagus racemosus* Willd.	C/W	Root	0
4.	Brahmi/neer-brahmi/vallarai	*Bacopa monnieri* (L.) Wettst./*Centella asiatica* (L.) Urb.	C/W	Leaf, whole plant	0
5.	Punarnava/raktapunarva	*Boerhavia diffusa* L.	W	Root, whole plant	40
6.	Akhaphoot	*Calotropis procera* (Aiton) Dryand.	W	Flowers	0
7.	Malkangani/bavanthibeeja/valuzhuvai	*Celastrus paniculatus* Willd.	W	Fruit (Seed)	0
8.	Daruharidra/mara manjal	*Coscinium fenestratum* (Goetgh.) Colebr.	W	Stem	90
9.	Kali musli/talamuli	*Curculigo orchioides* Gaertn.	W	Roots	0
10.	Aaldi, karimanjal/haridra	*Curcuma longa* L.	W/C	Rhizome	0
11.	Musta/nagarmotha	*Cyperus rotundus* L.	W	Rhizome	0
12.	Bhringraj/bhiranraja	*Eclipta prostrata* (L.) L.	W	Whole pla nt	0
13.	Vidanga	*Embelia ribes* Burm.f.	W	Fruit	75
14.	Langali/kalihari/kalappakilangu	*Gloriosa superb* L.	W/C	Rhizome	50
15.	Gudmaar/sirukurinjan	*Gymnema sylvestre* (Retz.) R.Br. ex Sm.	W	Leaf	0
16.	Anatmool/sariwa/sarasaparilla/svetasariva	*Hemidesmus indicus* (L.) R. Br. ex Schult.	W	Root	0
17.	Vasa/adusa/adhatoda	*Justicia adhatoda* L.	W/C	Leaf	0
18.	Champaka	*Magnolia champaca* (L.) Baill. ex Pierre	W/C	Flower	0
19.	Nagakesar/nagakesari/nagkeshar	*Mesua ferrea* L.	W	Flower	33
20.	Isapgul	*Plantago ovate* Forssk.	W/C	Seeds	0
21.	Citraka/chitrak	*Plumbago zeylanica* L.	W	Bark/stem	25
22.	Manjantthi	*Morinda citrifolia* L.	W	Fruit	100
23.	Arjuna/arjun/maruthapattai	*Terminalia arjuna* (Roxb. ex DC.) Wight &Arn.	W/C	Bark	0
24.	Behda/Bibhitaki/thandrekai	*Terminalia bellirica* (Gaertn.) Roxb.	W/C	Fruit	0
25.	Harda/himaj/kadukkai	*Terminalia chebula* Retz.	W/C	Fruit	0
26.	Amrithaballi/guduci	*Tinospora cordifolia* (Willd.) Miers	W	Stem	20
27.	Vettiver/lavancha	*Chrysopogon zizanioides* (L.) Roberty	W/C	Roots	0
28.	Neergundi/nocchi/renuka	*Vitex negundo* L.	W/C	Whole plant	0
29.	Asvagandha/ammukira	*Withania somnifera* (L.) Dunal	C/W	Root, whole plant	0
30.	Shunti/ardaka/chukka	*Zingiber officinale* Roscoe	C/W	Rhizome	0

W WILD, *C* cultivation

CTAB techniques for Genome DNA extraction from plant tissue

- CTAB buffer:100ml
  - 2.0 g CTAB (Hexadecyl trimethyl-ammonium bromide), 10.0 ml 1 M Tris pH 8.0, 4.0 ml 0.5 M EDTA pH 8.0 (EthylenediaminetetraAcetic acid Di-sodium salt), 28.0 ml 5 M NaCl, 40.0 ml H_2O, 1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) Mw 40,000) and Adjust all to pH 5.0 with HCL and make up to 100 ml with H_2O.
- 1 M Tris pH 8.0
  - Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust pH to 8.0 by adding 42 ml of concentrated HCl. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume to 1 L with H_2O. Sterilize using an autoclave.
- 5x TBE buffer
  - 54 g Tris base 27.5 g boric acid 20 ml of 0.5M EDTA (pH 8.0) Make up to 1L with water. To make a 0.5x working solution, do a 1:10 dilution of the concentrated stock.
- 1% Agarose gel
  - 1 g Agarose dissolved in 100 ml TBE

CTAB TECHNIQUES

- Grind 200 mg of plant tissue to a fine paste in approximately 500 µl of CTAB buffer.
- Transfer CTAB/plant extract mixture to a microfuge tube.
- Incubate the CTAB/plant extract mixture for about 15 min at 55o C in a recirculating water bath.
- After incubation, spin the CTAB/plant extract mixture at 12000 g for 5 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.
- To each tube add 250 µl of Chloroform : Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13000 rpm for 1 min.
- Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.
- To each tube add 50 µl of 7.5 M Ammonium Acetate followed by 500 µl of ice cold absolute ethanol.
- Invert the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution.
- Alternatively the tubes can be placed for 1 hr at -20 o C after the addition of ethanol to precipitate the DNA.

CTAB TECHNIQUES

- Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution.
- The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate.
- To wash the DNA, transfer the precipitate into a microfuge tube containing 500 µl of ice cold 70 % ethanol and slowly invert the tube.
- Repeat. ((alternatively the precipitate can be isolated by spinning the tube at 13000 rpm for a minute to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70 % ethanol)).
- After the wash, spin the DNA into a pellet by centrifuging at 13000 rpm for 1 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min).
- Do not allow the DNA to over dry or it will be hard to re-dissolve.
- Resuspend the DNA in sterile DNase free water (approximately 50-400 µl H_2O; the amount of water needed to dissolve the DNA can vary, depending on how much is isolated).
- RNaseA (10 µg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 µl RNaseA in 10ml H_2O).
- After resuspension, the DNA is incubated at 65o C for 20 min to destroy any DNases that may be present and store at 4° C.
- Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

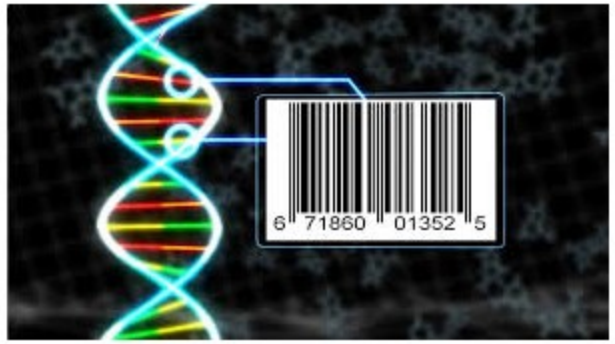
DNA barcode of biological reference material (BRM)

- Genomic DNA was extracted from leaves of 124 BRM samples representing the 30 medicinal plant species and their 14 allied species.
- PCR amplification was conducted using the universal primers, namely complete nr-ITS region, ITS1-TCCGTAGGTGAACCTGCGG; ITS4-TCCTCCGCTTAT TGA TAT GC and psbA-trnH region, psbA-GTT ATG CAT GAA CGT AAT GCTC; trnH-CGC GCATGGTGGATTCACAAATC spacer region.

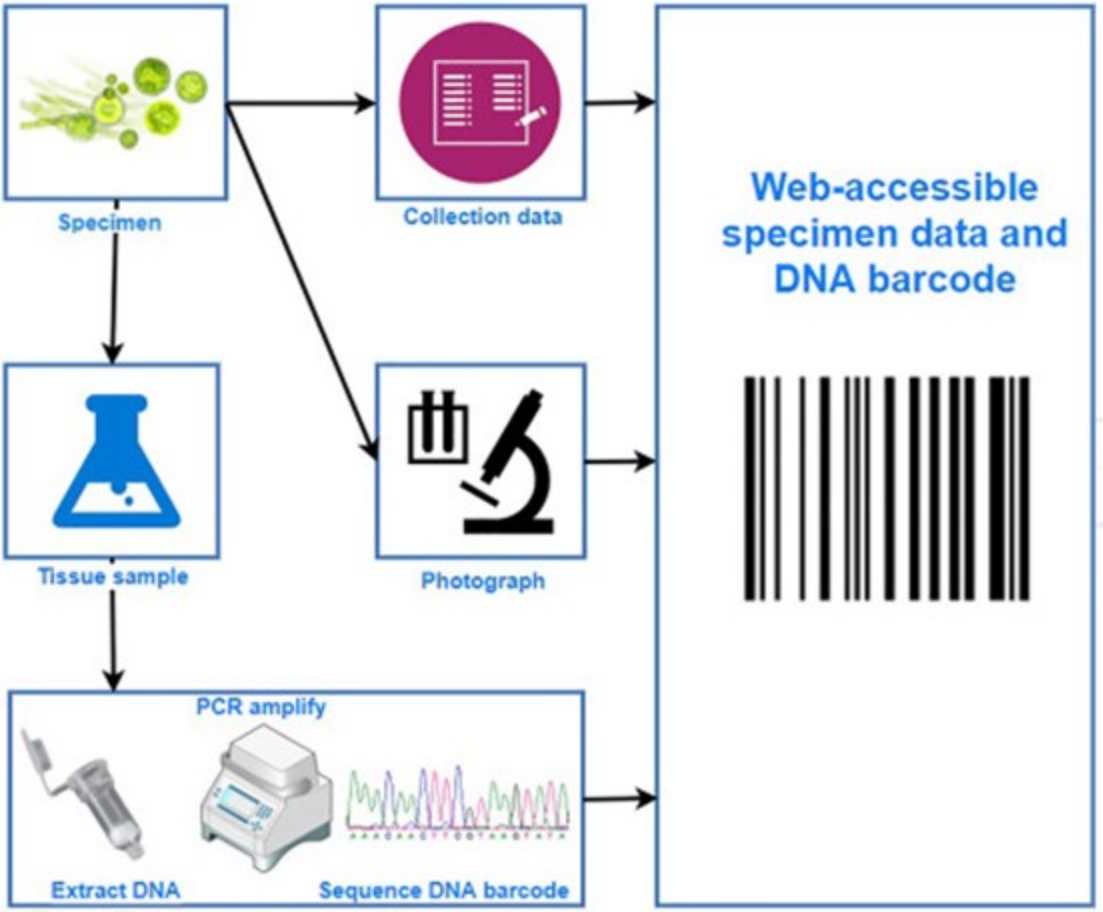
PCR amplification

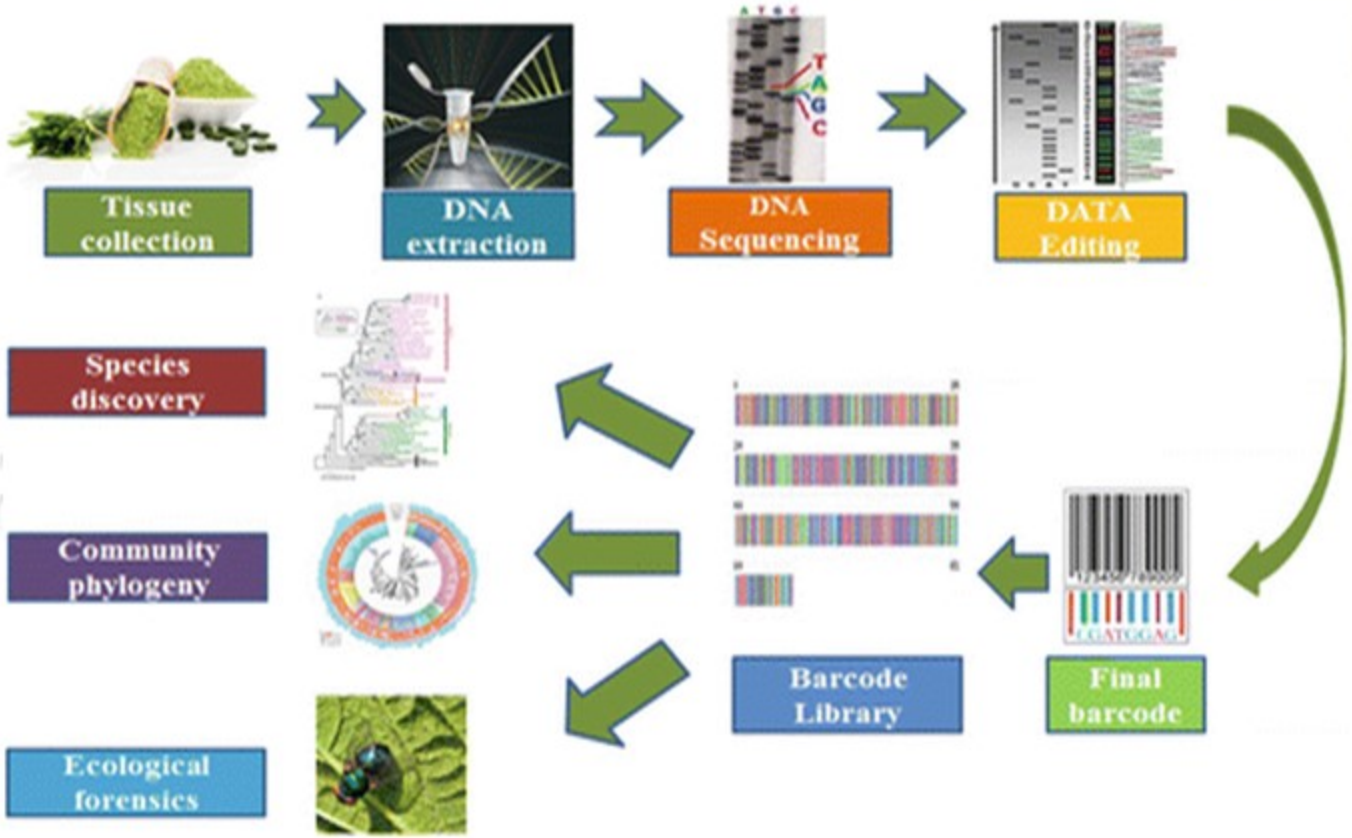
- PCR amplification was carried out in 25 µL reaction volume which consisted of template concentration of 80 ng/µL, 2.5 µL of 10X Taq buffer(Genie), 1 µL of 2 mM MgCl2, 2.5 µL of 1 mM dNTP's mixture, 5 pM of 1.5 µL each primer, 1.5 U Taq DNA polymerase(Genie) and sterile distilled water.
- PCR was performed in an Eppendorf Mastercycler Gradient (Hamburg, Germany).
- The amplification profile was 94 °C for 4 min followed by 30 cycles of 94 °C for 60 s, 55 °C for 45 s, 72 °C for 90 s with a final extension step at 72 °C for 10 min.
- The amplified products were sequenced in unidirectional.

Sequencing



- Direct sequencing of the gel-purified amplicon yielded a sequence length between 640 and 750 bp and 380 and 450 bp for nr-ITS and psbA-trnH spacer region, respectively.
- The obtained sequence results were edited manually using the BioEdit software (Version 5.0.6).
- The sequences of the nr-ITS region and psbA-trnH spacer region have been deposited at GenBank.
- The obtained BRM sequences were used as a query sequence in BLASTn in GenBank to identify the best matching sequences.
- Those sequences with the best match in blast search were downloaded as FASTA format from the GenBank and were included in the analysis


Specimen
Collection data
Tissue sample
Photograph
PCR amplify
Extract DNA
Sequence DNA barcode
Web-accessible specimen data and DNA barcode


Tissue collection
DNA extraction
DNA Sequencing
DATA Editing
Species discovery
Community phylogeny
Ecological forensics
Barcode Library
Final barcode

Assessment of adulteration in raw herbal trade market:

- To assess the extent of adulteration, if any, the raw herbal drug samples obtained from different locations in the South India were processed for determining the identity of the species using the BRM barcode as the reference.
- The plant material obtained from the shop was either in the form of stem, leaf, root or bark.
- This material was randomly separated into three parts representing three replicates with each of the part containing roughly one-third of the original material.

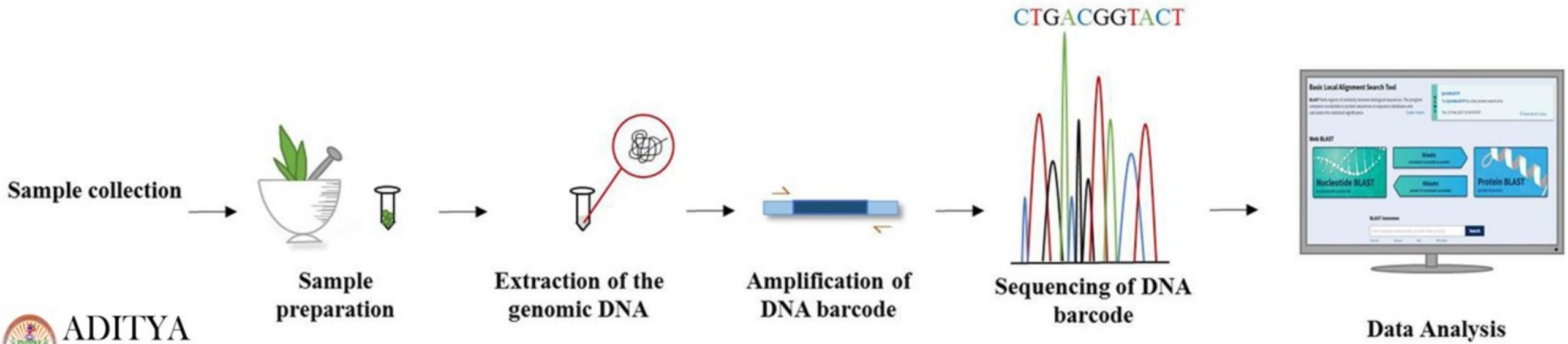
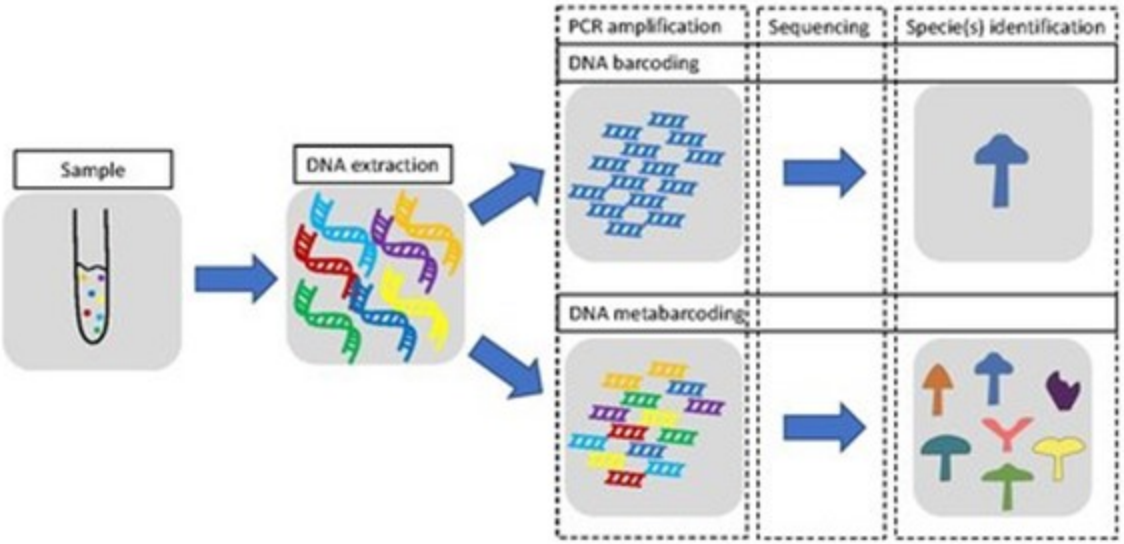


Table 2 List of sample code, vernacular name, simple BLAST results and final identifications based on the DNA barcoding

Sl. no.	Trade name	Market samples code	ITS_blast Vs BRM	ITS_blast Vs NCBI	PsbA_blast Vs BRM	PsbA_blast Vs NCBI	Identification by DNA barcoding approach
1	Bilva/bael	HAS 8, 52, 66, 78, 91, 113, 147, 232, 242, 252, 269	*Aegle marmelos*	*Aegle marmelos*	*Aegle marmelos*	*Aegle marmelos*	*Aegle marmelos*
2	Kalmegh/nila-vembu	HAS 41, 157, 201, 494, 520, 571	*Andrographis paniculata*	*Andrographis paniculata*	*Andrographis paniculata*	*Andrographis paniculata*	*Andrographis paniculata*
3	Shatavari	HAS 45, 110, 156, 186, 210, 278, 287	*Asparagus racemosus*	*Asparagus racemosus*	*Asparagus racemosus*	*Asparagus racemosus*	*Asparagus racemosus*
4	Brahmi/neer-brahmi/vallarai	HAS 36,117, 499, 528, 569	*Bacopa monnieri*	*Bacopa monnieri*	*Bacopa monnieri*	*Bacopa monnieri*	*Bacopa monnieri*
		HAS 85	Not matching	*Centella asiatica*	Not matching	*Centella asiatica*	*Centella asiatica*
		HAS 146	Not matching	*Centella asiatica*	Not matching	*Centella asiatica*	*Centella asiatica*
		HAS 174	Not matching	*Centella asiatica*	Not matching	*Centella asiatica*	*Centella asiatica*
5	Punarnava/rakta-punarva	HAS 162	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*
		HAS 205	Not matching	*Boerhavia erecta*	Not matching	*Boerhavia erecta*	*Boerhavia erecta*
		HAS 501	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*
		HAS 537	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*	*Boerhavia diffusa*
		HAS 563	Not matching	*Boerhavia erecta*	Not matching	*Boerhavia erecta*	*Boerhavia erecta*
6	Akhaphool	HAS 512, 572	*Calotropis procera*	*Calotropis procera*	*Calotropis procera*	*Calotropis procera*	*Calotropis procera*
7	Malkangani/bavanthibeeja/valuluvai	HAS 517, 522, 556, 587	*Celastrus paniculatus*	*Celastrus paniculatus*	*Celastrus paniculatus*	*Celastrus paniculatus*	*Celastrus paniculatus*

Sample analysis

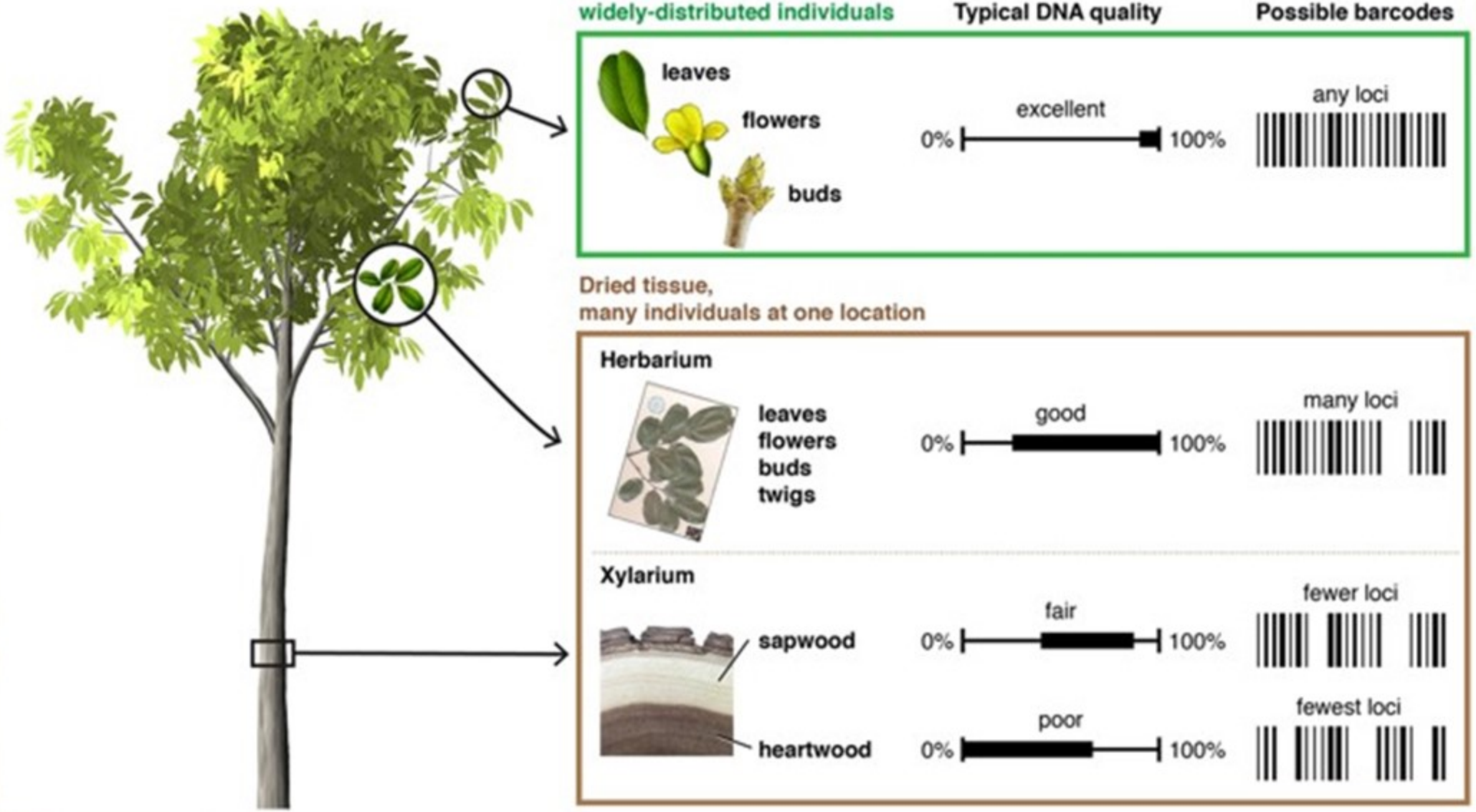
- The selected samples were ground, using liquid nitrogen and the genomic DNA was extracted from each of these three replicate samples using the Qiagen kit following the manufacturer's instructions (Cat No./ID 69104).
- Extracted DNA was purified using commercially available kits (Qiagen, Cat No./ID 28604).
- Genomic DNA was amplified using nr-ITS region and psbA-trnH spacer and sequenced as described as earlier.
- The sequences obtained from the herbal drug samples and the sequences downloaded from GenBank were analyzed along with the reference barcode library (BRM) as single query sequence.



- Differences in the standard methods for DNA barcoding & metabarcoding.
- While DNA barcoding points to find a specific species, metabarcoding looks for the whole community

Matching analysis

- All the BRM sequences matched either with the same species or the same genera.
- The amplification and sequencing of the barcode regions for the trade samples were relatively difficult compared to the BRM samples and required multiple attempts to obtain good sequences.
- The sequences obtained were compared with those obtained from the BRM DNA barcode library.


Fresh tissue,
widely-distributed individuals
Typical DNA quality
Possible barcodes
leaves
flowers
buds
excellent
0%
100%
any loci
Dried tissue,
many individuals at one location
Herbarium
leaves
flowers
buds
twigs
good
0%
100%
many loci
Xylarium
sapwood
heartwood
fair
0%
100%
fewer loci
poor
0%
100%
fewest loci

Common problem

- A common problem encountered while barcoding using nr-ITS was contamination with fungal flora, that were probably present in the raw drug samples.
- It may overcome by either scraping the outer tissues using a fine razor blade and or cleansing the outer surface using ethanol and/or by repeated extraction and amplification.

- This technique is independent of the type of tissue collected and also the geographical source of the material.
- Unlike animal systems, where the mitochondrial COI is regarded as the universal bar code, in plants there is as yet no consensus on a universal barcode.
- A number of authors have shown that the chloroplastic gene regions such as psbA-trnH and nuclear region such as nr-ITS have been widely used in raw herbal drug authentication.

REFERENCE

- https://agriculture.uq.edu.au/files/5650/plant-genomic-dna-extraction-by-ctab-2-fiona.pdf
- J. U. Santhosh Kumar, V. Krishna, G. S. Seethapathy, R. Ganesan, G. Ravikanth, R. Uma Shaanker, Assessment of adulteration in raw herbal trade of important medicinal plants of India using DNA barcoding, 3 Biotech (2018) 8:135.