BRITISH PHARMACOPOEIA COMMISSION

Working Party (DNA): Identification Techniques

SUMMARY MINUTES

A meeting of this Working Party was held at 151 Buckingham Palace Road, London, R-Y 335 on the 10th of July 2015.

Present: Dr K Helliwell (Chairman), Dr J A Hawkins, Dr E Mee, Prof. A. Slater and Prof. E M Williamson.

In attendance
NIBSC: Dr C Howard, Mrs C Lockie-Williams
BP Secretariat: Dr P Holland, Dr R Pask-Hughes and Mr M Whaley.

An apology for absence was received from Dr I Feavers

24 **General**

Members were reminded of the confidential nature of the papers, discussion and the minutes of the meeting. The Secretariat reminded members that the papers of the meeting were accessible from the BP website.

Declaration of Interests Prof. Slater declared interests in one or more agenda items and appropriate action was taken.

I MINUTES

25 The minutes of the meeting held on Wednesday 21st of January 2015 were confirmed.

II MATTERS ARISING FROM THE MINUTES

26 A list of matters arising from the minutes of the meeting of the WP (DNA) held in January 2015 was circulated together with the papers for this meeting. A copy is appended.

27 **Revised Terms of Reference** WP (DNA) (15)12

A revised Terms of Reference (ToR) of the Working Party to reflect the extension of the herbal project, taking into consideration comments by the Chairman, was received. The ToR focused on public health protection, pharmacopoeial standards for herbal medicines and looked to address the Pharmacopoeia's global standing alongside accountability and traceability. To demonstrate accountability of resources employed for the herbal project, members' acknowledged that it was essential to demonstrate transparency and where feasible to aim towards generating a return on the investment.

Members' attention was drawn to the work programme of the BP Commission Expert Advisory Group (EAG) on Herbal and Complementary Medicines (HCM). They were invited to consider the lists and to comment and advise on which of the herbal drugs would be good candidates for DNA barcoding either because of toxicity issues, as for instance *Aristolochia* species, or where there were adulteration concerns. Such information would be helpful to the

prioritisation of the on-going work.

The Chairman explained the current classifications defined in the published *Ph. Eur.* general monograph for Herbal Drug Extracts. He provided members with background information on the development of the monograph. A greater understanding of the nature of the different extracts defined in the *Ph. Eur.* general monograph should inform the rationale of investigating the application of DNA barcoding to commercially available extracts. Members agreed that applicability of DNA barcoding to commercial extracts should be assessed at the appropriate time in the near future.

Members also discussed the use of barcoding herbal substances on the MHRAs banned list. It was accepted that a DNA barcoding test would be beneficial if the sample was toxic because it would negate the need to work with toxic reference substances within the Laboratory. Dr Helliwell gave *Atropa belladonna* as an example of a toxic species where barcoding might be a useful method of identification. It was pointed out that adverse long term effects of herbal drugs may not yet be reported for some herbal drugs.

It was concluded that it was unlikely that barcoding in its current form would be found to be an acceptable pharmacopoeial test method for herbal medicinal products but inclusion of methods for herbal drug preparations would be useful. Members endorsed the revised Terms of Reference.

28 **Guidelines - The use of DNA barcoding as an identification method in BP monographs** WP (DNA) (15)12b

Draft revised guidelines on the use of DNA barcoding as an identification method in BP monographs were received and discussed. The revised document contained changes reflecting comments made by EAG HCM at its meeting in June 2015. The action for the BP – NIBSC Herbal Laboratory to explore the feasibility of applying barcoding to *Aristolochia* species had been deferred pending further consideration by both EAG HCM and the WP (DNA). The revised document will be presented to the BP Commission in the near future.

29 **Herbal Project – Progress Report** WP (DNA) (15)13

An extract from a paper on 'Herbal and Complementary Medicines' presented to the BP Commission at its meeting in July 2014 was presented for information. Dr Howard informed members of the positive comments that had been made by Commissioners. The Secretariat thanked the Working Party for their hard work and input. The Chairman stated that the extension of the project displayed confidence in the project. The question was raised whether additional expertise was required to complement the current membership in view of the revised Terms of Reference of the Working Party and also the extension of the project timeline. The Chairman commented that the current membership more than adequately covered the required areas of expertise.

30 **Deoxyribonucleic Acid (DNA)-Based Identification Techniques for Herbal Drug Preparations and Herbal Medicinal Products** WP (DNA) (15)14

Copies of the report titled 'A review of the New York Attorney General's Herbal Supplement Investigation' and a full report by the BP-NIBSC Herbal Laboratory on DNA identification of *Passiflora incarnata* Ker Gawl. together

with two separate published papers titled ‘The application of a DNA-based identification technique to over-the-counter herbal medicines’ by Kazi *et al*. 2013, Fitoterapia and ‘The Capabilities and Limitations of DNA Barcoding of Botanical Supplements’ published by Authentechnologies in March 2015 were received. A thorough background information into events that had occurred in the USA on the use of DNA barcoding to investigate herbal products instigated through the New York Attorney General’s office was provided at the meeting.

Members had commented earlier in the year by correspondence on the full report of the DNA analysis of the *Passiflora incarnata* aqueous extract which concluded that DNA was not successfully extracted from the aqueous extract sample. Although some amplicons had been produced, these were most likely caused by environmental contamination and did not correlate well with the starting material used. It appeared that the processing used to create the aqueous extract had damaged or removed the DNA of the *Passiflora incarnata* herbal drug. It was noted that key points for consideration when working with herbal extracts were that DNA may not be present or may be too degraded for standard polymerase chain reaction (PCR) to take place. The report on the *Passiflora incarnata* extract was a good demonstration of such a situation.

There was consensus amongst members that an evidence-based and rational approach was paramount to the success of the herbal project. The idea was raised regarding amplification of small amplicons of approximately 80 base pairs, which would be more easily amplified and more resistant to degradation. The idea of designing methods against known plant mini barcodes was also discussed.

Members noted the published paper by Kazi *et al*. and the results within it, showing the utility of short amplicons for finished product testing. This led to an in-depth discussion by members. It was agreed that short amplicons were more resistant to degradation although there was evidence that work on ancient samples had yielded DNA fragments as short as 30 base pairs. Smaller amplicons would contain fewer DNA base pairs for analysis which could heighten the possibility of cross species amplification, showing the importance of validating any methods developed. The use of full barcode regions to design subsequent tests should minimise this possibility. It was considered whether different extracts should be tested to see any comparisons and differences.

A standardised presence or absence test was seen to be of benefit for the end-user, requiring less molecular expertise and experience from the analyst and being more user-friendly. The idea of using a bio-analyser and qPCR machine was also raised as a possibility. It appeared that a published paper where effect of boiling of Ginseng at various different time points had been investigated and appeared to be the current extent of reported data in the scientific literature regarding the presence and quality of DNA after processing.

It was highlighted that an emphasis on selecting the right species was important due to the amount of time that would be dedicated by the BP-NIBSC Herbal Laboratory and the information generated could be extremely useful. The question was raised whether it was desirable to focus on the finished product rather than the plant material used within it and the

suitability. A plan detailing a work programme to assess the applicability of DNA testing to variety of herbal preparations on the market at an appropriate time in the near future.

31 **Operational and Strategic Plans** WP (DNA) (15)15

It was agreed that the initial objectives of the herbal project had been achieved, namely the publication of the appendix method 'Deoxyribonucleic Acid (DNA)-Based Identification Techniques for Herbal Drugs' in the BP 2016 and the production of a reference material aimed to aid users in the application of DNA-based identification methods. It was noted that the timescale of the herbal project had been increased by an additional three years.

Tentative plans for work to be done at the BP – NIBSC Herbal Laboratory to end of 2016 were presented for discussion. A copy of the published *Ph. Eur* monograph for Black Cohosh was received for information.

Authentication of herbal drugs It was agreed that work should continue on the authentication of herbal drugs used for the development of test methods and where applicable assay methods for the inclusion in BP monographs.

Development of Quantitative Methods The discussion focused on qPCR and the justification of using Holy Basil as the first candidate. Prof. Slater had queried the benefits of analysing Holy Basil leaf by qPCR at the meeting in January 2015. It was confirmed that Holy Basil leaf was not a good candidate because there were no corresponding licensed preparations or products. Members considered Black Cohosh as a probable test candidate. subject to a review of available data. It was agreed that whilst Holy Basil was likely to give a quick proof of principle results and the likelihood that Black Cohosh would prove more challenging, the latter would ultimately be more beneficial to public health.

Priority projects At the meeting of the Working Party in January 2015, it had been agreed that it would be worthwhile demonstrating the applicability of DNA identification methods as a tool for solving problems due to adulteration, contamination and/or toxicity. It was noted that for *Echinacea spp.* preliminary work done at CAMAG had indicated that the chemical profiles of samples sourced in Europe had changed in recent years. Dr Reich had provided the BP - NIBSC Herbal Laboratory with samples of *Echinacea* and the analysis was planned for August 2015.

32 **Supplementary Chapter**
DNA-Based Identification Methods
for Herbal Drugs WP(DNA) (15)16

A draft of a new Supplementary Chapter on DNA-Based Identification Methods for Herbal Drugs will be prepared before the next meeting of the Working Party (DNA) and will be progressed by correspondence.

33 **Barcode Reference Material – Update** WP (DNA) (15)17

A report on the Fitness for purpose testing of the BPNARM was received. Details of the original design of the reference material and the reasons for modifying the design were discussed. It was agreed that the BP-NIBSC Laboratory would continue fitness for purpose testing and amend the instructions to be provided to the user with the reference material as deemed necessary.

Convention on Biodiversity; Nagoya Protocol It was confirmed that the BPNARM material was outside the scope of both legislations.

34 ***Ocimum tenuiflorum*** ***Ocimum gratissimum*** WP (DNA) (15)18

A report was received from De Montfort University confirming that the BP 2016 Appendix XI V method was suitable for separating *O. tenuiflorum* and *O. gratissimum*. It was accepted that, if required, a barcode could be published for *O. gratissimum* with no changes to the published protocol in the BP 2016. It had been difficult to source the required number of batches to progress the chemical analytical work at the BP Laboratory. A report showing the DNA sequence differences of *O.gratissimum* was also received. Dr Howard reported that it had been observed at the BP-NIBSC Laboratory that *O. gratissimum* was a closer relation to *O. tenuiflorum* than *O. basilicum*. There were only a few base differences between the DNA sequences of *O. gratissimum* and *O. tenuiflorum* but there was also a large insertion within *O. tenuiflorum* which set it apart from *O. gratissimum*. It was accepted that it was possible to use DNA barcoding of the *trnH-psbA* region to separate these two *Ocimum* species.

A member offered to examine the *O. gratissimum* samples with the intention of providing information on both the macroscopical and microscopical descriptions. The DNA barcode data generated for *O. gratissimum* would be included in the BP at an appropriate time subject to availability of samples and the generation of chemical analysis data.

35 ***Drimia maritima*** (Squill) WP(DNA) (15)19

A report on the DNA analytical work on *Drimia maritime* (L.) Stearn. to date was received. It was explained that 14 samples of *D. maritima* had been received. Two of the samples had been selected to trial DNA extraction methods. The normal practice at the BP-NIBSC Herbal Laboratory was to take four samplings for DNA extraction from a potentially mixed sample. Advice was specifically sought on the sampling procedure for Squill because of the structure of the bulb. The wider issue of sampling was further discussed. For the Squill samples, it was agreed that samples should be milled/ground prior to extraction and that the results of analysis of the milled samples could be compared to pre-milled samples. The BP- NIBSC Herbal Laboratory was currently in the process of identifying a suitable metal grinder with ease of decontamination that will be robust and effective for milling.

It was noted the BP-NIBSC Herbal Laboratory had identified a source of *S. bifolia* and *S. sibirica* seeds, both were on sale from an established seed supplier. Germination of the seeds from these species would be attempted

for inclusion in the DNA analytical work. A member offered to provide herbarium samples to include closely related species of *Drimia* spp. for inclusion in the DNA analysis.

The DNA verified samples would be used for the development of test methods and if justified for the development of an assay method. The resulting outputs should enable the Secretariat to revise the published BP monographs.

36 **Phellodendron Amurense Bark**
Phellodendron Chinese Bark WP(DNA) (15)20

The two separate BP 2016 monographs for Phellodendron Chinese Bark and Phellodendron Amurense Bark were received for information. Members were reminded that a full report on the DNA barcoding analysis on *Phellodendron amurense* and *Phellodendron chinense* had been discussed at the meeting of the Working Party held in January 2015. The possibility of including the barcoding for both *P. chinense* and *P. amurense* in the published monographs had been considered. It was accepted that the agreed morphological and chemical tests approved for publication by EAG HCM adequately differentiated between the two species. The barcoding data would be incorporated into a Supplementary Chapter on DNA barcoding analysis intended for inclusion in material for publication in the BP 2017.

37 **Completed Projects:** WP(DNA) (15)21
Anethum Graveolens Sowa
Ophiopogon japonicus

Reports on DNA analysis of *Anethum Graveolens* Sowa and *Ophiopogon japonicus* were received. It was noted that the template of the report forms had now been standardised.

***Anethum graveolens* Sowa** Seed samples, some of which had been germinated and grown to maturity to produce vouchers and high quality DNA samples had been used for the work. For comparison commercial samples of fresh dill were used. Members were informed that DNA extraction was extremely efficient from all samples analysed. The DNA isolated from the seeds required dilution but no further purification was needed. Attention was drawn to the results and the analysis of the data. In regard to barcode selection, it was noted that no published sequences were publicly available for Anethum Sowa, the previous botanical name for *A. graveolens* Sowa. All samples analysed were described in the Kew report as a 'good match' making it problematic for the analysis of different DNA regions to find a species-specific sequence as there would be no comparator. The problem had been exacerbated by the lack of published sequence information from related species. Only 3 useful published sequences were found, making the assessment of applicable barcode regions problematic. Dill and Dill weed samples were incorporated in the analysis to provide a measure for the *A. graveolens* Sowa samples. It was noted that the *A. graveolens* Sowa classification was relatively recent and might account for the absence of specific information on the barcoding of the subspecies. The *rbcL*, *matK* and *trnL-F* regions all amplified well. Members considered the stepwise analysis of the data generated at the BP-NIBSC Herbal Laboratory in comparison with that published and agreed with the

conclusion and outputs stated in the report. That is both the *trnH-psbA* and the ITS region could be used to identify *A. graveolens* and closely related *Anethum* species. Within the ITS region two base positions were specific to the *A. graveolens* Sowa subspecies. The conclusion had been based on the analysis of very few non-Sowa sequences. Members endorsed the suggestion for the BP-NIBSC Laboratory to analyse additional samples known to be *A*. *graveolens* but not the Sowa subspecies. It was also accepted to continue the analysis of the current samples to ensure that the contig sequences all cover the key bases for identification which would be best achieved using the ITS2 sub-region. Members would be informed of the results of the further work to be done at the earliest opportunity.

Ophiopogon japonicus The phylogeny of *O. japonicus, O. bodinieri* and *Liriope spicata* was discussed. The DNA extraction had been performed on the outer material from individual rhizomes. The extraction of the DNA was 50% successful and the failure to extract DNA from the other 50% of the samples was believed to be due to the processed nature of the samples. Whilst it would be helpful to have information on the processing method that had been used, the likelihood was very slim. The sequencing of *trnH-psbA* and *trnL-F* had been unsuccessful for almost all the samples analysed. This was attributed to a combination of a technically difficult sequence and low quality of template DNA. Due to the lack of data from BP samples these two regions were not analysed further. The *rbcL*, *matK* and *ITS* regions were also evaluated. The *ITS* region appeared to separate *O. japonicus* from closely related species. It was accepted the *ITS2* sub region might amplify easier. Further samples will be procured for analysis.

38 **Sanger vs. NGS - *Phyllanthus amarus*** WP(DNA) (15)22

Members were informed of progress of a trial run using Next Generation Sequencing (NGS). The rationale for using NGS technology was also discussed. The cost - time benefit of NGS was raised and the differences between NGS and the Sanger method were also discussed. It was commented that the high volume of data generated by NGS and the associated sensitivity of NGS would mean less sampling would be required. In view of the sensitivity of NGS it was concluded that different thresholds would be required for contamination, especially when at very low level. The suggestion was made that the number of NGS 'reads' for a species could be used as a quantitative analysis technique. Concerns were raised regarding the use of the ITS region because of its many copies and pseudogenes, though it was noted that this region is useful for identification purposes.

Questions on NGS were raised regarding the read length which is limited to 250 or 300. Due to this, an amplicon of over 600 base pairs might result in an absence of sequence data between the two sequences (paired reads). It was confirmed that the *matK* region had not been explored with NGS for this reason but the other candidate regions (*rbcL, trnH-psbA, trnL-F* and ITS) had been examined. It was also commented that NGS was a better quantitative method compared with PCR. Members were very interested to receive further data from investigations at NIBSC and commended the research into a new technology.

39 **Work In Progress at the NIBSC Herbal Laboratory** WP(DNA) (15)23

The BP-NIBSC Herbal team updated members on the current work in progress at the BP-NIBSC Herbal Laboratory.

Myristica Fragrans (Nutmeg) 10 samples had been examined; 9 of whole kernels and 1 powdered sample. DNA samples had been amplified for all the candidate regions (*rbcL, trnH-psbA, trnL-F, matK* and ITS). Amplification was successful for the *trnH-psbA, trnL-F and matK regions* for 8 samples and *rbcL* for 7 samples. Problems with the ITS region were highlighted with ITS amplifying poorly and ITS2 showing multiple bands, which would need separation before Sanger sequencing.

Vitex negundo 13 samples of dried leaf of *Vitex negundo* had been examined together with 1 sample of *Agnus castu*s fresh leaf. The samples had been amplified successfully using the *rbcL* and *trnLF* protocols. The work was on-going and members would be updated of the results at the earliest opportunity.

Gallum aparine; Tribulus terrestris

Work was scheduled to commence on both *G. aparine* and *T. terrestris* once the on-going work on both *M. fragrans* and *V. negundo* had been completed. A programme of work would be presented at the next meeting of the Working Party.

Sampling

The work was on-going to identify a best approach for sampling and milling samples. In regard to this the BP-NIBSC Herbal Laboratory were looking to identify a suitable grinder and members were invited to provide any appropriate information they might have on this.

40 **Any Other Business**

Programme of Meetings in 2016

Wednesday, 28th of January
Wednesday, 20th of July 2016

MATTERS ARISING FROM THE MINUTES

Minute 13.0 Membership Process	Members have registered for access to the BP website.
14.0 Herbal Project **Actions** (c) The BP-NIBSC herbal laboratory to explore the feasibility of designing a DNA-based test method for aristolochic acid containing plants in herbal drugs.	A report on the feasibility of designing a DNA-based test method for aristolochic acid containing plants in herbal drugs will be presented at the meeting scheduled for January 2016.
19.0 Holy Basil Leaf	Members will be updated on the approach to adopt for revising the BP 2016 monograph at the earliest opportunity.

Acronym/Synonym	Name
APhI	Ayurvedic Pharmacopoeia of India
ATA	Ayurvedic Trade Association
BHomP	British Homoeopathic Pharmacopoeia
BP	British Pharmacopoeia
BP (Vet)	British Pharmacopoeia (Veterinary)
BP Commission	British Pharmacopoeia Commission
BPCx	British Pharmaceutical Codex
BPCRS	British Pharmacopoeia Chemical Reference Substance
BS	British Standard
BHMA	British Herbal Medicine Association
BOLD	Barcode of Life Database
CMPACC	Chinese Medicinal Plants Authentication and Conservation Centre (Kew)
CEP	Certification Procedure for the European Directorate for the Quality of Medicines
CHM	Commission on Human Medicines
CP	Pharmacopoeia of the People's Republic of China
CRS	Chemical Reference Substance
DNA	Deoxyribonucleic Acid
EAG	Expert Advisory Group
EPC	European Pharmacopoeia Commission
EPCRS	European Pharmacopoeia Chemical Reference Substance
EU	European Union
FDA	Food and Drug Administration
FIP	International Pharmaceutical Federation
FoI	Freedom of Information
GC	Gas chromatography
HAB	German Homoeopathic Pharmacopoeia
HCM	Herbal and Complementary Medicines
HKCMMS	Hong Kong Chinese Materia Medica Standards
HMPC	Herbal Medicinal Products Committee
ICH	International Conference on Harmonisation
ICMM (China)	Institute of Chinese Materia Medica
IR	Infrared

ISO	International Organisation for Standardisation
JP	Japanese Pharmacopoeia
LC	Liquid chromatography
LD	Licensing Division
LGC	Laboratory of the Government Chemist, Teddington
LR	BP Laboratory Report
MAIL	Medicines Act Information Leaflet
MHRA	Medicines and Healthcare products Regulatory Agency
MPNS	Medicinal Plant Names Services - Royal Botanic Gardens, Kew
NIBSC	National Institute for Biological Standards and Control
NPA	National Pharmacopoeial Authority
OMCL	Official Medicines Control Laboratory
Ph Eur	European Pharmacopoeia
PMU	Pharmacy Medicines Unit – to be confirmed
QSIMP	Quality Standards of Indian Medicinal Plants
SOP	Standard Operating Procedure
SPC	Special Product Characteristics
TGA	Therapeutic Goods Administration, Australia
THMPD	Traditional Herbal Medicinal Products Directive
TLC	Thin layer chromatography
UK	United Kingdom
UKD	United Kingdom Delegation [to the European Pharmacopoeia]
USP	United States Pharmacopeia
UV	Ultraviolet
WHO	World Health Organization