# Overview of results obtained from HPLC analyses of selected Theaceae leaf samples

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**Abstract**

Chemical analyses of some 171 *Camellia* species, 35 *Camellia* cultivars and varieties and 12 Theaceae genera (represented by 21 replicates), were carried out, using High Performance Liquid Chromatography (HPLC) techniques and protocols. The results of the aqueous extractions, LC-MS and LC-MRM procedures, the preparation of standard curves and of quantitative analyses, were evaluated utilising Cluster Analysis and Ward Linkage protocols. The results suggest a taxonomic complexity which was not reflected in the current morphology based descriptors for *Camellia* and known Theaceae genera. The implications of these new findings, based on the analyses of caffeine, L-theanine and a further eight secondary metabolites, indicate the incompatibility of the new data when compared to the morphology and molecular derived information. The newly obtained data sets show that the creation of a new taxonomic system, based solely on the HPCL derived knowledge, is achievable. Thus the modifications to the existing *Camellia* and Theaceae taxonomies, is made possible, and based on the use of the HPLC methods and protocols, may contribute towards the resolution of some perennial taxonomic problems.

***Key words:*** *Camellia*, epigenetics, heredity, metabolism, taxonomy

## Introduction

Discussions about the potential of using alternative scientific methods and techniques to elucidate some of the long disputed, specific and sectional issues of Theaceae and *Camellia* L. taxonomy have been conducted in a desultory manner for some time. The attaining and incorporation of novel and reliable data has been highly desirable, but until recently not practicable, this being due mostly to the lack of a sufficiently wide cross-section of materials (leaf samples) and of techniques thought to be suitable for this purpose.

Finally the HPLC analysis of *Camellia* leaf samples was identified by the author of this paper as the best of the possible methods available. Thus the analysis of secondary metabolites – the analysis of the intermediate products of a plant's metabolic reactions catalysed by various enzymes that naturally occur within plant cells – was adopted. The aim of the Orel & Curry 2017-2018 preliminary experiments was to ascertain the nature and extent of relationships between the published morphology-based taxonomic works for Theaceae and genus *Camellia* L. and the newly obtained data sets derived from the preliminary HPLC analyses. A relatively high degree of concomitance between the morphology-based and the HPLC methods-derived data had been found. This new evidence allowed for the continuation and extension of the project presented here.

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## Materials and Methods

Due to the necessary brevity of this paper it was not possible to enumerate all of the 227 Theaceae replicates used. **Table 1** shows the numbers of *Camellia* and Theaceae leaf samples utilised in this work. All *Camellia* materials were identified on the generic and specific level, e. g. *Camellia dongnaiensis* Orel. However, not all Theaceae replicates could be thus identified, e. g. some *Polyspora* sp. These samples were collected from vigorous and in many cases actively growing, disease-free specimens. The provenance details and the dates of collections were duly recorded. All samples were pressed *in situ* and in the long term the herbarium materials were stored in unsealed paper envelopes, in dry conditions and at an ambient temperature. The average sample size used for the HPLC work fluctuated between 1.0 g and 1.5 g. The following substances were targeted: L-theanine; caffeine; (+)-catechin; (-)-epicatechin; (-)-epigallocatechin; (-)-epigallocatechin gallate; (-)-gallocatechin; (-)-gallocatechin gallate; (-)-epicatechin gallate and (-)-catechin gallate).

**Table 1** The identification and the number of *Camellia* and Theaceae leaf samples used in this work.

| | |
|---|---|
| *Camellia* species | 171 |
| *Camellia* varieties, selections and cultivars | 35 |
| Theaceae species (12 genera) | 21 |
| TOTAL REPLICATES | 227 |

### A short summary of methods used

The LC-MRM (Liquid Chromatography Multiple Reaction Monitoring) procedures were used for quantitative proteomic analysis and high-throughput biomarker detection. Multiple channels were set in a single measurement, thus providing a high quality of information about the target molecular profiles in given complex systems. The HPLC UV (High Performance Liquid Chromatography UV) methodology was used to detect and identify the analyte in specific samples. These samples were then identified by measuring the sample's absorption of light at different wavelengths. High resolution and the sensitivity of these methods ensured the determination of the minimal amounts of substances in the given samples.

A small part of each *Camellia* leaf was excised from the middle of each leaf sample. This was then manually reduced into smaller fragments. Samples that did not fragment easily were cut with a sterile scalpel blade to generate the desired size. Some 10.0 mg of each sample was transferred into a 1.5 mL tube. Each sample was weighed and the exact mass was duly recorded. Each leaf sample was extracted by the addition of boiling ultra-pure water to a final concentration of 10 mg/mL. The aqueous extracts were incubated for

30 minutes shaking at 95°C and then spun at 100 RPM. The extracts were prepared for LC-MS/MS analysis by filtering through a 0.22 mm PES syringe filter into an HPLC vial.

Analysis of the aqueous extracts of each *Camellia* sample was performed, initially using 1 mL of each 10 mg/mL extract. The analysis was repeated using a 5 mL injection of the 10 mg/mL extracts. The increased injection volume was used to quantify low concentrations that did not generate accurate values in the smaller injection volume. In addition, the Green Tea quality control sample was run at the start and the end of the complete analysis. The Green Tea QC sample was not included within the run as the initial sample demonstrated significant quantities of the analytes which could have carried over into other *Camellia* leaf samples. To facilitate quantitation and comparison of the Green Tea QC sample, additional 1:10 and 1:100 dilutions were analysed to generate peak areas within the range of the standard curves. Successful quantitation of the majority of the L-theanine, Caffeine and Catechins was achieved.

Relative Quantitation LC/MS/MS experiments were performed using Multiple Reaction Monitoring (MRM) on a SCIEX QTRAP 6500 (hybrid Triple Quadrupole Linear Ion Trap Mass Spectrometer, AB Sciex, Chromos, Singapore). 1ul of each of the extracts used for HPLC-UV analysis was injected onto a reversed phase High Performance Liquid Chromatography (HPLC) column (Phenomenex Synergi Polar-RP, 4um, 80A, 150 x 4.6mm) at 1 ml/min in 95% mobile phase A (0.1% Formic Acid in Water) and 5% mobile phase B (0.1% Formic Acid in Acetonitrile). LC separation was carried out on a Shimadzu Nexera X2 UHPLC system (Shimadzu, Rydalmere NSW, Australia) as per the following conditions. Following the injection, an isocratic hold was used for 1 minute at 5% B followed by a linear ramp to 80% mobile phase B over 6 minutes, a further ramp to 95% B held for 1 minute was used as a washing step before the column was re-equilibrated for 2 minutes at initial conditions.

Datasets were generated using either LC-MRM or LC-UV methods. In the case of the UV data, the 280 nm and 210 nm readings were merged to generate one dataset. Both the UV and MRM data were transformed using log(X+1) **(Figure 1)**. To analyse the resemblance between each sample, a Euclidean distance metric was created. A Euclidean metric is used to generate the 'ordinary' straight line distance between two fixed points, and was therefore chosen for this dataset because of the fixed nature of the mass (or UV) readings. Finally, a hierarchical cluster analysis was conducted on each dataset to generate graphs and visualise sample clustering **(Figure 1** and **Figure 2)**.

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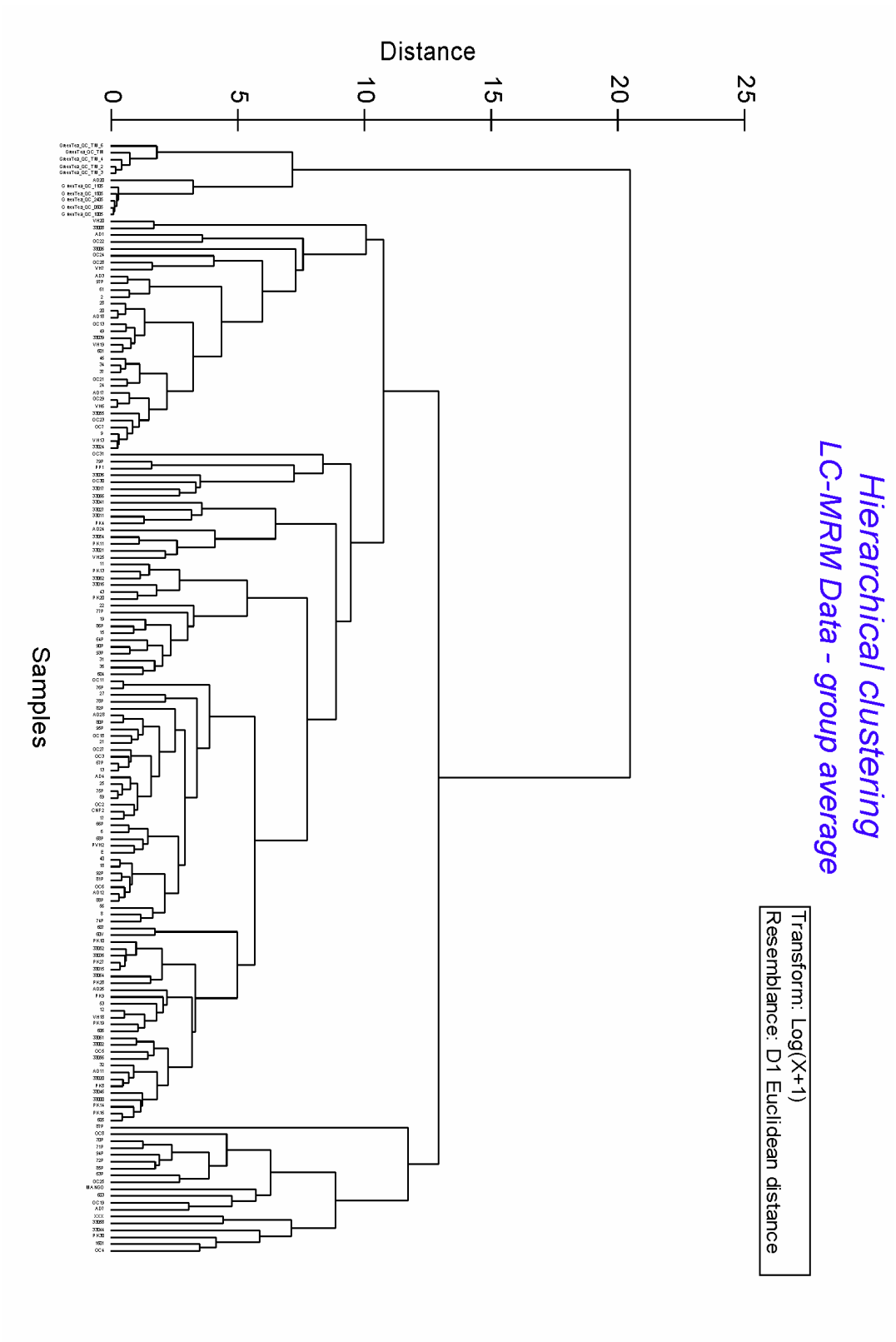

**Figure 1** An example of hierarchical clustering, LC-MRM data. *Camellia* replicates only. Individual *Camellia* replicates identified on sectional level only.

**Table 2** The number of *Camellia* replicates in each of the 12 main clusters in **Fig. 1**. Individual clusters designated A to L.

| Cluster | A | B | C | D | E | F | G | H | I | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No of replicates | 11 | 34 | 7 | 9 | 6 | 11 | 2 | 19 | 15 | 29 | 1 | 18 |
| TOTAL 12 clusters | 162 replicates | | | | | | | | | | | |

**Table 3** Dendrogram 'A', horizontal axis. Division of *Camellia* replicates, of all sections, across the 12 clusters of the Dendrogram. Maximum values highlighted in red. Figures not adjusted, all values in %. Dendrogram 'A', vertical axis. Total of *Camellia* replicates in each individual cluster. Maximum values highlighted in red. Figures not adjusted, all values in %.

| Camellia sections | | | | | Cluster ID | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **A*** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** | **L** |
| *Thea* | 100 | 8.8 | **14.3** | 11.1 | | 9.1 | | | | | | 6 |
| *Camellia* | | 8.8 | 14.3 | 22.2 | 50 | **54.5** | | 21 | | 10.3 | | 33 |
| *Paracamellia* | | 5.9 | 14.3 | 11.1 | **16.7** | | | 16 | 7 | 10.3 | | |
| *Heterogenea* | | **8.8** | | | | | | | 7 | | | |
| *Oleifera* | | 5.8 | | **11.1** | | | | | | 10.3 | | |
| *Archecamellia* | | 8.8 | | | | 9.1 | | | | **10.3** | | 6 |
| *Corallinae* | | 2.9 | | | | | | 5.3 | | | | **6** |
| *Eriandria* | | 2.9 | **28.5** | | | | | | 14 | 7 | | 5 |
| *Theopsis* | | 18 | 14.3 | 44.5 | | 9.1 | **50** | 5.3 | 30 | 14 | | |
| *Chrysanthae* | | **21** | | | | 9.1 | | 16 | 7 | 18 | | |
| *Calpandria* | | 2.9 | | | | | | | | | | **6** |
| *Piquetia* | | 2.9 | | | | | | | | | | **22** |
| *Dalatia* | | 2.9 | 14.3 | | **33.3** | | | 5.3 | | | | 11 |
| *Heterogenea* | | | | | | | | | | | **100** | |
| *Stereocarpus* | | | | | | **9.1** | | 5.3 | 7 | 3 | | |
| *Pierrea* | | | | | | | **50** | | | | | |
| *Furfuraceae* | | | | | | | | **10** | 7 | 3 | | |
| *Tuberculatae* | | | | | | | | 11 | **14** | 10.3 | | |
| *Pseudocamellia* | | | | | | | | 5.3 | **7** | | | 5 |
| *Bidupia* | | | | | | | | | | **3.5** | | |
| **Total sects. 20** | | | | | | | | | | | | |

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* Cluster A: *C. sinensis* vars. used as controls

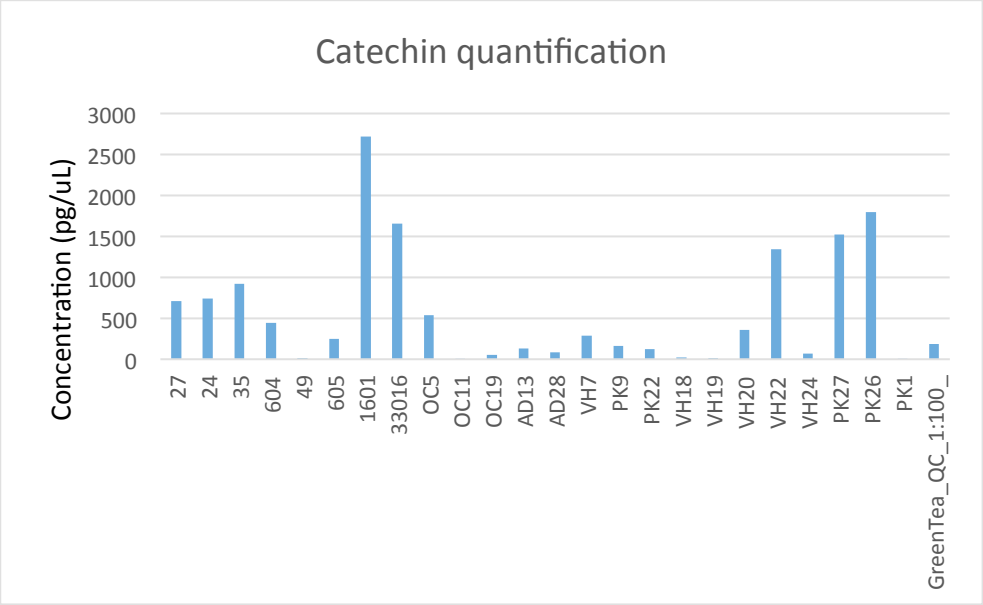


**Figure 2** Total catechins quantification in 24 *Camellia* replicates tested. Individual concentrations in pg/uL.

**Discussion**

As previously pointed out, the current morphology based taxonomic systems for genus *Camellia* (and family Theaceae) may be described as imprecise and unsatisfactory. The results published so far of a number of molecular studies tend to support the well-tested morphology-based data imperfectly and in some cases fail to do so almost completely, thus creating ambiguities which in some cases cannot be reconciled.

Currently, subject to future scientific advances, these differences cannot be explained by simple linear reasoning as the arising issues are multilayered, historical, methodological and epistemological and therefore in many instances extremely challenging. Some of the discrepancies between the two systems may be due to the still rather imperfect molecular methods and protocols currently used. This is despite some advances in molecular methods (sequencing and fingerprinting, etc.

Thus, the experiments, described in this work sought to explore the scientific merits of secondary metabolite-derived data and to use this new knowledge for the possible novel taxonomic segregation of *Camellia* species and Theaceae genera. The new research was undertaken despite the possibility that the newly obtained data would not indicate any taxonomic divisions at all, as the contents of the secondary metabolites, in the majority of materials tested, may have been identical or almost identical when subjected to detailed comparisons.

To begin with, the question of the production, or otherwise, of secondary metabolites *via* the agency of epigenetic processes, had to be explored. In their recent study, Kooke et al., (2019), tested a number of epigenetic recombinant inbred lines (epiRILs) of *Arabidopsis thaliana* L. (Brassicaceae). These were used to assess the impact of the

epigenetic variation on the composition of *Arabidopsis*' secondary metabolites. These experiments have established that epigenetic mechanisms impacted the metabolic diversity in *Arabidopsis*, possibly via small RNAs (molecules of 50 to 250 nucleotide lengths. Temel (2015) in her study has also shown that the coordinated expression of genes is under epigenetic control. This may be accomplished by different mechanisms, which include DNA methylation, histone modifications and chromatin *re*-modelling. It appears that these modifications alter the state of chromatin and by this alteration, induce or represse gene expression. Seed development, flowering, sexual reproduction and leaf senescence seem to be controlled by epigenetic mechanisms, as are the plant's stress responses to the adverse environment.

The review of environmentally mediated epigenetic regulation in plants was also undertaken by Baulcombe and Dean (2014). Here, two metabolic processes were scrutinized. One of these was the vernalization, or the exposure of plant materials to the prolonged cold of winter r to an artificial equivalent. The other process involved virus-induced silencing involving trans-generationally inherited epigenetic modifications. The results of these experiments have shown that:

*Heritable epigenetic marks may result in heritable phenotypic variation, influencing fitness, and so be subject to natural selection* (Baulcombe and Dean, 2014).

Perhaps the following statement, although referring to humans, is quite instructive, as it encapsulates the above expressed scientific opinions:

*Epigenetic modifications, such as DNA methylation and chromatin assembly states, reflect the high plasticity of the genome and contribute to stably alter gene expression without modifying genomic DNA sequences. Consistent components of complex traits, such as those linked to human stature/height, fertility and food metabolism or to hereditary defects, have been shown to respond to environmental or nutritional condition and to be epigenetically inherited* (Trerotola et al., 2015).

It could be argued that the conclusions of these two citations have a profound impact on the interpretation of data obtained from this enquiry.

Thus, what was found and what is the meaning of the newly obtained results within the terms of reference established for this work? It should be noted that the 'raw results' clearly indicate a rather detailed, secondary metabolite-based segregation of *Camellia* and Theaceae replicates into autonomous and distinct clusters. It should also be noted that the consecutive divisions of main clusters into smaller sections were given careful consideration, because in taxonomic terms, the results of repeated and detailed HPCL analyses of relationships between the individual *Camellia* replicates **(Figure 1)**, did not correspond to any of the known *Camellia* or Theaceae taxonomies, derived from the currently available morphological data.

The complete lack of any comprehensive and/or reliable taxonomic systems, based entirely on molecular data, prevented comparisons with the new HPLC-based findings to be made. However, some of the available molecular-based taxonomical studies offer a degree of guidance which may be of value.

Consequently, in order to gain a wider perspective, taxonomic developments in another large plant Family, namely Magnoliaceae Juss., should be considered. Parallels

between the developments in the taxonomic status of Theaceae and Magnoliaceae may be quite instructive. In Magnoliaceae, just like in Theaceae, the general morphology-based taxonomy of the family seems to be in a state of discord.

At least three dissimilar taxonomic systems are in evidence; perhaps the most used one is the morphology-based system of Zeng. The relatively recent morphological research and the observations of live plants, together with the advances in molecular techniques and systematics, indicate that Magnoliaceae are best described as a family containing only one genus, *Magnolia*. Evidence suggests that this re-classification may clarify a number of long disputed issues. It appears that this new re-classification is gaining validity and is currently in the process of being adopted. A good example of this is the mentioning of the new system in the current *Flora of China*.

Some of the past molecular- and the current HPLC-based studies, which are presented in this work **(Figure 1**, **Table 2**, **Table 3)**, strongly indicate the viability of the creation of a similar taxonomic arrangement for Theaceae, with *Camellia* being the sole genus. Then, in parallel with genus *Magnolia*, this sole remaining genus *Camellia* may be further sub-divided into a number of subgenera, sections and subsections. It is the opinion of the author of this work that such a new taxonomic system would reflect taxonomic relationships within genus *Camellia* with a heightened degree of realism and in time may unite the results of the morphological, molecular and HPLC studies into a workable, hybrid system.

To conclude, one more comment should be made. The HPLC-based results presented here were achieved by building on the attainments of the modern science of the 21$^{st}$ Century. It should also be remembered that the current sciences of chemistry, genetics, botany and taxonomy were founded on the great works of all those scientists who over the past centuries contributed to our modern knowledge.

**Acknowledgements:**

The author of this work would like to extend his thanks to the following: the leadership and members of the Otomo-Haydon Research Fund Board for making this research project possible; the leadership and staff of the University of Newcastle, City of Newcastle, NSW, Australia, for performing the HPLC-related laboratory procedures; and Mr Phuong Mai, Professor Emeritus Clifford R. Parks, Mr Robert 'Bob' Cherry, Mr John Robb, Mr Tony Curry and Messrs Clayton and Charles Harland for supplying some of the leaf materials.

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