論文題目　　　Morphological and genetic characterization of mulberry (genus *Morus*)
　　　　　　　（クワ（Morus属）の形態学的および遺伝的特性評価）

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Introduction

Mulberry belongs to the genus *Morus* and the family of Moraceae with over 68 species recognized (Datta, 2002). After the first classification of mulberry that unveiled seven species (Linnaeus, 1753), several studies on mulberry classifications have proposed different number of mulberry species such as 24 species and one subspecies (Koidzumi, 1917), 35 species (Hotta, 1958), 16 species (Zhou and Gilbert, 2003) and the need for correct reclassification still continues for breeding and utilizing mulberry all over the world. This diversity and classification have elucidated a long debate over time due to relatedness in morphological features as a result of the heterogeneous nature and influence of environment (Machii et al., 2001). Contribution through enhanced morphological and genetic studies have been done in previous studies (Sharma et al., 2000; Tikader et al., 2001; Bhattacharya and Ranade, 2001; Awasthi at al., 2004; Kafkas et al., 2008; Vijayan et al., 2008; Chikkaswamy et al., 2012), however, it remains a challenge as they focused on either morphological or genetic markers with minimum number of species and varieties as well as different technologies. This further prompted me to contribute towards knowledge base of mulberry since understanding the relationship in mulberry varieties among the *Morus* species is crucial to enhance the benefits of breeding, conservation and utilization.

My research objective is to contribute to addressing the above mentioned issues by revealing phenotypic and phylogenetic relationship existing among 56 diploid mulberry varieties belonging to seven different *Morus* species (*M. alba, M. indica, M. bombycis, M. acidosa, M. latifolia, M. kagayamae*, and *M. rotundiloba*) and one unspecified *Morus* species ('Enbu') by combining both morphological and genetic markers, as well as unravelling the whole genome sequence of 'Enbu'.

Results and discussions

1. Morphological characterization of 56 mulberry varieties

Morphological characterization has been indispensable in plant taxonomy over time; however, the nature of plant phenotypes and diversity make it challenging to distinguish between closely related varieties among species of the same family. I used the 56 diploid varieties selected at NARO Genebank (Tsukuba) with aim of evaluating their morphological features which may be used for classifying them into species category. The varieties consist of *Morus alba* (8 varieties), *M. acidosa* (6), *M. bombycis* (13), *M. indica* (4), *M. kagayamae* (4), *M. latifolia* (13), *M. rotundiloba* (4), interspecific hybrids (3) and *Morus* sp. 'Enbu' (hereafter, 'Enbu'). In order to evaluate morphological characteristics of 56 varieties, I measured the quantitative and qualitative morphological features of individual varieties using three replicates; the quantitative ones are blade length, blade width, apex length and petiole length of mulberry leaves (**Fig. 1(a)**) and the qualitative ones are appearance of leaf blade, shoot structure and orientation.

The result revealed diversity among mulberry at variety level and within the same species exist in the morphological features (**Fig. 1(b) - (i)**). In the blade length and blade width, clear differences were observed between species with higher values observed in 'Enbu', *M. latifolia*, *M. kagayamae* and interspecific hybrids ('Rohachi', 'Shinichinose' and 'Kairyou Ichinose') while smaller values in species *M. alba*, *M. indica* and *M. acidosa*, respectively. However, similar values were observed among some species, making it difficult to isolate the species clearly. The apex length differs among the species separating three groups of long apex (*M. kagayamae*, *M. acidosa*, and *M. rotundiloba*), medium apex group ('Enbu', *M. indica*, *M. bombycis* and interspecific hybrids) and finally the small group category (*M. latifolia* and *M. alba*). The petiole length also grouped the species into long group (*M. latifolia*, *M. kagayamae*, the interspecific hybrids, and *M. alba*), medium group (*M. bombycis*, *M. indica* and 'Enbu') and the short group (*M. rotundiloba* and *M. acidosa*) respectively. Overall, similar values were observed among some species in each feature, making it difficult to isolate the species clearly. However, the leaf apex maybe useful for differentiating some mulberry varieties compared with other features. Among the mulberry varieties, 'Enbu' seemed to be having big leaf blade, medium apex and short petiole compared to other species phenotypes (**Fig. 2**). The results prompted necessity for further analysis using SNP markers in order to bring out the phylogenetic relationships among them through genetic markers.

Fig. 1. Diversity among the mulberry varieties and species. (a) Morphological features of blade length, blade width, apex length and petiole length were evaluated. (b-i) Photos of *M. alba*, *M. acidosa*, *M. bombycis*, *M. indica*, *M. kagayamae*, *M. latifolia*, *M. rotundiloba*, and 'Enbu', respectively.

Fig. 2. Variance among the mulberry species in blade length, blade width, petiole length and apex length.
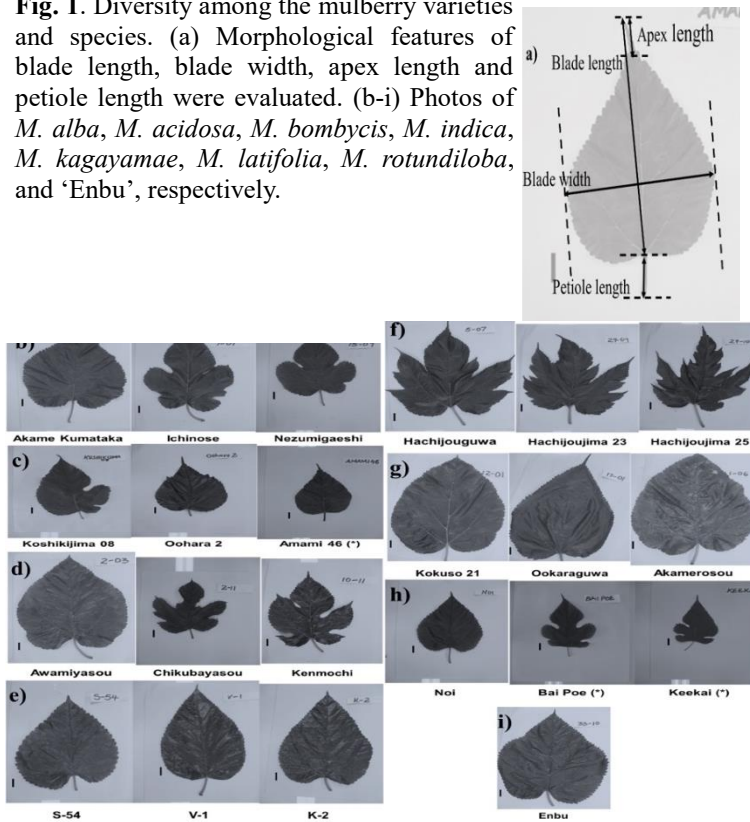
2. Genome-wide SNP marker discovery and phylogenetic analysis of mulberry varieties using double-digest restriction site-associated DNA sequencing

Due to difficulties observed in differentiating the mulberry species by morphological phenotypes, further investigations focused on genetic interrelationship among the varieties was conducted using double digest restriction site associated DNA sequencing (ddRAD-seq). Genome-wide 2,229 homozygous SNPs were identified among the 54 mulberry varieties in the eight species by ddRAD-seq. The results of the phylogenetic analysis revealed existence of three clear monophyletic clades in two Japanese native species, *M. acidosa* and *M. kagayamae*, distributed in different geographically isolated islands, and a Thai native species, *M. rotundiloba*, whereas the other species were non-monophyletic (**Fig. 3**). Varieties of *M. bombycis*, another Japanese native species, were roughly classified into three groups. Of these, two groups were monophyletic with *M. acidosa* and *M. kagayamae*, respectively, while another group was not monophyletic. Furthermore, varieties of *M. indica*, an Indian native species, were classified into two different monophyletic clades. Of these, one clade was clearly monophyletic with an indigenous variety in Kenya, 'Enbu', while another clade was monophyletic with *M. rotundiloba* varieties and one *M. latifolia* variety. There were no clear monophyletic clades within *M. alba* and *M. latifolia* varieties, which could be a result of several hybridization events after their introductions from China to Japan. These results suggested that it was difficult to clearly classify the hybridized mulberry varieties even with genome-wide DNA markers. Having observed that the leaf apex could contribute towards differentiating the varieties, I evaluated relationship between the structure of the phylogenetic and morphological characteristics of mulberry leaves. The results revealed that leaf tip ratio (LTR), calculated by apex / blade length, may correlate to genetic difference among the two *M. bombycis* groups in monophyletic clades and another *M. bombycis* group in non-monophyletic clades, suggesting that LTR might be used for evaluating hybridization level of *M. bombycis* varieties. Overall, these results may provide new insights into taxonomic debate of mulberry species.
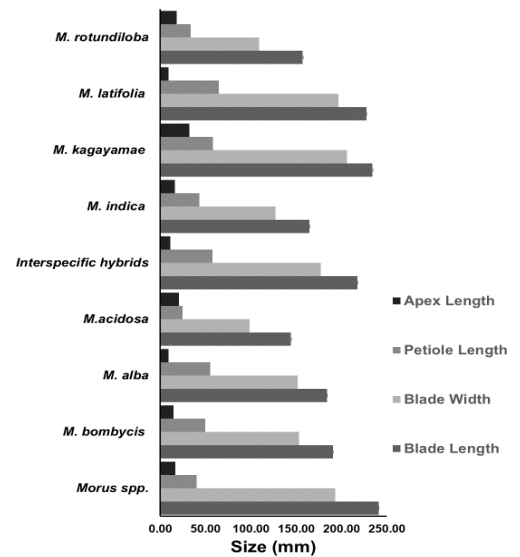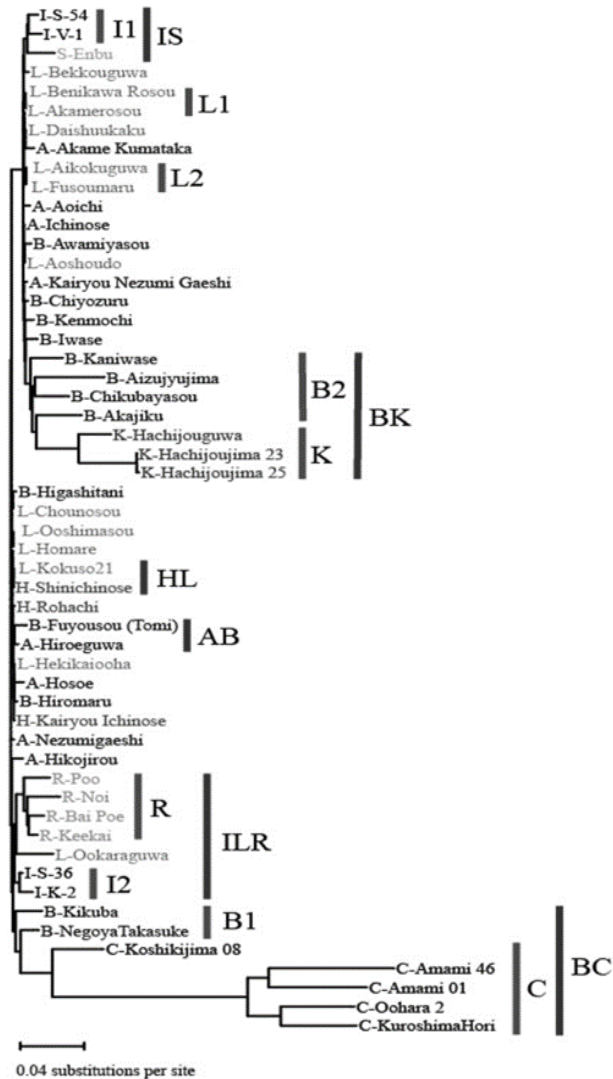

Fig. 3. Neighbour joining (NJ) phylogenetic tree of 54 mulberry varieties where **C**: *M. acidosa*; **I**: *M. indica*; **R**: *M. rotundiloba*; **K**: *M. kagayamae*; **B**: *M. bombycis*; **L**: *M. latifolia*; **A**: *M. alba*; **S**: 'Enbu'.

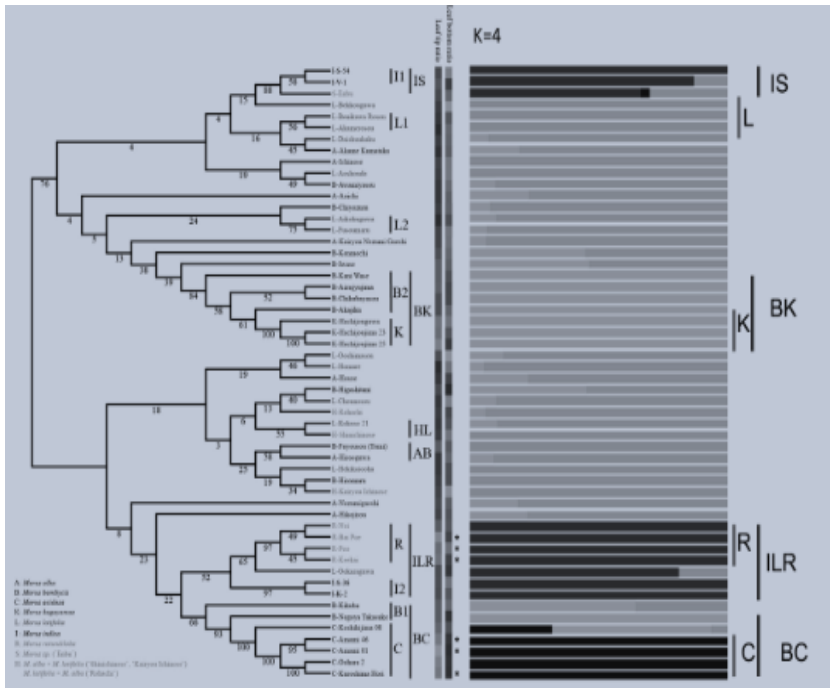## 3. Admixture analysis of 54 mulberry varieties

Fig. 4. Neighbour joining (NJ) phylogenetic tree combined with admixture analysis result of 54 mulberry varieties where C: *M. acidosa;* I: *M. indica;* R: *M. rotundiloba;* K: *M. kagayamae;* B: *M. bombycis;* L: *M. latifolia;* A: *M. alba;* S: 'Enbu'.

Interesting upon conducting the admixture analysis on varieties from the documented 7 known species plus one unknown species, there is indication of varietal interactions among the species resulting to creation of three clear clades of native species of **C**: *M. acidosa*, **K**: *M. kagayamae* and introduced **R**: *M. rotundiloba* as had been observed in phylogenetic analysis. However, the results further reveal presence of admixtures that may have occurred among the varieties in their growing niches resulting to similarities among the phenotypes after introductions of **L**:*M. latifolia* and *M. indica* and *M. alba. M. latifolia* seems to be most affected by the hybridization among the species.

4. Genome sequencing of Enbu variety of mulberry with comparison to Chinese mulberry Chuansang (*Morus notabilis*)

Whole genome sequencing of 'Enbu' was performed in order to give insight into the genome of 'Enbu' in comparison to existing genome sequences of *Morus notabilis* in MorusDB with target of finding out the similarities and differences among the species. Highly improved genome assembly of 'Enbu' compared with the existing genome assembly of *M. notabilis* was constructed by de novo assembly using PacBio long reads with scaffolding by Dovetail's Chicago method. The results revealed that the size of 'Enbu' genome assembly is 349.92 Mb, (N50 is 1,17 Mbp). In the genome assembly, 35,483 predicted gene sequences were identified in 32,989 loci. Functional annotation for the predicted genes using several public databases such as NCBI-nr, InterPro, Gene ontology, and KEGG were performed.

Conclusion

Morphological features alone despite being indispensable and accessible cannot differentiate the varieties among the species clearly. My result of combining both morphological features and genetic markers may offer a promising future in differencing varieties, however, in highly hybridized varieties there is need to go deeper into single copy nucleotides markers and finger typing for proper placement of varieties among the species. The use of SNP markers and some morphological features such as LTR could help solve the dilemma in classification of mulberry species. Furthermore, exploration of the genome sequence and comparison to public databases offers an opportunity to proper utilization of the mulberry bioresource by other researchers and farmers in breeding varieties that meet their needs.