Elucidation of Japanese pepper (*Zanthoxylum piperitum* De Candolle) domestication using RAD-Seq

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Abstract

Japanese pepper, *Zanthoxylum piperitum*, is native to Japan and has four famous varieties: Asakura, Takahara, Budou, and Arima, named after their production area or morphology. Restriction-site associated DNA sequencing (RAD-Seq) was used to analyse 93 accessions from various areas, including these four varieties. The analysis of the single nucleotide variants was used to classify the plants into eight groups: each of the Asakura and Arima varieties has two groups, each of the Takahara and Budou varieties has one group, and two additional groups are present. In one group of the Asakura varieties and two groups of the Arima varieties, the plants were present in agricultural fields and mountains, representing the early stage of domestication of the Japanese pepper. The second group of the Asakura varieties was of genetically close plants present in various areas, which represents the second stage of domestication of this plant because, after early domestication, genetically related varieties of excellent traits spread to the periphery. These results demonstrate that the domestication of the Japanese pepper is ongoing. In addition, this study shows that spineless plants are polyphyletic, despite the spineless variety being considered a subspecies of the Japanese pepper.

Introduction

Zanthoxylum species belongs to the family Rutaceae, commonly known as the citrus family. Many *Zanthoxylum* species are distributed throughout the world, particularly in Asian countries, where they are used for culinary and medicinal purposes. For example, the Āyurvedic traditional medicine in India and Sri Lanka uses *Z. tetraspermum* and *Z. rhetsa* ("*Katu Keena*")[1]. Sichuan pepper is an essential ingredient in the cuisines of the Sichuan province of China and contains several species of *Zanthoxylum*, including *Z. simulans*. In traditional Chinese medicine, *Z. simulans* is used for its stomachic, analgesic, and anthelmintic properties[2].

Zanthoxylum piperitum is native to Japan (except for Ryukyu) and southern Korea. In Japan, it is mainly used for culinary purposes. In English, it is known as Japanese pepper; in Japanese, it is called sanshō. This plant is considered to be highly medicinal in various uses[3,4,5,6,7,8,9,10]. Although *Zanthoxylum piperitum* is widely consumed and distributed throughout Japan, varieties have been formed based on the production area and morphology of this plant. The Asakura, Arima, and Takahara varieties are classified based on the place of production, while the Budou variety is classified based on its morphology.

The Asakura variety is believed to have originated in the Asakura district of Yabu city in central Hyōgo Prefecture. A typical feature of the variety is the absence of spines. For this reason, the spineless plant is called the Asakura variety in Japan. This spineless plant has been identified as a subspecies of *Z. piperitum* and named *Z. piperitum* (L.) DC forma inerme (Makino) Makino[11]. However, there is no evidence that all spineless plants are either from Asakura or are monophyletic. In Yabu city, *Zanthoxylum piperitum* grows as wild plants in mountainous areas and cultivated plants in agricultural fields and gardens.

The Arima variety, also known as the Rokko variety, is another variety of Japanese pepper found in Kobe city in southern Hyōgo Prefecture. The flowers of these plants typically used as an ingredient in Arima cuisine.

The Takahara variety is grown in Takayama city in northern Gifu Prefecture. These plants are grown at high altitudes of 800 m, in contrast to the other varieties, which are cultivated on the plains. These plants bloom in mid-May, while other varieties bloom in late April. The wild Takahara variety is difficult to find growing in the mountains of this region.

The Budou variety (Budou means “grape” in Japanese) is categorised based on its morphology. This variety is cultivated mainly in Kainan city in Wakayama Prefecture. It bears clusters of fruits that resemble grapes.

These four varieties are well known, and *Zanthoxylum piperitum* is cultivated all over Japan, except for Ryukyu. Some varieties are present both in the mountains and in agricultural fields. Therefore, the domestication of Japanese pepper may be an ongoing progress, and recent reports have stated that the domestication of other crop species, such as olives in Morocco[12] and dragon fruits/pitaya in Mexico[13], is also ongoing. Both olives and dragon fruits undergo two common steps of domestication: firstly, the *in situ* conservation of wild plants, and secondly, the introduction of wild plants into agricultural fields. These plants were selected as desirable plants for cultivation. Genetic studies on these crops have confirmed their ongoing domestication by distinguishing between the wild and cultivated genotypes and discriminating genotypes between different areas. In the present study, this analysis will be used to distinguish the stages of domestication of the varieties of Japanese pepper and elucidate their origins.

Studies on the genetic diversity of *Zanthoxylum* species have focused on: (1) the differences between subspecies[14], (2) the relationship and diversity between wild and

cultivated plants[15], and (3) the genetic structure of cultivated plants[16]. A previous study[15] compared wild plants to cultivated plants at the DNA level and detected genetic similarities between cultivated and wild *Z. armatum*. Although this finding may be relevant to the domestication of *Z. armatum*, the study only used a small number of markers and was unable to examine the species domestication details.

Genetic diversity studies of *Zanthoxylum* species have been based on the analyses of a small number of markers, such as sequence-related amplified polymorphism markers[15] and simple sequence repeat (SSR) markers[16]. The genetic analyses of ongoing domestication of olive and dragon fruit have also used SSR markers[12,13]. Compared to genetic studies using a small number of markers, high-throughput sequencing can generate many single nucleotide variants (SNVs) suitable for analysing intraspecific differences[17,18].

Restriction site-associated DNA sequencing (RAD-Seq)[19] is a high-throughput sequencing method. Initially, RAD-Seq cleaves individual genomes with a selected site-specific restriction enzyme. Then, DNA fragments adjacent to the restriction site are sequenced using a high-throughput sequencer. The double-digest RAD-Seq (ddRAD-Seq)[20] is a variant of RAD-Seq, which uses two restriction enzymes to cleave the genome. These methods allow for a reduced representation of individual genomes. Moreover, these methods do not require prior knowledge of the genome sequence[20]. Our group has previously used the original RAD-Seq and ddRAD-Seq methods to analyse citrus[21,22,23], loquat[24], and firefly[25].

In the present study, ddRAD-Seq was used to elucidate the occurrence of domestication in well-known and widely consumed varieties of Japanese pepper.

Results

Variant detection by *de novo* mapping of RAD-Seq data

Double digest RAD-Seq generated more than 11.9 gigabases of data for a total of 235.2 million raw single-end reads of 51 bp. Quality-based filtering resulted in an average of 2.5 million reads (maximum of 4.3 million and a minimum of 0.4 million) in 93 samples (Supplementary Table 1). Stacks program built loci *de novo* with an average coverage depth of 27.32 times (Supplementary Table 2). We performed the following analysis using data containing 4,334 variant sites.

Correlation of genetic similarity with geographic locations

Based on the principal component analysis (PCA) (Figure 1) and geographical information (Figure 2, Table 1), 93 samples were classified into seven groups. PC1 divided group A from the other groups (Figure 1a); PC2 divided groups D and E from the other groups (Figure 1a); PC3 (Figure 1b) identified groups C and F and reconfirmed the presence of group E; PC4 identified group B (Figure 1c) and reconfirmed the presence of groups C and F; PC5 reconfirmed the presence of groups B and F (Figure 1d); and PC6 identified group G (Figure 1e).

Group A may be one group of the Asakura variety because the distributed area of the group A plants coincided with the production area of the Asakura variety in Yabu city, central Hyogo Prefecture (Figure 2, Table 1). All plants in group A were spineless. Group A was widely distributed and cultivated in other areas, including Kobe and Kami in Hyogo Prefecture, Ayabe, and Kyōtamba in Kyoto Prefecture, and Gojō in Nara Prefecture (Table 1). Interestingly, plants 42, 43, 44, 45, 46, 47, 48, 49, 50, and 51 formed tight clusters from Yabu, Ayabe, Gojō, Kyōtamba, Yabu, Kobe, Yabu, Gojō, Gojō, and Kami, respectively. The other plants in group A, 35, 36, 37, 38, 39, 40, and 41, were from Yabu, Kyōtamba, Kobe, Yabu, Yabu, Kyōtamba, and Kyōtamba, respectively.

The analysis identified group D as the second group of the Asakura variety. The group D plants were from Yabu city only (Figure 1.a, Figure 2, Table 1), except for plant 74 from Kami. Unlike group A, which had more cultivated plants, group D contained many wild plants found in mountainous areas. Most, but not all, of the group D plants were spineless. Plant 58 was 100 years old and was growing in an agricultural field. When we observed plant 58 in 2017, this plant was spineless. As of 2020, this plant is about to die, and its spines were visible.

The analysis identified group E as the Takahara variety. Group E (Figure 1.a) was an isolated group with plants only from Takayama city, located in the highland region of northern Gifu Prefecture (Figure 2, Table 1). This group consisted of only cultivated plants. Most of the group E plants were spineless (Table 1).

The analysis identified group C as the first group of the Arima variety. Group C plants were from Kobe and Asago cities in southern Hyogo Prefecture (Figure 2, Table 1), where the Arima variety is produced. There is an agricultural field in Asago city for the preservation of the Arima variety. All plants in group C were spinal and transplanted from Mt. Rokko (Table 1).

The analysis identified group F as the second group of the Arima variety. All plants in group F (Table 1) were from the Arima variety production area in Asago and Kobe (Figure 2). Group F contained spinal plants. Three plants cultivated at the Fruit Tree Research Institute came from Mt. Inariyama, located near Arima. PCA did not clearly separate group F from other plants.

The analysis identified group B as the Budou variety. Group B was a group from Kainan city in Wakayama Prefecture (Figure 2), where the Budou variety is produced (Table 1). Group B is the grape-like Budou variety with a different phenotype than the other varieties. The plants in group B plants were all cultivated and had spines.

We designated group G as an unclassified variety. This plant group included plants from different locations across Japan (Table 1), including Adachi, Akita, Agamachi, Ayabe, Kami, Kobe, Nagahama, Nangoku, Noto, Toyooka, and Tsuruoka. The plants in group G were both cultivated and wild (Table 1).

Tight cluster and additional group revealed by cluster analysis

We performed cluster analysis using a pairwise distance matrix. The cluster analysis re-identified seven groups and showed that the plants 42, 43, 44, 45, 46, 47, 48, 49, 50, and 51 formed tight clusters.

In addition, cluster analysis identified an eighth group (Figure 3), named group H. Group H (Figure 3) contained the plants from Kochi Prefecture (Figure 2, Table 1). This was named “unforeknown Kochi group”. The plants in this group were spinal. Plants 1 and 87 were cultivated, and plant 2 was wild. These plants are used ingredients to make rice cakes in this region (Table 1). The other plant (number 92) from Kochi Prefecture did not belong to group H.

Regional groups and their exceptions elucidated through phylogenetic analyses

To further establish the above classifications, we performed phylogenetic analyses using the maximum likelihood (ML) method (Figure 4) and a method based on the coalescent model (Figure 5). Both phylogenetic trees classified plants into eight groups. The phylogenetic trees (Figures 4 and 5) also showed a tight cluster in group A, containing plants 42, 43, 44, 45, 46, 47, 48, 49, 50, and 51, present as a subclade within clade A with high bootstrap values. The branch lengths of the ML tree (Figure 4) indicated that these nine plants have a higher degree of genetic similarity.

There were some exceptions to the eight groupings. Two trees separated plant 14 from other plants of group E. The ML tree separated plant 13 from other plants in group D. Indeed, in PCA, these two plants were closer to the plants in the other groups (Figure 1.a and 1.b).

The three analysis methods, one cluster analysis, and two phylogenetic analyses, showed that group G had two regional groups in common: (1) plants 78 and 79, and (2) plants 81, 82, and 83. Plants 78 and 79 in the first group were from Agamachi, Niigata Prefecture, and were wild spineless plants. In the second group, plant 81 was from Yamagata Prefecture, plant 82 was from Akita Prefecture, and plant 83 was from Tsuruoka, Yamagata Prefecture; these two prefectures are next to each other. However, plant 76 from Tsuruoka, Yamagata Prefecture, did not belong to this regional group.

In group G of both phylogenetic trees, plants 88, 92, and 93 were isolated from other plants. In group G of the coalescent tree, plants 77 and 80 were separated from other plants of the same group. Thus, the grouping of group G is ambiguous, and may be due to the fact that plants in various locations belonged to group G.

Admixture history of eight groups and the tight cluster of group A

Admixture analysis was used to estimate ancestral history and calculated cross-validation (CV) errors to estimate possible values of K (number of ancestral populations) = 1–12 (Supplementary Figure 1). $K = 2$ was the most likely because the error value of the cross-validation was minimal. We presented the results of the admixture analysis from $K = 2$ to $K = 7$ (Figure 6).

In the case of $K = 2$ (Figure 6), except for plant 42, the tight cluster of group A (plants 43, 44, 45, 46, 47, 48, 49, 50, and 51) formed a single population, shown in yellow. The colour red corresponds to plants other than those in group A. The rest of the group A

plants were admixed groups of the two populations. Thus, at $K = 2$, the group A members were clearly separated from the other plants.

The analysis of $K = 3–7$ separated six groups A, B, C, D, E, and H (Figure 6). However, the analysis did not separate group F, which is consistent with the results of PCA, which did not clearly separate group F from other plants.

The admixture analysis also showed that the grouping of group G was ambiguous. Reflecting this observation, the pattern of colours in group G was not consistent across several *K* values. Thus, the plants in the group G plants may be admixtures of a variety of groups.

Conservation of heterozygosity

Asexual reproduction was confirmed by checking heterozygosity conservation using pairwise alignments. Plants 8 and 9 of group F were grafted from the same tree (Table 1). Between these two plants, 2288 of 3699 sites were conserved heterozygous sites (61.9%) (Supplementary Fig. 2). Likewise, plants 33 and 34 of group C were grafted from the same tree (Table 1). Between these two plants, 1947 of 3097 sites were conserved heterozygous sites (62.9%) (Supplementary Fig. 3). Thus, this degree of heterozygosity conservation was found among asexually reproduced plants when analysing the data using *de novo* mapping.

Between plants 3 and 4 of group B, 2198 of 3879 sites were conserved heterozygous sites (56.7%) (Supplementary Fig. 4), between plants 40 and 41 of group A, 2140 of 3414 sites were conserved heterozygous sites (62.7%) (Supplementary Fig. 5), and between plants 38 and 39 of the group A, 1700 of 3502 sites were conserved heterozygous sites (48.5%) (Supplementary Fig. 6). Thus, each of 3 and 4, 40 and 41, and 38 and 39 was identified as asexually propagated plants, although no records have been kept. We

examined the conservation of heterozygosity for tight clusters in group A (plants 42, 43, 44, 45, 46, 47, 48, 49, 50, and 51). The degrees of conservation ranged from 50.4% to 68.9% (Supplementary Fig. 7, Supplementary Table 3). These levels of conservation were absent between the plants of the tight clusters in group A and other plants (plants 35, 38, and 40) (Supplementary Fig. 7, Supplementary Table 3). Therefore, the members of the tight cluster in group A are asexually propagated plants derived from the same tree.

Statistical interpretations of the classifications

In our statistical analysis (Table 2, Table 3, Supplementary Table 4), we removed the plants (13 and 14) with uncertain results and the plants in group G that caused the ambiguity. Although groups C and F belonged to the Arima varieties, the Fst value between them was high (0.110, Table 2). Thus, the difference in the pairwise Fst values between these two groups may indicate two places of origin.

Similarly, the pairwise Fst value between groups A and D of the Asakura variety was high (0.0803, Table 2). The reason for the higher Fst value may be the presence of a tight cluster in group A. Therefore, we performed a second statistical analysis by modifying the grouping. In the new grouping, we designated the tight cluster in group A as A2. We denoted the plants that remained in group A as A1. As expected, the pairwise Fst values were high between group A2 and each of the other groups (Table 3). Table 3 shows that group A1 was genetically closer to group D (0.0539). Thus, the higher genetic distance between groups A and D in Table 2 was due to the tight cluster.

Discussion

Our study classified *Zanthoxylum piperitum* into several groups based on DNA sequences and geographic information. Each group showed interesting characteristics,

some of which may be related to domestication. The domestication of *Zanthoxylum piperitum* can be describe in two steps. The first step is the cultivation of wild plants in agricultural fields, in which case the wild and cultivated plants are one genetically similar group. In the second step, the varieties of excellent traits spread from their place of origin to the surrounding areas, in which case the genetic diversity of individuals within the varieties should be low. In crops such as fruit trees, grafting is one of the means of spreading.

Group D plants showed the first step of domestication, because the cultivated plants probably originated in the Yabu Mountains. Group D was comprised of both wild plants in mountainous areas and cultivated plants in agricultural fields (Table 1).

The spineless Asakura variety (*Z. piperitum* (L.) DC forma inerme (Makino) Makino), first described 90 years ago[11], may be found in group D. This 90-year-old documentation[11], based on a much older book, states that the origin of the Asakura variety was on a cliff near the Konryuji temple. Plant 90 belonging to group D grows in a place similar to the place of origin and is spineless. In addition, spineless wild plants 53, 54, 60, and 69 were collected near the Konryuji temple.

Group A plants show the second domestication step, because they include varieties with excellent traits and show spreading. Group A contains a tight cluster comprised of plants 42, 43, 44, 45, 46, 47, 48, 49, 50, and 51. This cluster shows minimal genetic diversity (Figure 4). Furthermore, the examination of the conservation of heterozygosity showed that these plants were propagated by grafting (Supplementary Fig. 7, Supplementary Table 3). These plants were also found to be widely cultivated in many cities (Yabu, Ayabe, Gojo, Kyotamba, Kobe, Kami), indicating that they have spread. Plants 42 and 48 are highly productive trees in Yabu City, which supply scions for grafting. Thus, the plants in this tight cluster represent a variety of excellent traits.

There are two potential places of origin for group A: Yabu city, where the Asakura district is located, and another distant location. The following reasons may explain the first possibility: (1) many of the group A plants grow in Yabu City (plants 35, 38, 39, 42, 46, and 48); (2) a 90-year-old document[11] describes that the spineless plants originate from Asakura district, Yabu city; (3) all of the group A plants are spineless. Interestingly, we collected plants 35, 38, and 39 near the Konryuji temple, a potential place of origin for the Asakura variety. Therefore, we do not exclude the possibility that the spineless Asakura variety described 90 years ago[11] may represent group A plants. However, plants 35, 38, and 39 were found to be cultivated. Thus, a serious problem for the first possibility is the absence of wild plants in group A in the mountains of Yabu city.

The second possibility is that the group A plants are from another distant location; that is, the plants in the tight cluster of group A of unknown origin hybridised with the group D plants to form the rest of the group A plants. This possibility is consistent with the results of the admixture analysis of $K = 2$. However, it is worth noting that the results of the admixture analysis may be artificial since the second step of domestication formed the ancestral population containing the plants in the tight cluster. If the second possibility is true, the origin of the plants in the tight cluster remains unknown, although the origin of the rest of the group A plants can be explained by hybridisation.

In groups C and F of the Arima variety, we observe the first domestication step based on the records. According to records, the group F plants (plants 7, 8, and 9) were transplanted from Mt. Inariyama to Asago city (Table 1). Group C plants (plants 29, 30, 31, 32, 33, and 34) were transplanted from Mt. Rokko to Asago and Kobe cities. The other group F plants (plants 5, 6, 10, 11, and 12) should be of the same origin. According to PCA, the group F plants (plants 5, 6, 10, 11, and 12) were closer to the plants in the other groups. This proximity to the other plants may be due to hybridisation.

We did not find any records of the origin of the group E plants. In addition, we could not find the wild plants of this group in the mountains. If the group E plants have adaptations to high altitudes, the mountainous areas near Takayama city may be the origin of group E. Spineless plants are present in group E (plants 16, 17, 18,19, 20, 21, 22, 23, 24, 25, and 26), and are genetically different from the plants in group A and D in Yabu city, where the Asakura district is located.

In addition to these observations, the results for group G also suggest that domestication is underway in many areas. Ongoing domestication is associated with the cultivation of wild varieties on nearby agricultural lands and gardens. Group G was genetically different from the other plant groups undergoing domestication. Therefore, these plants may have been domesticated in their respective locations.

The ongoing domestication of Japanese pepper is not entirely similar to that of olives and dragon fruit. In the first step of olive domestication, wild plants are preserved *in situ*[12]. As a result, farmers continue to disturb the natural habitat of the olive when selecting wild populations. In Japanese pepper, wild plants are harvested from their natural habitat and transplanted to agricultural fields or gardens while leaving other wild plants intact in the mountains. The second step of the domestication of olives and dragon fruits occurs through the continuous introduction of wild plants into agricultural fields to further select preferred traits[12,13]. In Japanese pepper, wild plants are not frequently reintroduced into cultivated plant sites. The wild plants of dragon fruit are morphologically different from those currently cultivated[13]. In Japanese pepper, there is no morphological difference between wild and cultivated plants. Domestication syndrome changes the genomic, physiological, and morphological characteristics of cultivated plants from those of wild plants[26]. The genomic characteristics of the plants of the tight cluster in group A were different from those of wild plants. Except for this case, we did not observe any evidence

of domestication syndrome. Therefore, domestication in Japanese pepper is at an incipient stage compared to olives and dragon fruit.

Spineless plants are known as the Asakura variety, which is classified as a single subspecies, *Z. piperitum* (L.) DC forma inerme (Makino) Makino[11]. However, it is inappropriate to refer to the spineless plant as the Asakura variety or *Z. piperitum* (L.) DC forma inerme (Makino) Makino. In one group of the Asakura variety (group A), the spineless plants of the tight cluster with an unknown origin were present. Although the second group of the Asakura variety (group D) is from the recorded place of origin[11], it contained some spinal plants. In addition, the spineless trait is not unique to the Asakura variety, as some of the plants in the Takahara variety (group E) are also spineless. The majority of group G plants are spineless. Importantly, these spineless plants are from many locations. Therefore, our study does not support that the spineless variety is either from Asakura or monophyletic. Our results indicate that the spineless variety is not a single subspecies.

Some of the Fis values were negative (Supplementary Table 4). Notably, the tight cluster plants in group A (group A2) had the highest negative Fis value. Therefore, we speculated that some of these plants had been asexually reproduced by grafting, since asexual reproduction may cause negative Fis values[27,28]. The pairwise alignments support this possibility (Supplementary Fig. 7, Supplementary Table 3).

In this study, we elucidated the genetic diversity of Japanese pepper while tracing the process of domestication. Our results demonstrate that the conservation of plant genetic resources is an important agricultural practice. Several cultivated varieties of Japanese pepper were found to have closely related plants growing in the mountains, in contrast to other crops. On the other hand, several cultivated varieties of Japanese pepper have no closely related plants that grow in the mountains, which suggests that plants that grow in

the mountains may disappear. Therefore, there is a need to conserve both plants growing in the mountains *in situ* and plants growing in agricultural fields or preservation facilities *ex situ*. The results presented in this study will be useful in developing a plan for the conservation and breeding of Japanese pepper.

Methods

Plant materials

Ninety-three samples of *Zanthoxylum piperitum* collected from various parts of Japan were used (Table 1). Photographs of the materials were taken, and their leaves were stored in a freezer (–80°C).

DNA extraction and double digested restriction site amplified DNA sequencing (ddRAD-Seq)

DNA extraction and purification were performed using previously described methods[23]. The library for ddRAD-Seq was prepared based on the original protocol[20] with some modifications[29]. The first restriction site near the primer binding site, which reads the single-ended DNA sequence, was *Bgl*II. The second restriction site, adjacent to the binding site for reading the index sequence, was *Eco*RI. In two lanes of Illumina HiSeq2000 (Illumina, San Diego, CA, USA), the library had been sequenced with 51 bp single-ended reads in Macrogen (Seoul, Korea).

Processing and quality control of ddRAD-Seq data

The Stacks package[30] was used to analyse the ddRAD-Seq reads. The process_shortreads program of the Stacks package (version 2.4) was used to clean reads using -c (clean data, remove any read with an uncalled base), -q (discard reads with low-

quality scores), and -r (rescue barcodes) options. The cutadapt program (version 2.6)[31] removed adaptor sequences from reads using the -b (remove adapter anywhere, the sequence of an adapter that may be ligated to the 5' or 3' end) and -e 0.1 (maximum allowed error rate by keeping the default value 0.1 = 10%) options. Any low-quality bases in the single-end reads were trimmed using the trimmomatic program (version 0.39)[32] (LEADING:19 (cut bases off the start of a read, if below a threshold quality), TRAILING:19 (cut bases off the end of a read, if below a threshold quality), AVGQUAL:20 (drop the read if the average quality is below the specified level), MINLEN:51 (drop the read if it is below a specified length), and SLIDINGWINDOW:20:20 (performs sliding window trimming, clipping once the average quality within the window falls below a threshold).

De novo mapping and variant calling

The denovo_map.pl of the Stacks package (version 2.5), a wrapper script for ustacks, cstacks, sstacks, tsv2bam, and gstacks, was used to map reads *de novo* without using a reference genome. The options for denovo_map.pl were -M 4 (number of mismatches allowed between stacks within individuals (for ustacks)), -n 4 (number of mismatches allowed between stacks between individuals (for cstacks)), and -m 3 (number of identical reads required to initiate a new putative allele (for ustacks)). The genotyping data were created to specify all the samples under analysis by assigning one individual per population. After performing denovo_map.pl, the populations program of the Stacks package (version 2.5) created a vcf (variant call format) file[33], plink file[34], and phylip file with the -R 0.5 (minimum percentage of individuals across populations required to process a locus), --write-single-snp (restrict data analysis to only the first SNP per locus), --min-maf 0.05 (minimum minor allele count required to process a SNP), --vcf, --plink, -

-phylip-var-all options. The populations program was also used to create pairwise alignments between two individuals. In this case, the -R option was set to one.

Principal component analysis (PCA) and cluster analysis

Principal component analysis was performed based on the vcf file generated by the populations program. The SNPRelate program[35] in the R software environment (version 3.6.2) converted the vcf file to a gds (genomic data structure) file, drew the PCA diagrams, and calculated the contribution ratios for each principal component. This step used only bi-allelic loci. The SNPRelate program plotted the dendrogram considering identity by state (IBS) pairwise distances.

Admixture analysis

The plink program (plink 2, version 1.90p)[34] was used to create the admixture program's input files. The admixture program (version 1.3)[36] was used to determine the admixture history and the cross-validation (CV) error for the hypothetical runs from *K* (number of ancestral populations) = 1–12. The CV error plot was drawn using the received log data to determine the optimal *K* value. R software (version 3.6.2) was used to draw admixture plots using the Q estimates files created by the admixture programs.

Maximum likelihood phylogenetic tree analysis

The maximum likelihood (ML) phylogenetic tree was created using the raxmlHPC-PTHREADS-SSE3 program (version 8.2.12)[37]. The phylip file created by the populations program was used as the input file. The parameters for raxml were -f a (rapid bootstrap analysis and search for the best-scoring ML tree in one program run), -x 12345 (an integer number (random seed) and turn on rapid bootstrapping), -p 12345 (a random number seed

for parsimony inferences), -N 1000 (bootstrap value), -m (model of binary (morphological), nucleotide, multi-state, or amino acid substitution), and -GTRGAMMA (GTR + optimisation of substitution rates + GAMMA model of rate heterogeneity with estimate of proportion of invariable sites). Group H was used as the root.

Phylogenetic tree analysis under the coalescent model

Phylogenetic tree analysis was performed using SVDquartets[38] integrated into PAUP (version 4.0a) software[39]. The input file for PAUP was created by converting a phylip file containing all sites to the nexus file format. The parameters used for SVDquartets were quartet evaluation; evaluate all possible quartets, tree inference; select trees using QFM quartet assembly, tree model; multi-species coalescent, write quartets file; QMC format, and handling of ambiguities; and distribute. The number of bootstraps analyses was 1,000 replicates. Group A was used as the root.

Statistical analysis

Groups separated by PCA and cluster analysis were assigned as separate populations in the population map data required for the Stacks package. The denovo_map.pl was re-performed after modifying the data for the population map. The populations program was performed with the options -R 0.5, --write-single-snp, --min-maf 0.05, --fstats (SNP and haplotype-based F statistics), --Fst_correction (a correction to be applied to Fst values with p-value (p-value to keep an Fst measurement. default: 0.05)).

Acknowledgments

We are grateful to those who helped us collect samples at various locations. We thank Ms. Yuki Sano, Mr. Atushi Shirakawa, and Mr. Kazuma Kubo for the preparation of

DNA samples. This work was supported by the Urakami Foundation for Food and Food Culture Promotion.

Author contributions

N.F., K.Y., F.H., and Y.N. designed the research. N.F., H.M., N.I., and Y.N. collected samples. Y.N. performed DNA extraction. M.D.G.P.P. and Y.N. performed bioinformatic analysis. A.J.N. performed the RAD-Seq analysis. M.D.G.P.P., N.F., K.Y., F. H. H. M., N.I., and Y.N. analysed the data. M.D.G.P.P. and Y.N. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Data availability

Sequences are available at the DNA Data Bank of Japan Sequence Read Archive (https://www.ddbj.nig.ac.jp/dra/index-e.html; Accession no. DRA011046).

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Figure legends

Figure 1. Principal component (PC) analysis of *Zanthoxylum* accessions used in the study. Group A of the Asakura variety is shown in blue; group B of the Budou variety is shown in brown; group C of the Arima variety is shown in orange; group D of the Asakura variety is shown in violet; group E of the Takahara variety is shown in maroon; group F of the Arima variety is shown in green; group G is shown in violet. The letters indicating the sample number are also shown in the same colour. The contribution rate of each principal component is shown in parentheses.

Figure 2. Sampling points and locations of the varieties. The colour scheme is the same as in Figure 1.

Figure 3. Cluster analysis of *Zanthoxylum* accessions. The new group, H group, is shown in light blue. The colour scheme is the same as in Figure 1, with the additional colour.

Figure 4. Maximum likelihood phylogenetic analysis of *Zanthoxylum* accessions. The colour scheme is the same as in Figure 3.

Figure 5. Coalescent model phylogenetic tree of *Zanthoxylum* accessions. The colour scheme is the same as in Figure 3.

Figure 6. K = 2-7 admixture plots in admixture analysis of *Zanthoxylum* accessions used in this study. The horizontal axis shows the group names and the sample numbers. The

colour scheme for the group names and the sample numbers is the same as in Figure 3. The colour for the genetic cluster is different from that used to denote the group names and sample numbers.

Tables

Table 1. Information on *Zanthoxylum piperitum* accessions used in the study.

Plant Number	**Prefecture**	**City/Town**	**Spinal/Spineless**	**Information**	Group	Variety
1	Kochi	Sakawa	Spinal	Agricultural field, cultivated	H	Unforeknown Kochi group
2	Kochi	Nangoku	Spinal	Mountain, wild tree	H	Unforeknown Kochi group
3	Wakayama	Kainan	Spinal	Agricultural field, cultivated, Nagamine agricultural cooperative	B	Budou
4	Wakayama	Kainan	Spinal	Agricultural field, cultivated, Nagamine agricultural cooperative	B	Budou
5	Hyogo	Kobe	Spinal	Garden, cultivated	F	Arima
6	Hyogo	Kobe	Spinal	Garden, cultivated	F	Arima

7	Hyogo	Asago	Spinal	Agricultural field in fruit tree research institute for preservation purpose, cultivated, originally transplanted from Mt. Inariyama, agricultural field	F	Arima
8	Hyogo	Asago	Spinal	Agricultural field in fruit tree research institute for preservation purpose, cultivated, originally transplanted from Mt. Inariyama, agricultural field, grafted from the same tree as plant 9	F	Arima
9	Hyogo	Asago	Spinal	Agricultural field in fruit tree research institute for preservation purpose, cultivated, originally transplanted from Mt. Inariyama, agricultural field, grafted from the same tree as plant 8	F	Arima
10	Hyogo	Kobe	Spinal	Garden, cultivated	F	Arima
11	Hyogo	Kobe	Spinal	Garden, cultivated	F	Arima
12	Hyogo	Kobe	Spinal	Garden, cultivated	F	Arima
13	Hyogo	Yabu	Spineless	Garden, cultivated	D	Asakura
14	Gifu	Takayama	Spinal	Garden, cultivated	E	Takahara
15	Gifu	Takayama	Spinal	Agricultural field, cultivated	E	Takahara

16	Gifu	Takayama	Spineless	Agricultural field, cultivated	E	Takahara
17	Gifu	Takayama	Spineless	Agricultural field, cultivated	E	Takahara
18	Gifu	Takayama	Spineless	Agricultural field, cultivated	E	Takahara
19	Gifu	Takayama	Spineless	Agricultural field, cultivated	E	Takahara
20	Gifu	Takayama	Spineless	Garden, cultivated, used as mother tree for grafting	E	Takahara
21	Gifu	Takayama	Spineless	Agricultural field, cultivated, used as mother tree for grafting	E	Takahara
22	Gifu	Takayama	Spineless	Agricultural field, cultivated	E	Takahara
23	Gifu	Takayama	Spineless	Agricultural field, cultivated	E	Takahara
24	Gifu	Takayama	Spineless	Garden, cultivated	E	Takahara
25	Gifu	Takayama	Spineless	Agricultural field, cultivated, about 30 years old	E	Takahara
26	Gifu	Takayama	Spineless	Agricultural field, cultivated, about 25-26 years old	E	Takahara
27	Gifu	Takayama	Spinal	Agricultural field, cultivated	E	Takahara
28	Gifu	Takayama	Spinal	Agricultural field, cultivated	E	Takahara
29	Hyogo	Kobe	Spinal	Garden, cultivated, originally transplanted from Mt. Rokko	C	Arima
30	Hyogo	Kobe	Spinal	Garden, cultivated, originally transplanted from Mt. Rokko	C	Arima

31	Hyogo	Asago	Spinal	Agricultural field in fruit tree research institute for preservation purpose, cultivated, originally transplanted from Yubunedani of Mt. Rokko	C	Arima
32	Hyogo	Asago	Spinal	Agricultural field in fruit tree research institute for preservation purpose, cultivated, originally transplanted from Yubunedani of Mt. Rokko	C	Arima
33	Hyogo	Asago	Spinal	Agricultural field in fruit tree research institute for preservation purpose, cultivated, originally transplanted from Yubunedani of Mt. Rokko, grafted from the same tree as plant 34	C	Arima
34	Hyogo	Asago	Spinal	Agricultural field in fruit tree research institute for preservation purpose, cultivated, originally transplanted from Yubunedani of Mt. Rokko, grafted from the same tree as plant 33	C	Arima
35	Hyogo	Yabu	Spineless	Agricultural field, cultivated, near the Konryuji temple	A	Asakura
36	Kyoto	Kyōtamba	Spineless	Agricultural field, cultivated	A	Asakura

37	Hyogo	Kobe	Spineless	Agricultural field, cultivated, purchased as nursery stock of high-yielding Asakura sanshō	A	Asakura
38	Hyogo	Yabu	Spineless	Agricultural field, cultivated, near the Konryuji temple	A	Asakura
39	Hyogo	Yabu	Spineless	Agricultural field, cultivated, near the Konryuji temple	A	Asakura
40	Kyoto	Kyōtamba	Spineless	Agricultural field, cultivated	A	Asakura
41	Kyoto	Kyōtamba	Spineless	Agricultural field, cultivated	A	Asakura
42	Hyogo	Yabu	Spineless	Agricultural field, cultivated, used as high-yielding mother tree for grafting	A	Asakura
43	Kyoto	Ayabe	Spineless	Agricultural field, cultivated	A	Asakura
44	Nara	Gojō	Spineless	Agricultural field, cultivated	A	Asakura
45	Kyoto	Kyōtamba	Spineless	Agricultural field, cultivated	A	Asakura
46	Hyogo	Yabu	Spineless	Agricultural field, cultivated	A	Asakura
47	Hyogo	Kobe	Spineless	Agricultural field, cultivated, purchased as nursery stock of Asakura sanshō	A	Asakura
48	Hyogo	Yabu	Spineless	Agricultural field, cultivated, used as mother tree for grafting	A	Asakura
49	Nara	Gojō	Spineless	Agricultural field, cultivated	A	Asakura
50	Nara	Gojō	Spineless	Agricultural field, cultivated	A	Asakura

51	Hyogo	Kami	Spineless	Agricultural field, cultivated	A	Asakura
52	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
53	Hyogo	Yabu	Spineless	Mountain, wild tree, near the Konryuji temple	D	Asakura
54	Hyogo	Yabu	Spineless	Mountain, wild tree, near the Konryuji temple	D	Asakura
55	Hyogo	Yabu	Spinal	Agricultural field, cultivated, near the Konryuji temple	D	Asakura
56	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
57	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
58	Hyogo	Yabu	Spineless in 2017, spinal in 2020	Agricultural field, cultivated, 100 years old tree	D	Asakura
59	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
60	Hyogo	Yabu	Spineless	Mountain, wild tree, near the Konryuji temple	D	Asakura
61	Hyogo	Yabu	Spinal	Possible abandoned tree near an abandoned house	D	Asakura
62	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
63	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
64	Hyogo	Yabu	Spinal	Mountain, wild tree	D	Asakura
65	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura

66	Hyogo	Yabu	Spinal	Mountain, wild tree, near the Konryuji temple	D	Asakura
67	Hyogo	Yabu	Spinal	Mountain, wild tree	D	Asakura
68	Hyogo	Yabu	Spinal	Mountain, wild tree	D	Asakura
69	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
70	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
71	Hyogo	Yabu	Spineless	Mountain, wild tree	D	Asakura
72	Hyogo	Yabu	Spinal	Mountain, wild tree	D	Asakura
73	Hyogo	Yabu	Spinal	Mountain, wild tree	D	Asakura
74	Hyogo	Kami	Spineless	Garden, spontaneously grown tree	D	Asakura
75	Ishikawa	Noto	Spineless	Mountain, wild tree	G	Unclassified
76	Yamagata	Tsuruoka	Spinal	Garden, spontaneously grown tree	G	Unclassified
77	Shiga	Nagahama	Spineless	Agricultural field, cultivated	G	Unclassified
78	Niigata	Agamachi	Spineless	Mountain, wild tree	G	Unclassified
79	Niigata	Agamachi	Spineless	Mountain, wild tree	G	Unclassified
80	Hyogo	Kami	Spineless	Agricultural field, cultivated	G	Unclassified
81	Yamagata	Tsuruoka	Spineless	Garden, spontaneous grown tree, growing adjacent to plant 83	G	Unclassified

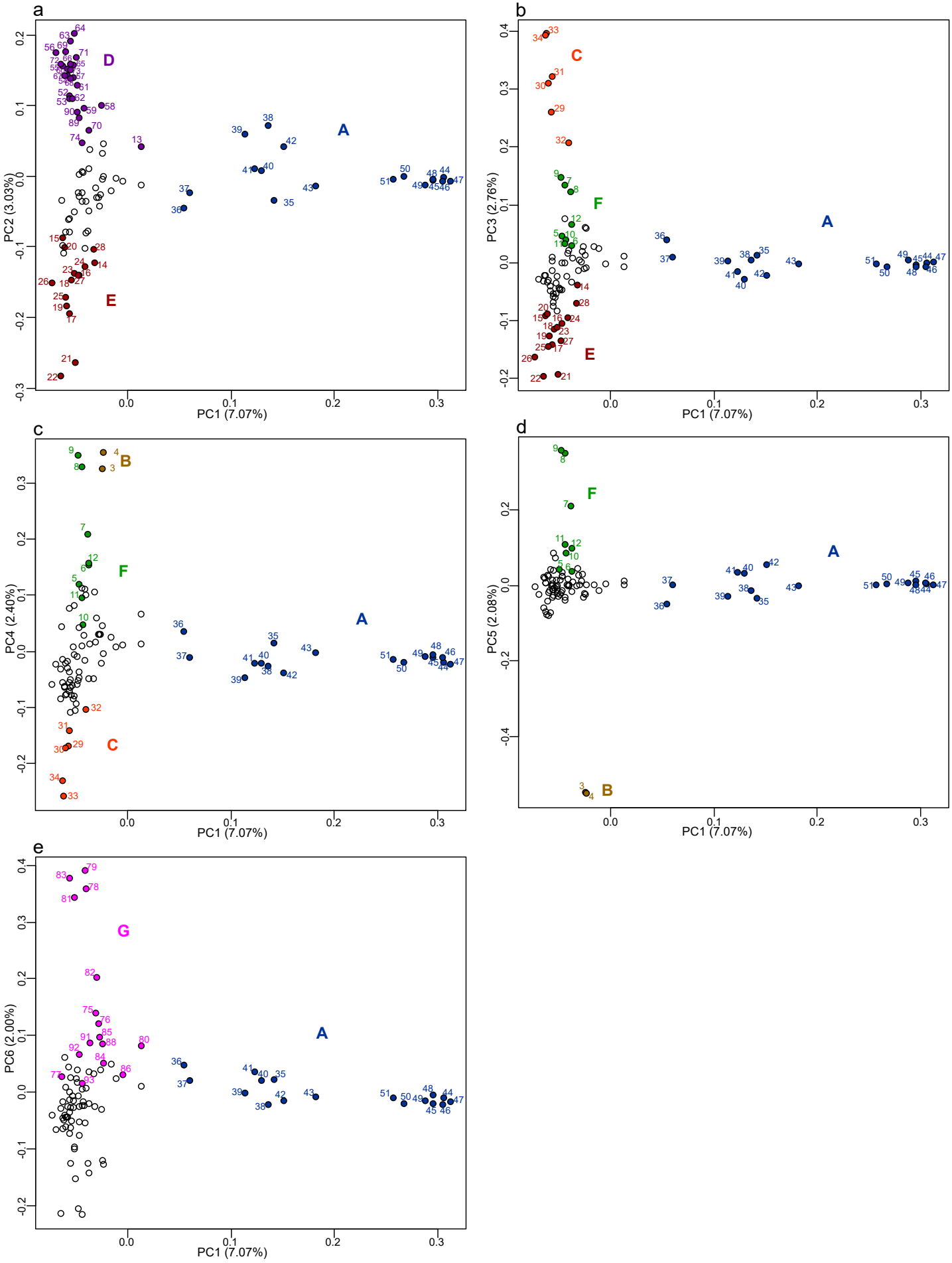
82	Akita	Akita	Spinal	Transplanted from mountain, wild tree	G	Unclassified
83	Yamagata	Tsuruoka	Spineless	Garden, spontaneous grown tree, growing adjacent to plant 81	G	Unclassified
84	Tokyo	Adachi	Spinal	Garden, cultivated from the seed of an old tree	G	Unclassified
85	Ishikawa	Noto	Spineless	Mountain, wild tree	G	Unclassified
86	Ishikawa	Noto	Spineless	Mountain, wild tree	G	Unclassified
87	Kochi	Sakawa	Spinal	Agricultural field, cultivated	H	Unforeknown Kochi group
88	Kyoto	Ayabe	Spineless	Agricultural field, cultivated	G	Unclassified
89	Hyogo	Yabu	Spineless	Garden, cultivated, near the Konryuji temple	D	Asakura
90	Hyogo	Yabu	Spineless	Mountain, wild tree, near the Konryuji temple, located in the recorded place of the origin of Asakura variety	D	Asakura
91	Hyogo	Toyooka	Spinal	Mountain, wild tree	G	Unclassified
92	Kochi	Nangoku	Spinal	Agricultural field, cultivated	G	Unclassified
93	Hyogo	Kobe	Spineless	Garden, spontaneously grown tree	G	Unclassified

Table 2. Fst values for each pair of groups A, B, C, D, E, F, H.

Group	B	F	E	C	A	D
H	0.307	0.120	0.0847	0.165	0.1080	0.0490
B		0.130	0.0950	0.191	0.125	0.0529
F			0.0689	0.110	0.103	0.0457
E				0.0874	0.0994	0.0464
C					0.126	0.0572
A						0.0803

Table 3. Fst values for each pair of groups A1, A2, B, C, D, E, F, H.

Group	B	F	E	C	A1	A2	D
H	0.268	0.109	0.0760	0.156	0.132	0.196	0.0464
B		0.127	0.0950	0.189	0.159	0.226	0.0531
F			0.0697	0.111	0.100	0.154	0.0454
E				0.0878	0.0807	0.132	0.0464
C					0.134	0.195	0.0582
A1						0.0990	0.0539
A2							0.0993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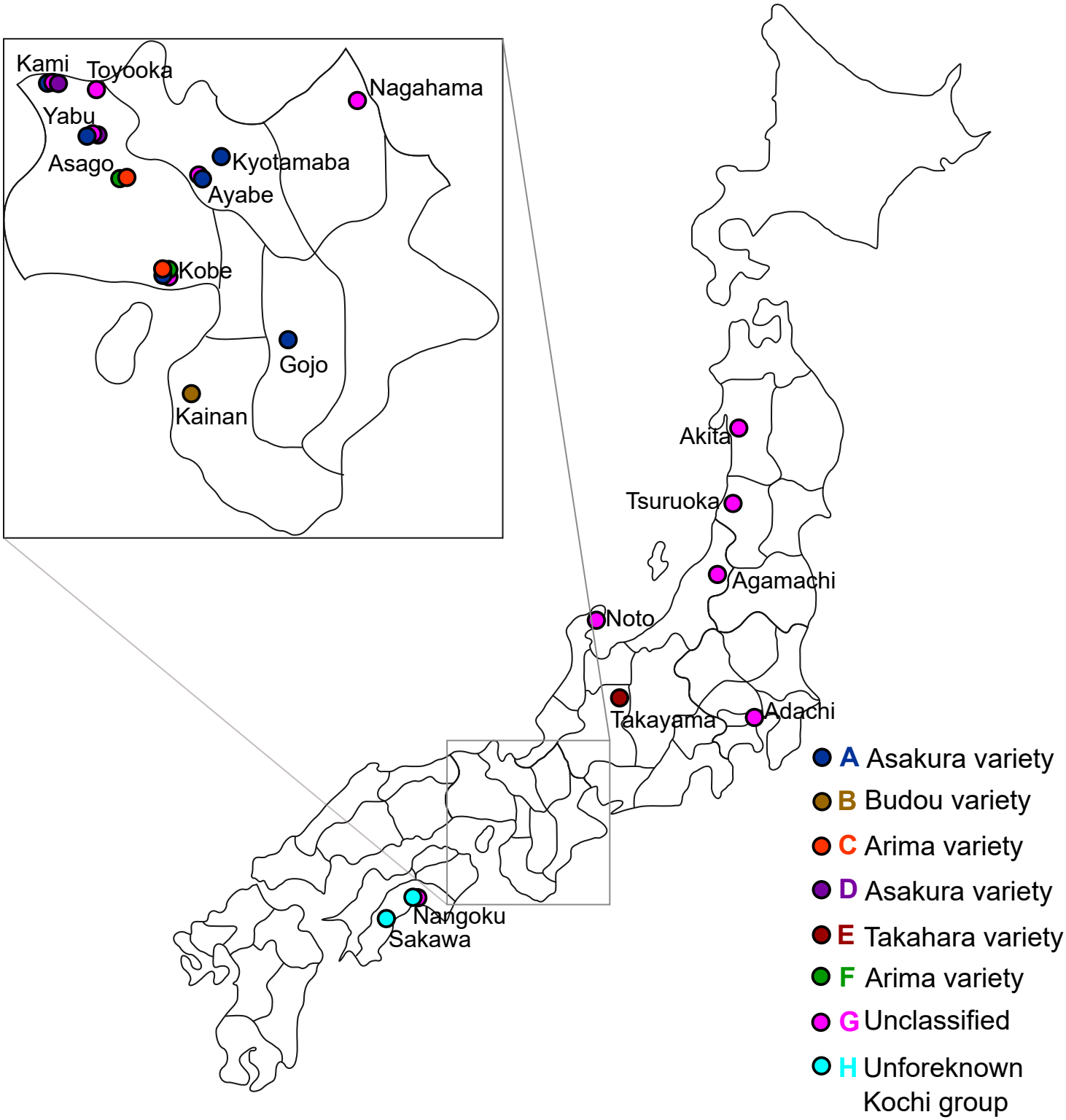
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Takayama
Adachi
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C Arima variety
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F Arima variety
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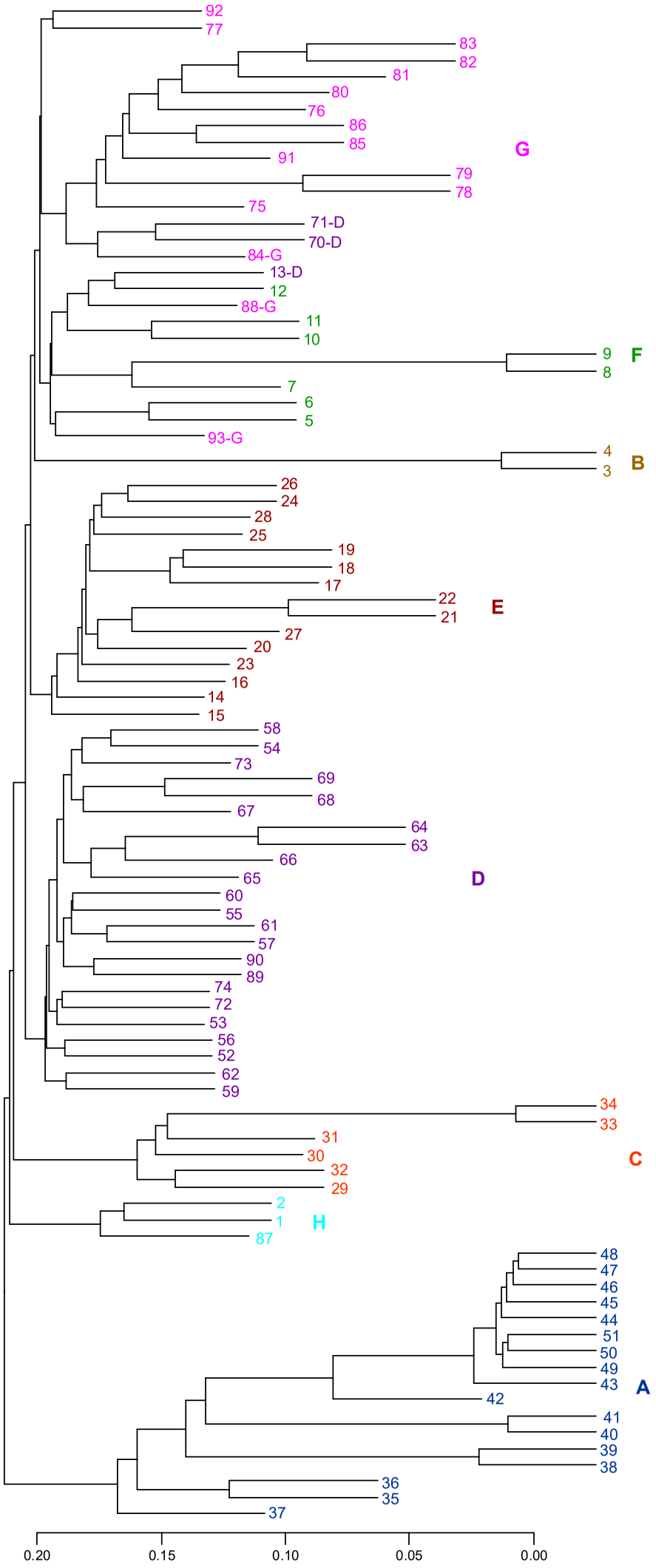
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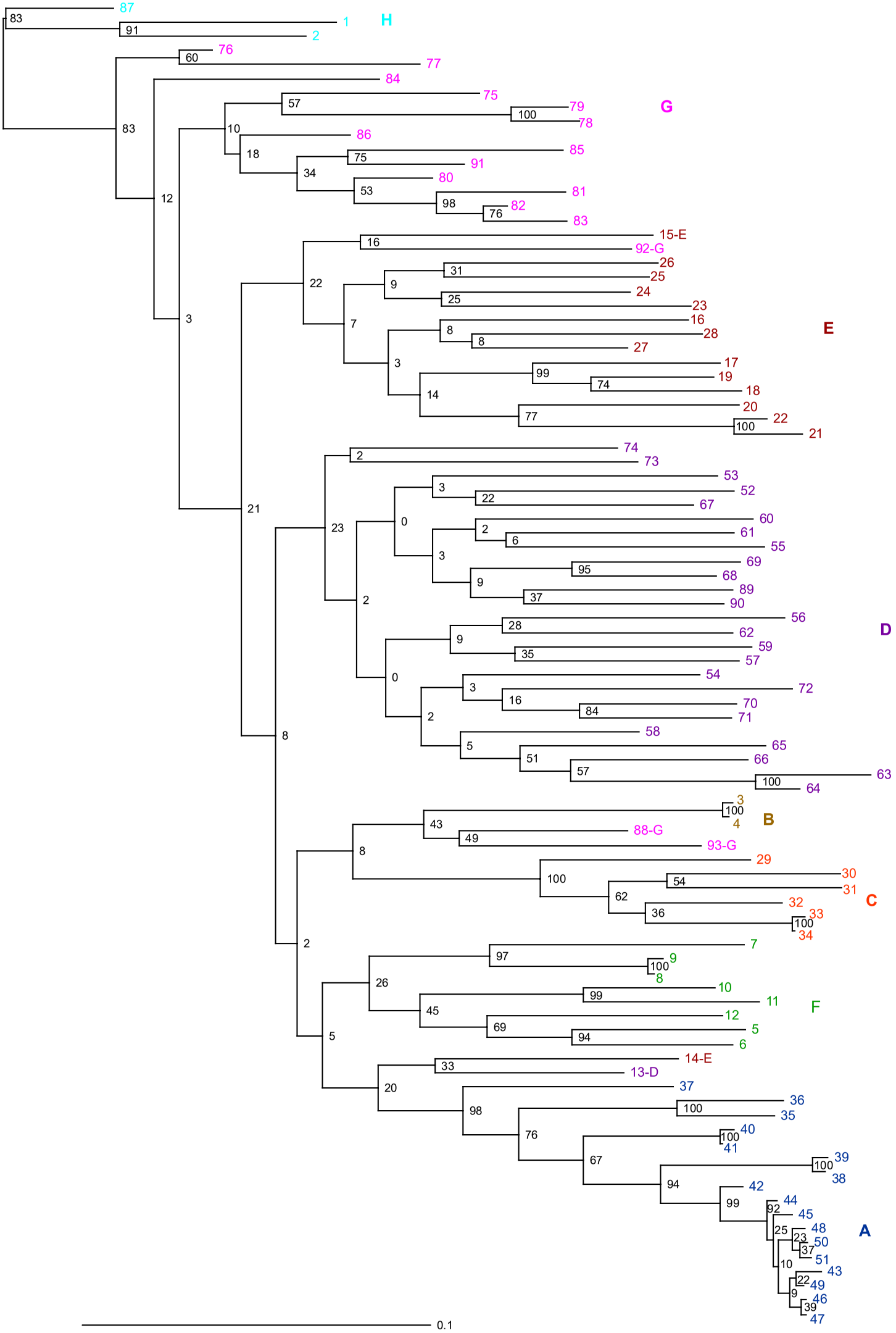
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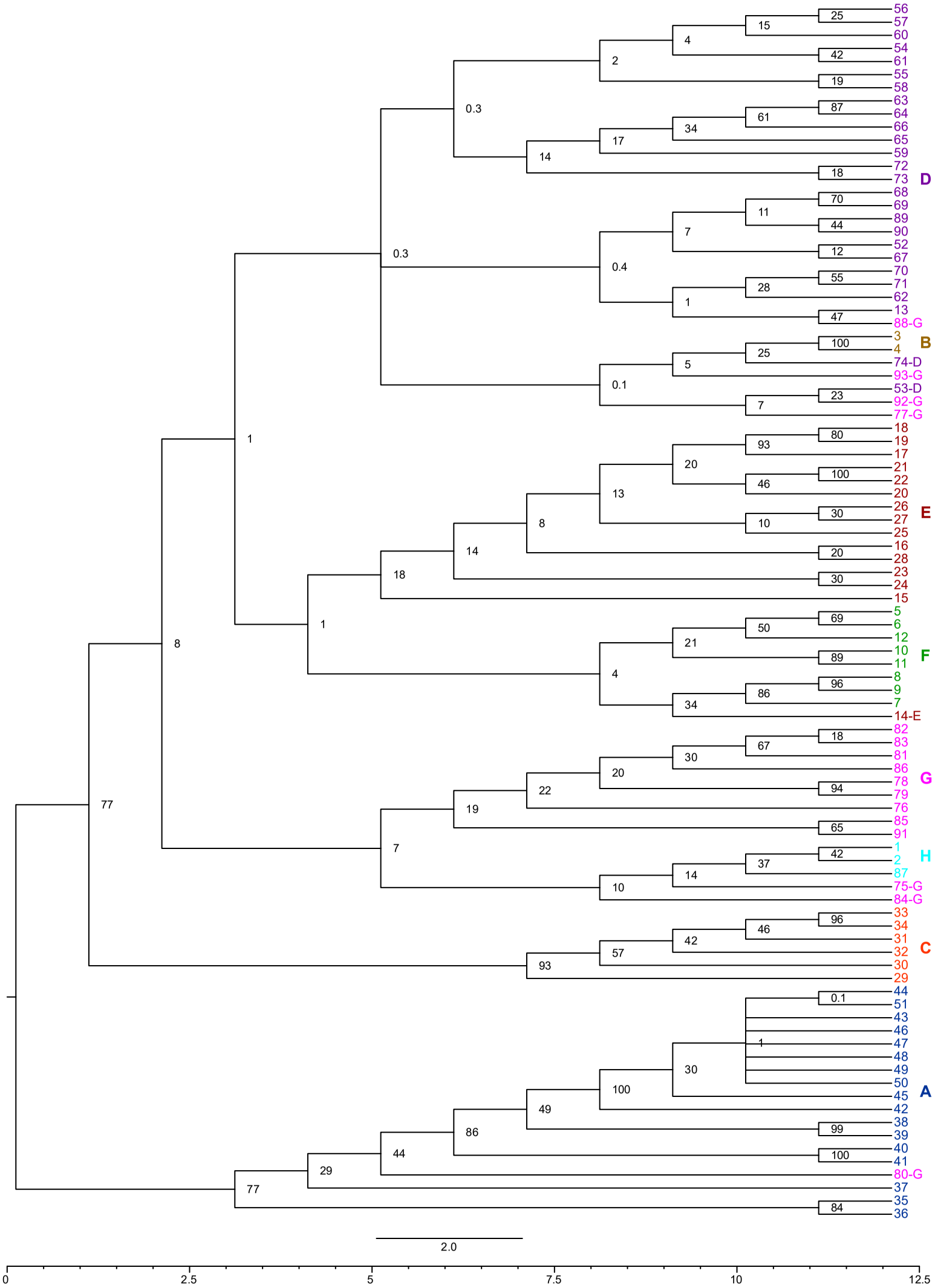

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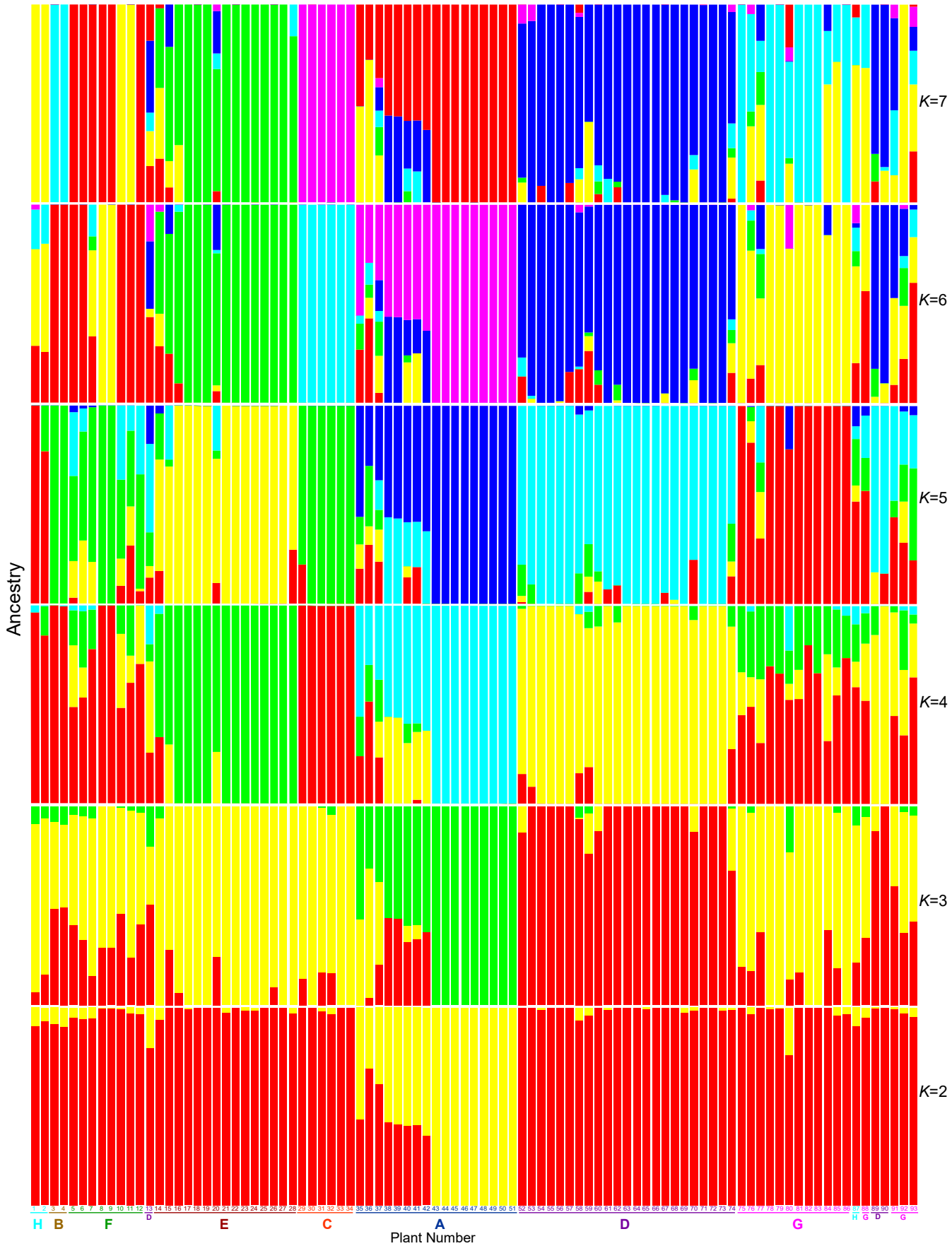
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