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The Identification 5S nrDNA Unit Classes in Genera of Plants and Selected Non-Vertebrate Animals and Their Potential for the Study of Species Relationships

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Abstract

We have investigated the utility of the 5S nrDNA, a conserved, multicopy gene family organized into tandem repeats as a tool for genetic analyses in a wide variety of genera. Previous work in the Triticeae (Poaceae) demonstrated that the prior identification of unit classes based upon the 5S nrDNA NTS, greatly facilitates analysis. We investigated the potential of defining unit classes in other plant genera and several animal genera as a step towards future phylogenetic analyses. Our results demonstrate that in in several plant and animal genera there are sufficient numbers of DNA accessions in GenBank™ to point to different unit classes within species and that in some genera several potential unit classes are found across species. These results justify both more in-depth sampling within species and more breadth in sampling within. Further investigation of additional DNA sequences will help to define true unit classes.

Keywords

5S nrDNA; unit classes; orthologous sequences

1. Introduction

The 5S nrDNA genes are organized into clusters of tandem repeats with each pair separated by a non-transcribed spacer (NTS) that contains sequence variation that may be useful for phylogenetic studies. However, many authors have expressed doubt about its usage. Over the last few decades, we and our collaborators have demonstrated that NTS can be used with success in the Triticeae, and with some Aveneae, through a process that assigns sequences to unit classes and then demonstrating that unit classes remain true to a genus and can even be detected in genera of hybrid origin.

The tribe Triticeae (Poaceae) contains many extensively-studied species of economic importance and analyses of the 5S nrDNA NTS sequences have illuminated genetic relationships within and between several important taxa. The first studies on Triticum aestivum [1], since extended to Hordeum [2, 3], demonstrated the diversity of NTS sequences that formed the data set for phylogenetic analyses of all the sequences in toto. Two size classes of the 5S nrDNA (gene + NTS) were identified, thus confirming previous findings in a number of Triticeae taxa [4]. Furthermore, sequences in these two size classes, historically named Short and Long but in fact demonstrating variability within the size class, can be classified into putative orthologous groups or "unit classes". Unit classes are nominally based on length (hence the historical names), but in reality, are based on nucleotide sequence patterns and variability within the NTS. While a unit class consists of orthologous sequences, different unit classes of orthologous sequences are paralogous. Unit classes can be used for the study of variation including population genetics and phylogenetic analysis as we have demonstrated for many species in serval genera within the Triticeae [3, 5-16].

Baum et al. [12], have outlined in much, more detail the methodologies employed to identify unit classes from different species, the study of phylogenetic relationships between them and the use of paralogous sequences from different unit classes to infer evolutionary relationships among repeat types. Briefly, CLUSTAL W is used to align all sequences in the data base of interest i.e., the 5S nrDNA sequences, created from as many DNA clones as possible collected from different locations within the geographic range of the species, and including at least one plant per location. Alignments are generated using CLUSTAL W [17] and refined with the aid of GeneDoc 2.4.002 [18] and manual editing [19]. Similar sequences are grouped and the process is repeated. This iterative approach of computer alignment and manual optimization reveals unit classes that can be distinguished one from the other [12].

Following upon the identification of unit classes and the ensuing phylogenetic analyses, a third type of analysis was possible. Since haplome (genome) content of taxa within the Triticeae has been extensively studied, it became possible to relate unit classes to haplomes based on the previous assignment of haplomes to genera and species [20, 21] based upon techniques such as intergeneric hybridization, Giemsa C-banding and fluorescence in situ hybridization. Thus, for each haplome we expected to find two unit classes, Long and Short. In the Triticeae there are genera such a Triticum and Hordeum that contain diploid, tetraploid and hexaploid species. If the tetraploid species is an allotetraploid with additional, different, haplomes, the resulting tetraploid is expected to contain four groups, i.e., two Long and two Short DNA with a Long and Short from

each ancestral, diploid species. In an allohexaploid species the combinations as predicted. Several examples of unit classes have been identified including Long S1, Short S1, Long S2, Long H1, Short H1, Long H2, Long Y1 and Long Y2 [3, 5-13, 22]. The terminology developed for unit classes follows the naming convention for haplomes of Á Love [21] and Dewey [20] and indicates genomic constitution of all taxa in the Triticeae. For example, diploid species with the designation H1 contain the Long H1 and, Short H1 unit classes and diploid species with the designation H2 contain the Long H2 unit class. While the Long H1 and Long S1 are paralogous, the Long H1 and Long H2 considered to be homeologous.

An additional advantage to this approach is that unit classes can then be used to predict genomic constitution of cytogenetically determined samples with unknown genomic constitution [13-15, 22]. Unit classes could be also be identified and assigned to haplomes in the genus Avena [23]. It is the ability to define unit classes and to assign them to haplomes that makes this approach successful as can be illustrated by an investigation of the 5S nrDNA NTS from the genus Dougladeweya. The alignment of sequences from the tetraploids Douglasdeweya deweyi and Douglasdeweya wangyi (see Figure 1, [22]) allowed the identification of 3unit classes, Long P1, Long S1 and Short S1, by phylogenetic analysis [22] reproduced here as Figure S1. This result is consistent with Douglasdeweya being an allotetraploid resulting from hybridization between Agropyron and Roegneria i.e., the PP genome from Agropyron and the StSt genome from Pseudoroegneria. Analysis of the 5S rDNA unit classes constitutes a powerful tool for genomic research especially in the Triticeae.

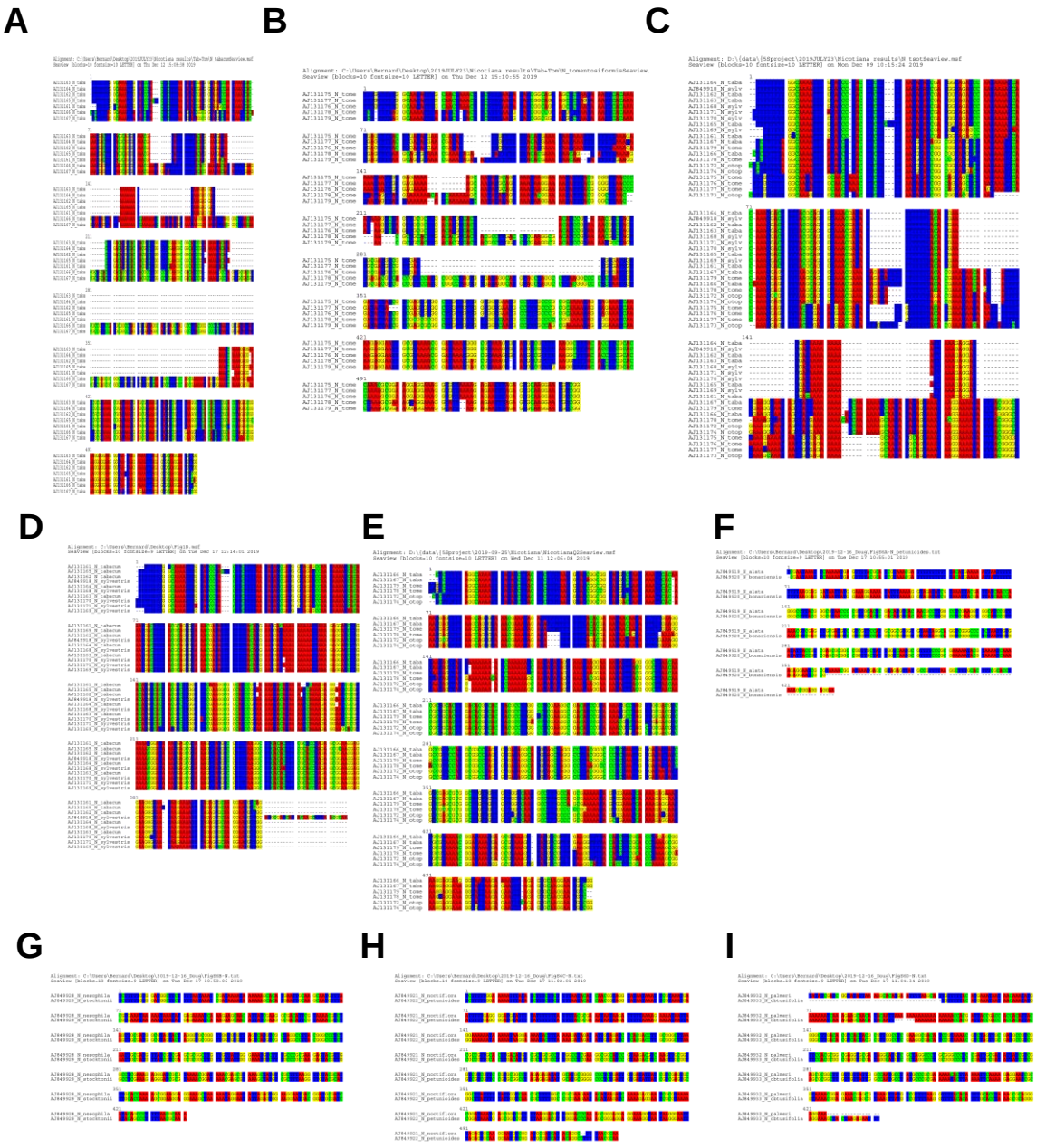

Figure 1 Identification of potential unit classes in Nicotiana subgenus Tabacum. A Identification of potential Long and Short unit classes in Nicotiana tabacum; B Identification of potential Long and Short unit classes in Nicotiana tomentosiformis; C Identification of potential unit classes common to four Nicotiana species: N. tabacum, N. otophora, N. sylvestris, N. tomentosiformis; D Identification of potential short unit class common to N. tabacum, N. sylvestris.; E Identification of potential long unit class common to N. tabacum, N. otophora, N. tomentosiformis. Identification of potential unit classes common to Nicotiana subgenus Petuniodes. F Alignment of Nicotiana alata (AJ849919) with Nicotiana bonariensis(AJ849920); G Alignment of Nicotiana nesophila (AJ849928) with Nicotiana stocktonii (AJ849929); H Alignment of Nicotiana noctiflora (AJ849921) with Nicotiana petunioides (AJ849922); I Alignment of Nicotiana palmeri (AJ849932) with Nicotiana obtusifolia (AJ849933).

The term "unit class" was derived from our studies mainly in the Triticeae which has been the focus of our efforts. But we also pondered whether we could apply these insights to the study of species in other Tribes and Families of plants [24-30] or even in animals [31-35] for which NTS have been analysed without first defining orthologous groups. While each study is unique to the species sampled, they follow a similar pattern. DNA from the many specimens collected from a variety of sites is amplified by 5S nrDNA-specific primers containing restriction sites to aid cloning, are cloned directly or following gel sizing and are sequenced to create databases of NTS sequences that can be aligned for phylogenetic analyses. Since 5S nr DNA are highly repeated and may be distributed among chromosomes, these databases include orthologous and paralogous sequences. The purpose of the present paper was to test two hypotheses, the first that two or more groups of sequences, i.e., unit classes of orthologous sequences, can be defined in genera not in Tribe Triticeae; and the second that unit classes are not restricted to a single species with a genus. To test these hypotheses we assembled data sets from existing GenBank™ accessions that although incomplete for our purpose could be used to test this approach, suggest experimental approaches that focus on the recovery of unit classes and point to future research directions. Even with limited data we could demonstrate that the hypotheses should be accepted.

2. Materials and Methods

2.1 Criteria for Data Selection

As a first step we identified several genera for study in which sequence acquisition was performed by a single group, using similar protocols and primers anchored within the coding regions of the tandem repeated sequences. Studies that utilized size fractionation by agarose gel electrophoresis to focus on a particular size class were discarded since one of the Long or Small classes may be diminished or even lost. This assemblage was reduced further based on the requirement that a large number of collections as possible from the area of distribution of a species was needed in order to search for different unit classes.

Based upon our experience, extensive analyses of 5S nrDNA sequences require not only a large number of DNA accessions per plant sample but a large number of plant accessions collected over the entire range of the species to get an accurate distribution of Long and Short unit classes. The number depends upon the nature and goals of the study. Within these general guidelines, some studies required large datasets. For a study of diversity within the genus Elymus, Baum et al. [15] created a dataset of "1,059 sequences used material from 128 accessions derived from 24 of the available 30 species." And an investigation of the relationships between Campeiostachys Drobov and Elymus L. species by Yang et al. [16] had a dataset of "271 5S nrRNA NTS cloned sequences from 28 accessions". However, a study of the Douglasdeweya revealed its genomic constitution and relationships even though only a very limited number of samples were available – "18 clones from D. deweyi and 9 clones from D. wangii" [22].

These criteria are typically not met, as in most studies accessions were found for multiple species with most sampled only a few times. We first analysed the data from one or a few species within a genus with the most accessions i.e., the largest data set available to identify potential Long and Short unit classes. Then sequences from other species within the same genus were investigated to determine whether they align with the previously determined Long or Short potential unit classes or perhaps identified possible new ones. To be clear, in many instances the

number of accessions was insufficient to allow identification of Long and Short groups of sequences and it is important to stress that at this stage of analysis these are only putative unit classes. For this reason, the absence of these putative unit classes for many species is not reported. In general, information on haplomes as is found in the Triticeae is not available.

2.2 Analysis of 5S nrDNA NTS Sequences

The details of this process as well as the justification for the steps can be found in Baum et al. [12]. The data deposited in GenBank™ contains sequences that are homologous and paralogous; thus, by first grouping sequences into potential unit classes we can focus analysis only on homologous sequences. Potential genera for study were initially identified by key word searches of GenBank (https://www.ncbi.nlm.nih.gov/books/NBK44863/) specifically "Entrez" Sequences or through Google Search (https://www.google.com/) to identify publications of interest. As different size classes were identified, the NTS of these regions were used in further BLAST searches to find successions missed by key word searches. All sequences then recovered from GenBank and rearranged to give the order NTS-5S gene sequence. Several accessions of Mytilus trossulus contained a tRNA-Arg gene within the NTS [34] that was removed before analysis.

We aligned the sequences of each species separately with CLUSTALW [18] then made refinements by moving single nucleotides or groups of nucleotides so as to maximize each of the alignments. The results were then subjected to another alignment by SEAVIEW [36]. No human participants, human data or human tissue were used in this study.

3. Results

In this exploratory study we are evaluating whether the methodology developed to determine unit classes within the tribe Triticeae could be extended to other genera, both plant and animal. We chose species from the following genera: Anemone, Nicotiana, Pinus and Populus for plants, and Ensis, Mytilus, and Pollicipes from the animal kingdom, for further investigation. Table S1 provides a list of the number of accessions per species used in this study for a total of 640 accessions. In most genera we were able to identify potential Long and Short sequences as seen in the Triticeae.

3.1 Identification of Potential Long and Short Unit Classes within Genera

Nicotiana (35 accessions from 20 species): N. tabacum and N. tomentosiformis [25] were first studied as they had the most accessions available. The sequences of the Long are different in length and pattern of the NTS from the Short sequences (Figures 1A-1B) with both potential classes found in each species. An alignment of accessions from three species within subgenus Tabacum (otophora, tabacum, tomentosiformis) with accessions from subgenus Petunioides (species sylvestris) show that potential Short and Long unit classes are shared between species (Figure 1C) Specifically the accessions from N. sylvestris align with the Short sequence of N. tabacum into one potential unit class (Figure 1D), that is different from the Short sequences of N. tomentosiformis. When the three accessions from N. otophora are included, they align with the Long sequences of N. tabacum and N. tomentosiformis possibly into one potential unit class (Figure 1E).

In several cases we were able to show nearly 100% identity between sequences from different species that had few accessions, e.g. N. alata AJ849919 with N. bonariensis AJ849920 (Figure 1F), N. nesophila AJ849928 with N. stocktonii AJ849929 (Figure 1G), N. noctiflora AJ849921 with N. petunioides AJ849922 (Figure1H), and N. palmeri AJ849932 with N. obtusifolia AJ849933 (Figure 1I). All 8 accessions are from Nicotiana subgenus Petunioides which may help to explain their conservation. (Figure 1F-1I). A very interesting observation from Nicotiana was that of the 15 species sampled only once, none aligned with the Long or Short sequences originally identified in N. tabacum and N. tomentosiformis suggesting that more diversity remains to be analysed.

These relationships demonstrate that within the genus Nicotiana some potential unit classes are shared between species. Further sampling is needed to determine whether this conclusion is true for all potential unit classes in Nicotiana and to determine whether these relationships can be used for phylogenetic analysis.

Pollicipes (113 accessions from 3 species): Initial alignments were performed separately with accessions from P. elegans, P. polymerus and P. pollicipes [35]. There are unequivocal differences between the Long and Short sequences in P. polymerus (Figure 2B). In P. elegans we detected only a Short sequence (Figure 2A); however, this was shorter that the Short sequence found in P. polymerus. And in P. pollicipes three of the 11 sequences (FR831828, FR831829, FR831830) were identified as Short (Figure 2C). Each one of these three is similar to one of the groups found in P. elegans and P. polymerus (Figure 2D). Although sampling in Pollicipes was greater than in Nicotiana, more sampling is still needed to clarify these relationships.

Anemone (203 accessions from 29 species): Accessions from A. acutiloba and A. multifida [28]: The sequences of the Long are different in length and pattern of their NTS from the Short sequences in A. acutiloba (Figure S2A, S2B). Note that the accessions defining Long sequences lack the run of Ts that normally indicated the start of the NTS suggesting that their actual sizes will be greater. In A. multifida, the differences between the Long and Short sequences are less pronounced but still exist within the pattern between the two groups (Figure S2C, S2D). These observations are based only on a few accessions that show similarity.

Ensis (66 accessions from 6 species): Accessions from E. directus, E. ensis, E. macha, E. magnus, E. minor and E. terranovensis [31, 32] were aligned. E. directus (Figure S3A) and E. macha (Figure S3B) had the largest number of accessions and for each species two groups could be identified. i.e. the Long and the Short sequences. In E. magnus (Figure S3C) and E. minor (Figure S3D) there were only 3 accessions each but the Long and the Short sequences were tentatively identified.

Mytilus (60 accessions from 5 species): Within the species M. californicus (Figure S4A), M. edulis (Figure S4B) and M. galloprovincialis (Figure S4C) [33, 34] we see clear differences between the Long and the Short sequences. In the remaining two species a Long sequence could be seen in M. trossulus (Figure S4D) and a Short sequence in M. coruscus (Figure S4E). Alignment of accessions with sizes close to 749 bp identified the same potential unit class in all three species (accessions Mytilus trossulus FN561819, Mytilus edulis AJ312085, Mytilus galloprovincialis AJ312078, AJ312079 and AJ312080) (Figure S4F).

Populus (52 accessions from 14 species): In the majority of the 14 species Populus [24] investigated, there are too few sequences available to detect differences. Even with limited sampling we are able to identify a potential unit class in P. euphratica, (Figure S45) and in P. fremontii (Figure S5B). Alignment of and both P. euphratica and P. fremontii (Figure S5C) suggests the sequences are members of the same unit class.

Pinus (111 accessions from 5 species): An alignment of all sequences from 5 species of Asian pine, P. bungeana, P. densata, P. massoniana, P. tabuliformis, P. yunnanensis [26], identified 2 possible unit classes (Figure S6), one containing sequences only from P. bungeana with a size ~510 bp and a second containing only sequences from the other 4 species with a size ~710 bp. This data suggests there may be two Long unit and one Short unit class.

Based on these few examples and the limited data sets available, we can conclude that identification of potential unit classes is possible and that in some cases their distribution spans species within a genus.

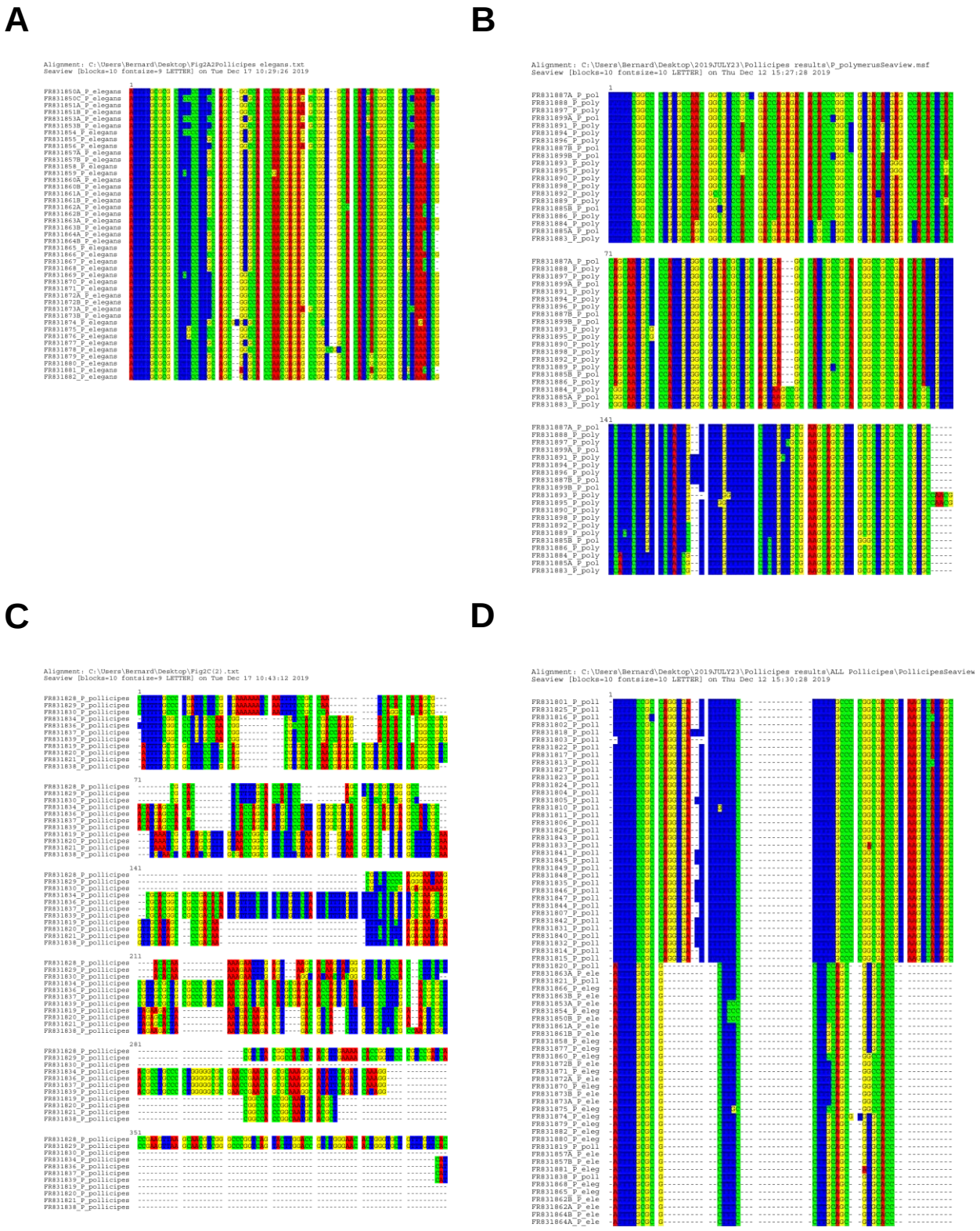

Figure 2 Identification of potential unit classes in Pollicipes. A Identification of potential Short unit class in Pollicipes elegans; B Identification of potential Long and Short unit classes in Pollicipes polymerus; C Identification of potential Long and Short unit classes in Pollicipes pollicipes; D Identification of potential unit class common to Pollicipes species.

4. Discussion

This study builds upon our extensive work in the tribe Triticeae that used the variation in the 5S nrDNA genes, mainly within the NTS, to answer questions relating to population genetics and phylogenetic analysis. We scanned GenBank accessions to create 5S nrDNA databases for a selection of genera including Anemone, Nicotiana, Pinus and Populus for plants, and Ensis, Mytilus and Pollicipes from the animal kingdom. In spite low number of accessions per species, we were able to achieve our first objective in that we captured putative Long and the Short unit classes from all seven genera where the differences between them depend not only on size (bp) but also nucleotide patterns within the NTS. With more sampling additional unit classes may de identified. We recognize that GenBank includes more 5S nrDNA accessions from genera that do not meet out criteria for inclusion and that these may be equally important for future analysis once more sequences are available.

To define a unit class, we need a large number of DNA sequence accessions. But a large number of accessions alone is insufficient. Liu et al. [26] extensively sampled 5S nrDNA sequences from Asian pines (Table S1) the sequences for P. yunnanensis, P. tabuliformis, P. massoniana, and P. bungeana were derived from a single tree while the sequences for P. densata were derived from two trees. Limited plant sampling may have contributed to the high sequence similarity they observed. Biased PCR amplification of different amplicon size classes may also lead to reduced representation of unit classes. In our previous work when two bands were detected both were cloned and several DNA sequences were analysed from each to account for diversity within allopolyploids. A description of unit classes for each species requires an extensive sample of diverse single individual plant/animal accessions representing a variety of geographical locations or from a large population.

Our second goal to investigate relationships between species within the same genus was limited to databases that we could recover from GenBank. Still, we could demonstrate that similar sequences can be found in several species within the genera Nicotiana and Pollicipes. In Nicotiana, the potential Long unit class (N. tabacum, N. tomentosiformis N. otophora) and Short potential class species (N. tabacum, N. tomentosiformis N. sylvestris) each contain accessions from three species. We were also able to identify accessions from four pairs of species with nearly 100%. Similarly, in Pollicipes, P. polymerus and P. pollicipes we have found similar Long and Short sequences -they are not equivalent to and should not be confused with the 7 the groups previously found in Pollicipes by Perina et al. [35] (their Figure 1, data presented as a maximum parsimony tree). Other genera containing accessions common to different species pointing to potential unit classes include Mytilus and Populus and. Calamus accessions have been recovered from 38 species, each species with limited representation [30]. We were able to identify sequences that may represent short unit classes: 5 accessions from C. castaneus (AJ242229 to AJ242233), 2 accessions from C. koordersianus (AJ242206, AJ242207) and 2 accessions from C. longispathus (AJ242208, AJ242209).

Can these unit classes be assigned to haplomes? Species in the. genus Nicotiana have chromosome contents between 2n=9 and 2n=24 [25, 37]. In the genus Anemone variation in chromosomes between 2n=14 and 2n=32 has been observed for 15 of 32 species [28] but only for 2 have genomes been defined (A. baldensis = AABBDD and A. multifida = BBDD) [38]. Chromosome assignments as found in Triticeae have not been attempted in either genus.

Extensive sampling may be used to assign additional sequences to these unit classes and to identify more unit classes. Going forward we can suggest ways to increase data collection by improved sampling. Gel purification of PCR products generated using "universal" primers within the 5S nrRNA genes in the tandem repeats may enrich for a Long or Short potential unit class(es) but cannot distinguish between different unit classes based upon size alone. A more direct approach would be to use nested primers that are within regions with higher sequence divergence thus selectively amplifying Long or Short or even two different Short (or Long) unit classes from each other as has been done for some species [39]. However, there is still a role for random selection of clones to discover new unit classes. We must emphasize that a large number of DNA accessions per plant sampled from a large number of plant accessions collected over the entire range of the species must be analyzed to get an accurate distribution of Long and Short unit classes.

5. Conclusion

The approach that we pioneered in the Triticeae using the 5S nrDNA NTS sequences grouped into potential unit classes or orthologous sequences to test hypotheses related to variation in populations and phylogeny was applied to various plant and animal species outside of the Triticeae. With only a limited number of data sets and accessions we were able to demonstrate the presence of Long and Short potential unit classes in most genera. In two cases, sequences in a potential unit class were found in different species within the same genus and in several cases similar, individual sequences were identified in different species. With more sampling more unit classes may be detected. And with more sampling guided by specific hypotheses our approach may prove useful in a number of genera, plant and animal.

List of Abbreviations

1. Allopolyploidy: A chromosome complement derived from the fusion of two or more chromosome sets of different species.
2. Autopolyploidy: A chromosome complement derived from the fusion of two or more chromosome sets of the same species.
3. BLAST: Basic Local Alignment Search Tool.
4. CLUSTALW2: Cluster Analysis of the Pairwise Alignments.
5. Haplome: A term coined by Löve (Baum et al. 2001) that refers to one of the chromosomes ("haploid genome"). A diploid would have two haplomes. In the Triticeae, the terms S1, S2, H1, H2, Y1, Y2 refer to haplomes initially found in specific species. Typically, each haplome contains two 5S nrDNA unit classes.
6. Homeologous: Homoeologous chromosomes are derived from a common ancestor, for example by duplication, but have diverged such that they rarely pair during meiosis.
7. 5S nrDNA NTS: 5S nuclear ribosomal DNA, non-transcribed spacer.

Author Contributions

Both authors contributed equally to this publication.

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Competing Interests

The authors have no competing interests, or other interests, that might be perceived to influence the interpretation of the article. All sequence data is available through GenBank™ using the accession numbers listed in Table S1.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1: GenBank accession numbers with data used in the present work§.
2. Figure S1: Phylogenetic analysis of the aligned Douglasdeweya 5S DNA sequences, demonstrating orthology status of three sets of sequences, i.e., unit classes—the long P1, long S1 and the short S1. a) NJ tree. b)
3. Figure S2: Identification of potential unit classes in Anemone.
4. Figure S3: Identification of potential unit classes in Ensis.
5. Figure S4: Identification of potential unit classes in Mytilus.
6. Figure S5: Identification of potential unit classes in Populus.
7. Figure S6: Identification of potential unit classes in Pinus: P. bungeana, P. densata, P. massoniana, P. tabuliformis, P. yunnanensis.

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