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Classification of Non-Animals and Invertebrates Based on Amino Acid Composition of Complete Mitochondrial Genomes

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Abstract

Amino acid compositions were predicted from data sets of 47 non-animal and 58 invertebrate animal complete mitochondrial genomes, which were chosen alphabetically based on scientific names without considering biological characteristics. Using Ward's clustering method with amino acid composition or nucleotide content as traits, non-animals were classified into Plantae, Chromalveolata, and Fungi, and invertebrates were classified into Animalia and primitive groups, Amoebozoa, Excavata, Protista, and Choanozoa. A combined sample set of primitive eukaryotes was also examined by cluster analysis using amino acid composition and nucleotide content. Some Amoebozoa comprised a single cluster, whereas other Amoebozoa were grouped with other organisms (Excavata, Prosista, Chromaleolata, Fungi and Plantae), indicating their close relationships. Choanozoa (choanoflagellates; *Monosiga brevicollis*), considered the closest living relatives of animals, were found to be instead closely related to Fungi (*Smittium culisetae*, *Pleurotus ostreatus*, and *Epidermophyton floccosum*) and Excavata (*Malawimonas jakobiformis*). Our results demonstrate that amino acid composition and nucleotide content are useful indices for characterizing non-animal and invertebrate complete mitochondrial genomes.

Keywords: Ward's clustering analysis, evolution, phylogenetic tree, complete mitochondrial genome, amino acid composition, nucleotide content, plant, animal

1. Introduction

Methodology for analyzing nucleotide gene sequences was first developed in 1975 (Sanger & Coulson, 1975; Maxam & Gilbert, 1977). A comprehensive analysis of *Haemophilus influenzae* was carried out in 1995 (Fleischmann et al., 1995), with a draft of the complete human genome obtained in 2001 (Lander et al., 2001; Venter et al., 2001). Because nucleotide mutations are associated with biological evolution, nucleotide and amino acid sequence data have been used to construct an enormous number of phylogenetic trees (Dayhoff, Park, & McLaughlin, 1977; Sogin, Elwood, & Gunderson, 1986; Doolittle & Brown, 1994; Maizels & Weiner, 1994; DePouplana, Turner, Steer, & Schimmel, 1998; Woese & Fox, 1977; Weisburg, Brns, Pelletier, & Lane, 1991) that have helped us to understand biological evolution. Because nucleotide and amino acid substitution rates differ among genes, however, universal phylogenetic trees accurately modeling true phylogenies cannot be reconstructed based on current knowledge levels. For instance, different analytical methods, such as Ward's clustering (Ward, 1963) and neighbor-joining (Saitou & Nei, 1987), have yielded different phylogenetic trees using the same data set, with analysis of different traits yielding different results (Sorimachi & Okayasu, 2013). Although we cannot presently construct phylogenetic trees that are universally representative of actual phylogenies, the scientific validity of phylogenetic trees cannot be denied.

Phylogenetic analyses have primarily utilized nucleotide and amino acid sequence data, with nucleotide content and amino acid composition rarely used to investigate biological phenomena such as evolution. Studies based on nucleotide or amino acid sequences are applicable to genes or regions of relatively small length, but not to entire genomes consisting of huge numbers of nucleotides and many genes. Nevertheless, simple comparison of sequence differences between genes, both within and among species, is of course still useful.

Sueoka (1961) was the first to analyze bacterial cellular amino acid composition. More recently, our laboratory

has independently analyzed cellular amino acid composition of bacteria, archaea, and eukaryotes (Sorimachi, 1999). Graphical representations and diagrammatic approaches to the study of complicated biological systems can provide intuitive pictures and useful insights (Chou, 1990; Qi X. Q., Won, & Qi Z. H., 2007). With the aid of certain graphical representations, simple patterns representing complicated organisms can be easily discerned from huge genomic data sets. For example, when radar charts are used to visualize cellular amino acid compositions, their star-shaped patterns are similar among various organisms, with any differences appearing to reflect biological evolution (Sorimachi, 1999). In addition, amino acid compositions deduced from complete genomes resemble those obtained from amino acid analyses of cell lysates (Sorimachi et al., 2001).

Intra-species nucleotide content was first analyzed by Chargaff, who reported that G = C, A = T, and (G + A) = (C + T). This rule has been dubbed Chargaff's first parity rule (Chargaff, 1950), and is understandable based on the double-stranded structure of DNA (Watson & Crick, 1953). This rule is also applicable to each single strand of nuclear DNA from individual species, a case that has been termed Chargaff's second parity rule (Runder, Karkas, & Chargaff, 1968). Because these rules are based on values normalized to 1 (i.e., G + C + A + T = 1), nucleotide contents are expressed by their ratios. The second parity rule is more difficult to understand, because it is difficult to imagine how G and C or T and A pairs are formed in a single DNA strand. This puzzle has recently been solved mathematically using similarity of the forward and reverse strands and homogeneity of the DNA strand over genome structure (Sorimachi, 2009). Although Chargaff's parity rules were originally formulated as intra-species phenomena, they can be expanded to encompass inter-species relationships using data from a large number of complete genomes (Mitchell & Brigde, 2006). These results indicate that nucleotide content, similar to amino acid composition (Sorimachi & Okayasu, 2003), can be used to characterize whole genomes (Sorimachi & Okayasu, 2004a).

We have recently demonstrated the existence of natural selection in vertebrate evolution using phylogenetic trees derived from amino acid composition or nucleotide content of complete mitochondrial genomes (Sorimachi & Okayasu, 2013). Vertebrate mitochondrial DNA contains 13 genes, whereas gene number varies in plant and invertebrate mitochondrial DNA. In the present study, we investigated evolution in other eukaryotes using phylogenetic trees constructed from amino acid composition or nucleotide content data.

2. Materials and Methods

Mitochondrial genome data were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/sites). In an earlier study, organisms were chosen according to the alphabetical order of their scientific names without considering their characteristics (Sorimachi & Okayasu, 2008). Nucleotide contents of complete mitochondrial genomes were calculated from their complete corresponding single-strand DNA (Sorimachi & Okayasu, 2008) and normalized to 1 (G + C + T + A = 1). Predicted amino acid compositions were estimated for mitochondrial genome coding regions. Ratios of individual amino acids to total amino acids were calculated as percentages of total amino acids, and the presentation order of each amino acid on radar charts was determined based on their HPLC elution orders (Sorimachi, 1999). In our previous studies (Sorimachi & Okayasu, 2013; Sorimachi, Okayasu, Ohhira, Masawa, & Fukasawa, 2013), phylogenetic trees obtained from Ward's clustering method (Ward, 1963) using amino acid compositions or nucleotide contents predicted from complete vertebrate mitochondrial genomes slightly differed from those obtained from neibor-joining method (Saitou & Nei, 1987) using 16S rRNA sequences. However, their basic results were consistent between two different methods. Therefore, Ward's clustering method was carried out in the present study. Classifications based on Ward's clustering method (Ward, 1963) were conducted using multivariate software developed by ESMI (Tokyo, Japan).

3. Results

3.1 Amino Acid Compositions Encoded in Mitochondrial DNA

In a previous study of vertebrate mitochondrial DNA, encoded amino acid compositions were similar among species. Contents (percentages) of some specific amino acids differed significantly among species, however, allowing classification of vertebrates into terrestrial and aquatic species (Sorimachi & Okayasu, 2013). To expand the scope of our investigation using amino acid compositions, we examined a wider range of eukaryote species, i.e., liverworts (*Marchantia polymorpha*), fungi (*Monoblepharella* and *Epidermophyton floccosum*), magnoliopsida (*Arabidopsis thaliana*), algae (*Cyanidioschyzon merolae*), and protists (*Phytophthora sojae*) (Figure 1). As shown in Figure 1, amino acid compositions differed among the various species examined. In every species, both Leu and Ile contents were high compared with other amino acids. Leu content was higher than that of Ile in some species, with the opposite true in other cases. In vertebrate mitochondrial DNA, Leu contents are usually higher than those of both Ile and Phe, giving rise in the case of Monoblepharella to a

"pennon shape" in the radar plot (Sorimachi & Okayasu, 2013). In addition, Asp and Ser contents were high; these high Asp and Lys contents, coupled with low Glu levels, were the cause of a characteristic "V-shape" pattern observed in representations of amino acid composition. These results indicate that non-animal mitochondrial DNA can be classified based on amino acid composition.

3.2 Cluster Analysis of Non-Animal Mitochondria

As new knowledge accumulated after Linnaeus formulated his well-known historical classification of organisms, five-kingdom (Whittaker, 1969), three-domain (Woese, Kandler, & Wheelis, 1990), and six-kingdom (Cavalier-Smith, 1998) theories were subsequently proposed. In the present study, we have primarily followed the six-kingdom classification of Cavalier-Smith (1998). Cluster analysis of non-animal mitochondria was carried out based on Ward's method using contents of 20 amino acids as traits. As shown in Figure 2, two major clusters were obtained. The first major cluster in the tree consists of three sub-clusters: (1) Chromalveolata (*Phytophthora sojae, Phytophthora ramorum*, *Saprolegnia ferax*, *Phytophthora infestans*, and *Rhodomonas salina*); (2) Fungi (*Smittium culisetae*, *Pleurotus ostreatus*, and *Epidermophyton floccosum*), and Plantae (*Cyanidioschyzon merolae* and *Chondrus crispus*); (3) Fungi (*Podospora anserina*, *Gibberella zeae*, and *Moniliophthora perniciosa*) and Chromalveolata (*Rhodomonas salina* and *Cafeteria roenbergensis*).

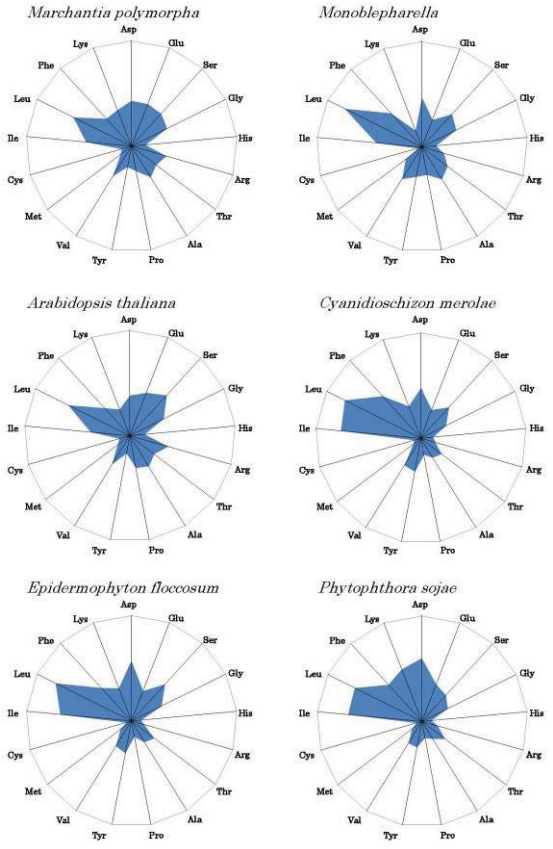

Figure 1. Radar charts of amino acid composition predicted from complete mitochondrial genomes. Values represent percentages of total amino acids

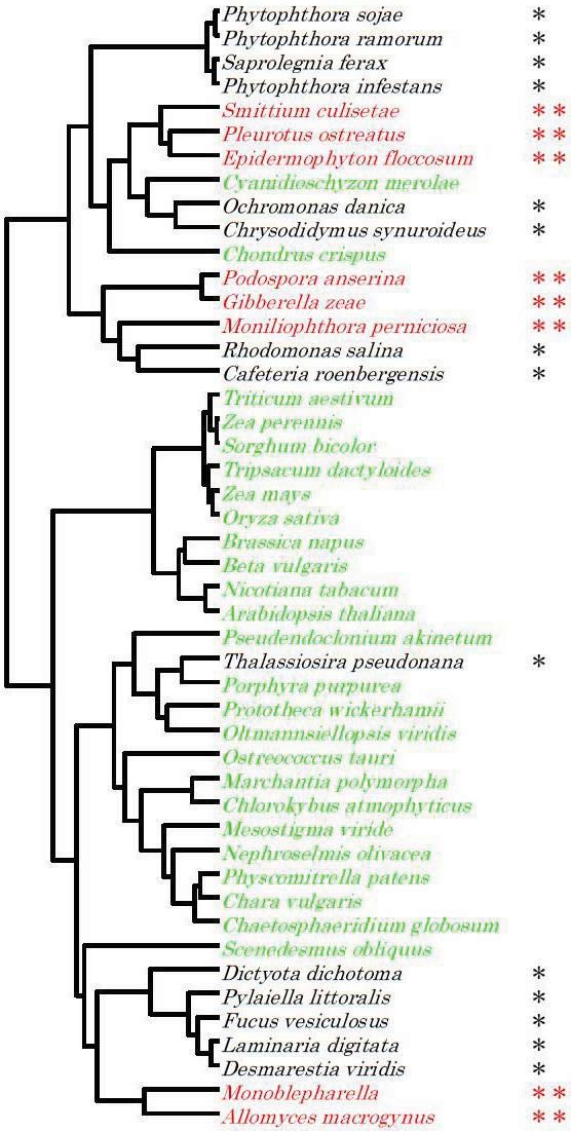

Figure 2. Phylogenetic tree based on Ward's clustering method using amino acid composition. Black (*), red (**), and green (null) characters represent Chromalveolata, Fungi, and Plantae, respectively

The second major cluster in Figure 2 consists of three sub-clusters. One sub-cluster comprises land plants (angiosperms or Magnoliophyta), which are classified into monocots or Liliopsida (*Triticum aestivum*, *Zea perennis*, *Sorghum bicolor*, *Tripsacum dactyloides*, *Zea mays*, and *Oryza sativa*) and into Magnoliopsida (*Brassica napus*, *Beta vulgaris*, *Nicotiana tabacum*, and *Arabidopsis thaliana*). Members of Magnoliopsida are completely separated from Liliopsida. The second sub-cluster consists of Plantae (*Pseudendoclonium akinetum*, *Thalassiosira pseudonana*, *Porphyra purpurea*, *Oltmannsiellopsis viridis*, *Ostreococcus tauri*, *Chlorokybus atmophyticus*, *Mesostigma viride*, *Nephroselmis olivacea*, *Chara vulgaris*, *Chaetosphaeridium globosum*, *Marchantia polymorpha*, and *Physcomitrella patens*). The third sub-cluster contains representatives of Plantae (*Scenedesmus obliquus*), Chromalveolata (*Dictyota dichotoma*, *Pylaiella littoralis*, *Fucus vesiculosus*, *Laminaria digitata*, and *Desmarestia viridis*), and Fungi (*Monoblepharella* and *Allomyces macrogynus*) – all of which are aquatic organisms except for moss.

3.3 Comparison of Amino Acid Compositions in Major Clusters

The sub-cluster in Figure 2 consisting of Protista (*Phytophthoraa sojae*) and Chromalveolata (*Phytophthoraramorum*, *Saprolegnia ferax*, and *Phytophthora infestans*) is characterized by higher Ile content

relative to Leu content, with radar plots displaying a "mountain-shape" because of high Phe, Lys, and Asp content and lower Glu, Ser, and Gly content (Figure 3). The sub-cluster comprising Fungi (*Smittium culisetae*, *Pleurotus ostreatus*, and *Epidermophyton floccosum*), Plantae (*Cyanidioschyzon merolae* and *Chondrus crispus*), and Chromalveolata (*Ochromonas danica* and *Chrysodidymus synuroideus*) is characterized by higher Leu content than Ile content and by the formation of a "V-shape" reflecting Asp, Glu, and Ser content. In the third sub-cluster, Fungi (*Podospora anserina*, *Gibberella zeae*, and *Moniliophthora perniciosa*) have lower Phe and Lys content than do Chromalveolata (*Rhodomonas salina* and *Cafeteria roenbergensis*).

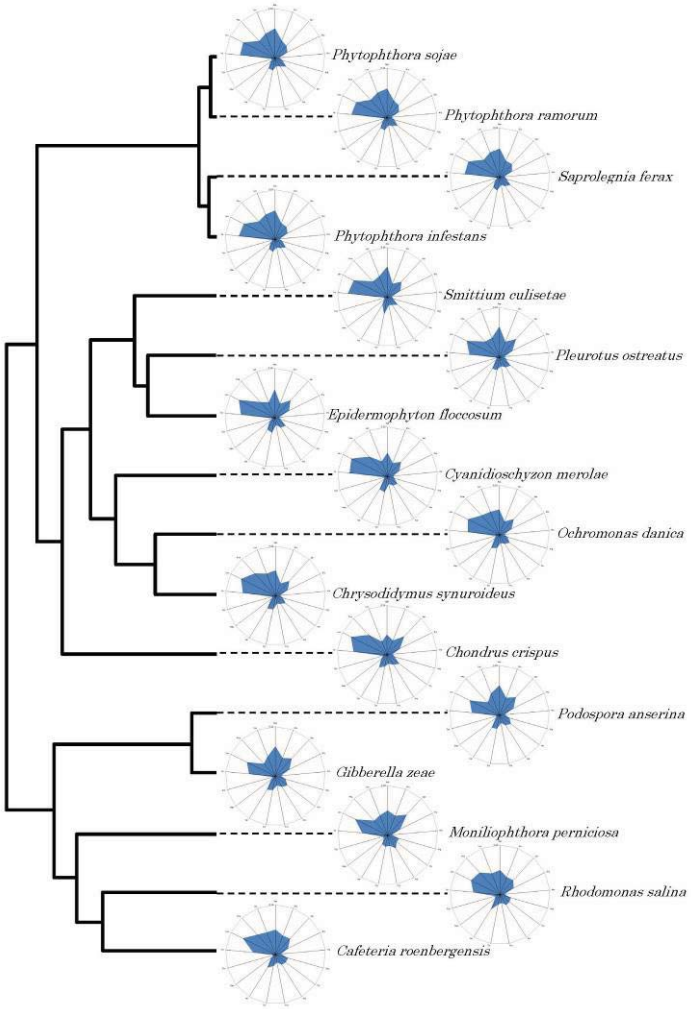

Figure 3. The first major cluster from Figure 2, a phylogenetic tree based on Ward's clustering using amino acid composition

The other major cluster of Figure 2 is made up of two sub-clusters. In the first sub-cluster, consisting of land plants divided into Liliopsida (*Triticum aestivum*, *Zea perennis*, *Sorghum bicolor*, *Tripsacum dactyloides*, *Zea mays*, and *Oryza sativa*) and Magnoliopsida (*Brassica napus*, *Beta vulgaris*, *Nicotiana tabacum*, and *Arabidopsis thaliana*) (Figure 4), Leu content is higher than Ile content, and Phe content is higher than Lys content.

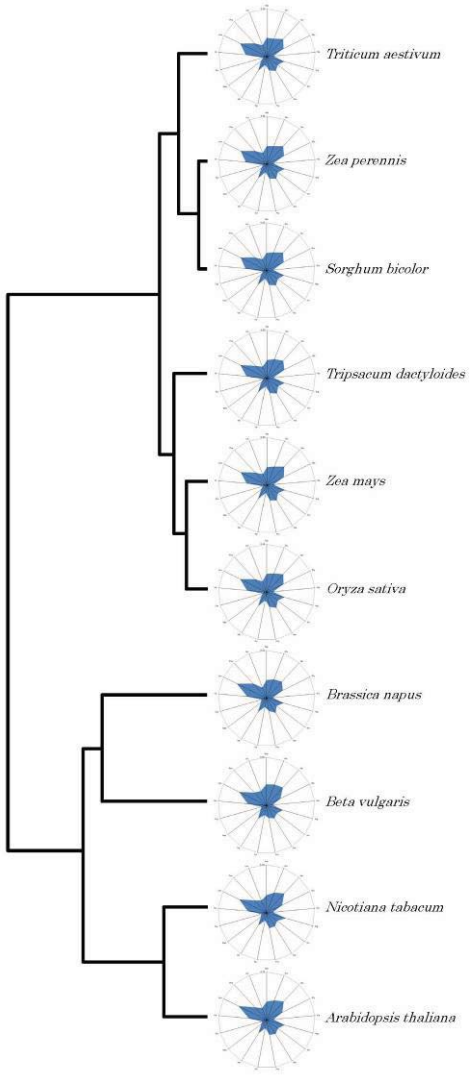

Figure 4. The second major cluster from Figure 2, a phylogenetic tree based on Ward's clustering using amino acid composition

The other sub-cluster, which includes Plantae (*Pseudendoclonium akinetum*, *Porphyra purpurea*, *Prototheca wickerhamii*, *Oltmannsiellopsis viridis*, *Ostreococcus tauri*, *Marchantia polymorpha*, *Chlorokybus atmophyticus*, *Mesostigma viride*, *Nephroselmis olivacea*, *Physcomitrella patens*, *Chara vulgaris*, and *Chaetosphaeridium globosum*) and a representative of Chromalveolata (*Thalassiosira pseudonana*), is characterized by higher Leu content than Ile content (Figure 5). In Fungi (*Monoblepharella* and *Allomyces macrogynus*), also included in this sub-cluster, Glu content is lower than that of both Asp and Ser, giving rise to a "V-shape" relationship among Asp, Glu, and Ser contents on the plot. Conversely, the "V-shape" is absent, with a weak convex shape noted in some cases, in Plantae and Chromalveolata of this sub-cluster *(Dictyota dichotoma*, *Pylaiella littoralis*, *Fucus vesiculosus*, *Laminaria digitata*, and *Desmarestia viridis*).

3.4 Cluster Analysis Based on Nucleotide Content

Using nucleotide content calculated from complete mitochondrial genomes, Ward's clustering method yielded results similar to those based on amino acid composition (Figure 6). Three major clusters were obtained: two clusters consisting of Chromalveolata, Fungi, and Plantae, and a clearly separated cluster comprising land plants and Fungi. This is consistent with results obtained using amino acid composition (Figure 2).

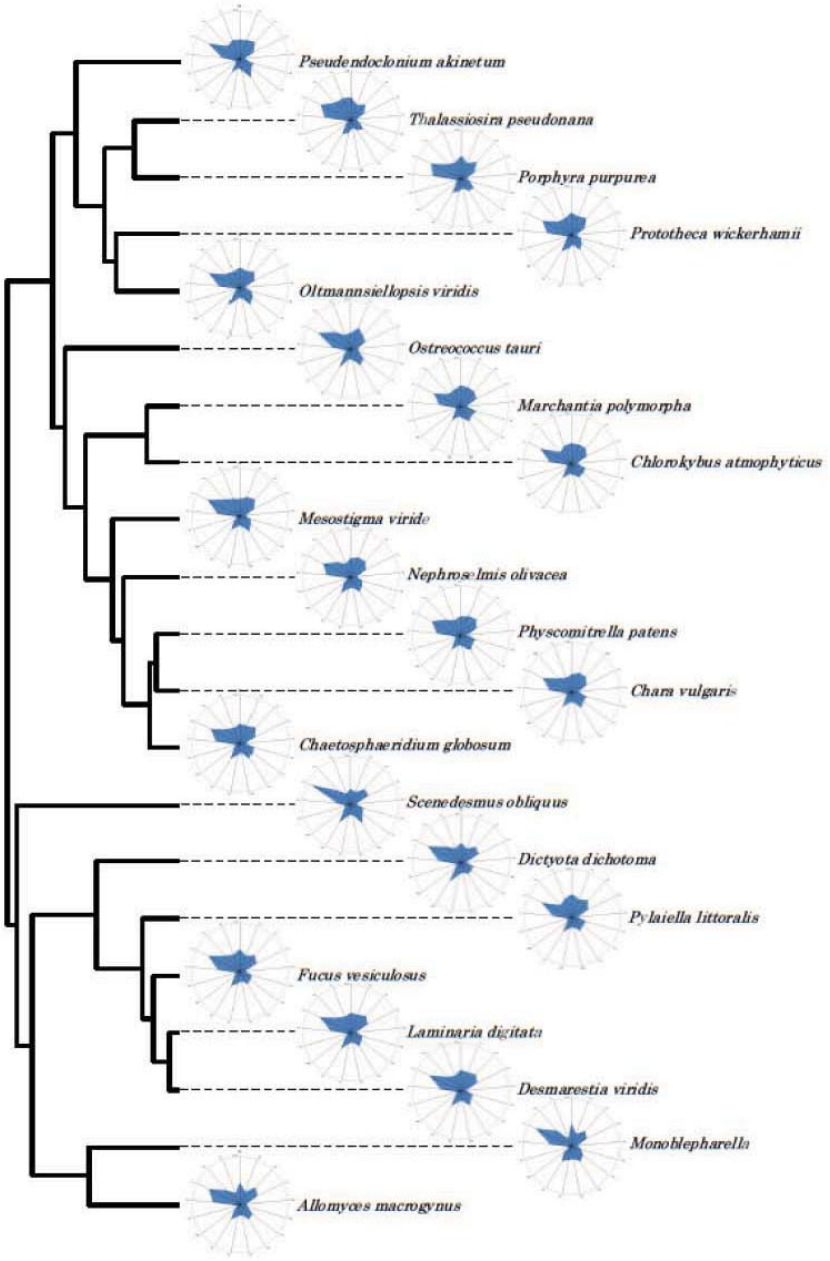

Figure 5. The third major cluster from Figure 2, a phylogenetic tree based on Ward's clustering using amino acid composition

3.5 Cluster Analysis Based on Nucleotide Content

Using nucleotide content calculated from complete mitochondrial genomes, Ward's clustering method yielded results similar to those based on amino acid composition (Figure 6). Three major clusters were obtained: two clusters consisting of Chromalveolata, Fungi, and Plantae, and a clearly separated cluster comprising land plants and Fungi. This is consistent with results obtained using amino acid composition (Figure 2).

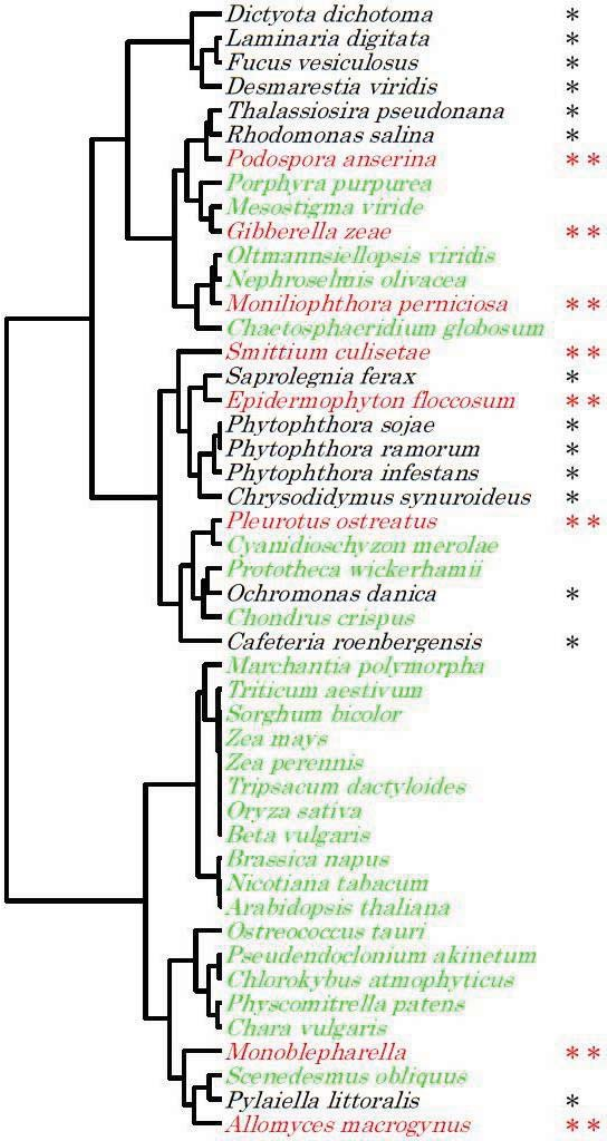

Figure 6. Phylogenetic tree based on Ward's clustering using nucleotide content. Black (*), red (**), and green (null) characters indicate Chromalveolata, Fungi, and Plantae, respectively

3.6 Amino Acid Compositions of Invertebrates

In our previous studies (Sorimachi & Okayasu, 2008), 58 invertebrates were chosen according to the alphabetical order of their scientific names without considering their biological characteristics. Amino acid compositions of seven species are represented in Figure 7. As shown in this figure, Protista (*Tetrahymena pyriformis*), Choanozoa (*Monosiga brevicollis*), Excavata (*Malawimonas jakobiformis* and *Reclinomonas americana*), and Amoebozoa (*Physarum polycephalum*, *Polysphondylium pallidum*, and *Dictyostelium discoideum*) are characterized by comparatively high Leu and Ile content. Ile content is significantly higher than Leu content in *Malawimonas jakobiformis* and *Physarum polycephalum*. Asp content is much higher than both Lys and Glu content, forming a "steep mountain-shape" in *Tetrahymena pyriformis*, Monosiga *brevicollis*, and *Malawimonasjakobiformis* and a "gentle slope mountain-shape" in *Physarum polycephalum*, *Reclinomonas americana*, and *Polysphondylium pallidum*.

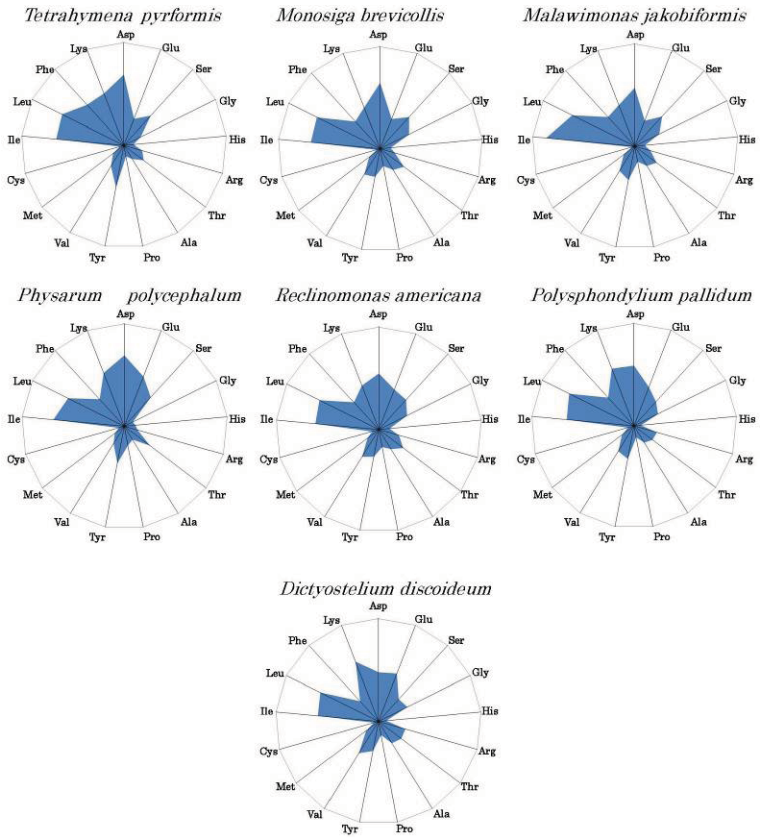

Figure 7. Radar charts of amino acid composition predicted from complete mitochondrial genomes. Values correspond to percentages of total amino acids

3.7 Cluster Analysis of Invertebrate Mitochondria

In the present study, the above seven species plus the remaining 51 invertebrates were categorized based on amino acid composition using Ward's clustering method (Figure 8 and Supplemental Figure 1). The set of 58 invertebrates was classified into two groups: kingdom Animalia, divided into clusters I-IV, and a group consisting of Amoebozoa (*Dictyostelium citrinum*, *Dictyostelium discoideum*, *Polysphondylium pallidum*, and *Physarum polycephalum*), Excavata (*Reclinomonas americana* and *Malawimonas jakobiformis*), Choanozoa (*Monosiga brevicollis*), and Protista (*Tetrahymena pyriformis*).

Within Animalia, Cluster I consists of Platyhelminthes (*Paragonimus westermani*, *Schistosoma mansoni*, *Echinococcus granulosus*), Nematoda (*Thaumamermis cosgrovei*, *Strelkovimermis spiculatus*, *Romanomermis culicivorax*, and *Caenorhabditis elegans*), and Arthropoda (*Varroa destructor*, *Bombyx mori*, and *Adoxophyes honmai*). Cluster II comprises Placozoa (*Trichoplax adhaerens*), Cnidaria (*Porites porites*, *Pavona clavus*, *Briareum asbestinum*), Porifera (*Geodia neptuni*), Mollusca (*Venerupis philippinarum*, *Lampsilis ornata*, and *Haliotis rubra*), Arthropoda (*Tigriopus japonicus*, *Tigriopus californicus*, *Lepeophtheirus salmonis*, *Vargula hilgendorfii*, *Ligia oceanica*, and *Daphnia pulex*) and Chordata (*Branchiostoma belcheri*). Cluster III is made up of Protozoa (*Plasmodium vivax)*, Mollusca (*Sepioteuthis lessoniana*, *Octopus vulgaris*, *Todarodes pacificus*, and *Dosidicus gigas*) and Arthropoda (*Tricholepidion gertschi*, *Reticulitermes hageni*, *Reticulitermes flavipes*, *Hutchinsoniella macracantha*, *Pagurus longicarpus*, *Eriocheir sinensis*, *Drosophila simulans*, *Drosophila melanogaster*, *Bactrocera oleae*, *Bactrocera dorsalis*, and *Anopheles gambiae*). Cluster IV includes Xenoturbellida (*Xenoturbella bocki*), Hemichordata (*Saccoglossus kowalevskii* and *Balanoglossus carnosus*), and Echinodermata (*Luidia quinalia*, *Asterias amurensis*, *Acanthaster planci*, and *Acanthaster brevispinus*).

Members of kingdom Animalia were completely separated from other kingdoms based on Ward's clustering (Figure 8 and Supplemental Figure 1), and amino acid compositions of Animalia differed accordingly from those of other groups consisting of Amoebozoa, Excavata, Choanozoa and Prosista. The latter group is characterized

by Ile content higher than or similar to Leu content, and by Asp content higher than that of Lys and Glu, with the exception of *Dictyostelium* (Figure 7). In animal mitochondria, high Leu, Ile, and Phe contents are characteristic. The highest observed amino acid percentages were those for Leu, giving rise to a "pen-point shape" for the representation of the content of these three amino acids. High Ser and Gly contents were also noted, resulting in a "knife-edge shape" in the plots (Supplemental Figure 1). Both characteristic shapes resemble a flying bird.

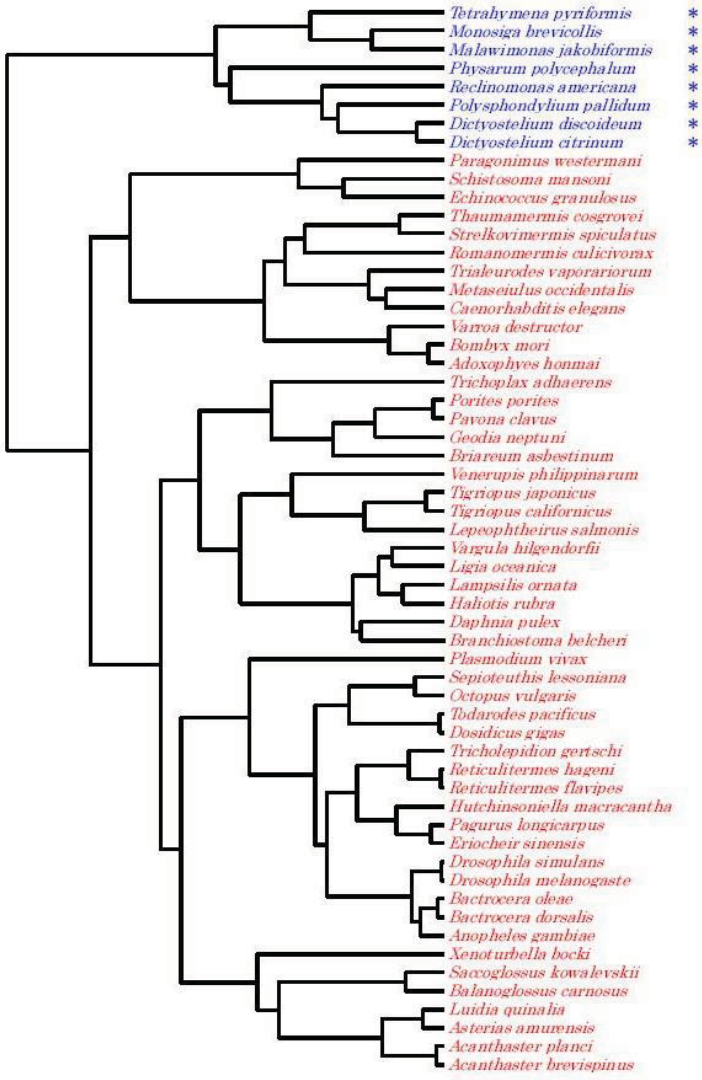

Figure 8. Phylogenetic tree based on Ward's clustering using amino acid composition. Blue characters (*) correspond to Amoebozoa, Excavata, and Choanozoa, and red (null) characters indicate Animalia

3.8 Cluster Analysis of Primitive Organisms

In the trees generated by cluster analysis, organisms belonging to one of the major clusters comprising Chromalveolata, Fungi, and Plantae are clearly separated from other plants (Figures 2 and 6), and the cluster consisting of Protista, Choanozoa, Excavata, and Amoebozoa is also separated from Animalia (Figure 8 and Supplemental Figure 1). Because these organisms seem to be primitive in Plantae or Animalia, Ward's clustering based on amino acid composition was carried out with a combined sample set (Figure 9). Amoebozoa (*Polysphondylium pallidum*, *Dictyostelium discoideum*, and *Dictyostelium citrinum*) formed a single cluster, while other Amoebozoa (*Physarum polycephalum*) and Excavata (*Reclinomonas americana*) fell into a cluster that included Fungi. Protista (*Tetrahymena pyriformis*), Choanozoa (*Monosiga brevicollis*), and Excavata (*Malawimonas jakobiformis*) were placed into another cluster consisting of Plantae (*Cyanidioschyzon merolae*) and Fungi (*Smittium culisetae*, *Pleurotus ostreatus*, and *Epidermophyton floccosum*). When a sample set

including many other plants was analyzed, similar results were obtained (Supplemental Figure 2).

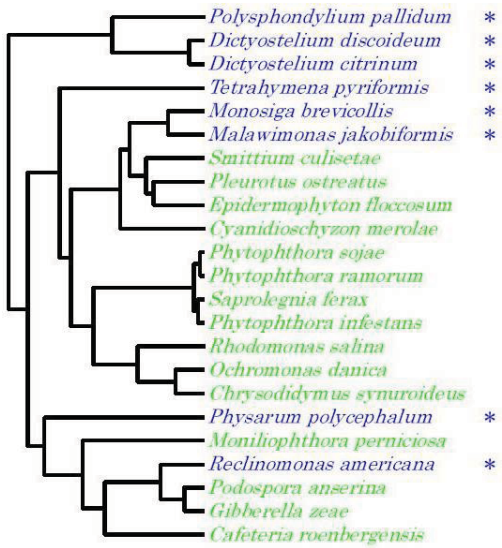

Figure 9. Phylogenetic tree based on Ward's clustering analysis using amino acid composition. Blue (*) characters designate Amoebozoa, Excavata, and Choanozoa, and green (null) characters correspond to Chromalveolata, Fungi, and Plantae

4. Discussion

In our previous study related to vertebrate evolution, vertebrates were clearly differentiated into two groups – terrestrial and aquatic – based on a data set chosen according to the alphabetical order of species names without considering species characteristics (Sorimachi & Okayasu, 2013). Species selection criteria are important for classification, because sampling affects classification results. We indeed found that when we varied which samples were chosen, differences were observed in the tree topologies recovered from the analyses (unpublished data).

Using amino acid composition as the examined trait in cluster analysis generated a better classification than did nucleotide content (Figures 2 and 6). The number of traits associated with amino acid composition (20) was greater than the number representing nucleotide content (4), resulting in good differentiation among organisms. Although amino acid composition provided better classification results than nucleotide content in the vertebrate evolutionary analysis, a significant separation between terrestrial and aquatic vertebrates was also obtained in the cluster analysis using nucleotide content (Sorimachi & Okayasu, 2013). In general, an increased number of traits reduce the probability of coincidental similarity in cluster analyses, resulting in better species classification. In contrast, an increase in the number of samples increases the probability of coincidental similarity, worsening species classification.

Terrestrial and aquatic vertebrates were completely separated in vertebrate phylogenetic trees based on amino acid composition or nucleotide content, with the exception of hagfish (*Eptatretus burgeri*), which fell into the terrestrial group (Sorimachi & Okayasu, 2013). This anomalous placement seems to be a consequence of its primitive characteristics (Janvier, 2010). Amino acid compositions predicted from complete vertebrate mitochondrial genomes were very similar to one another, although some amino acid contents differed significantly between the two groups and can thus be used to characterize them. Clear separation between terrestrial and aquatic vertebrates was thus obtained (Sorimachi & Okayasu, 2013). In contrast, species such as algae, mosses, and fungi are evolutionarily highly diverged, as can be seen by their large amino acid composition differences (Figures 1, 3-5). This means that evolutionary divergence in Plantae and Fungi may proceed in several different directions, resulting in multiple characteristic changes in amino acid composition. Consequently, the probability of coincidental similarity may increase, such that classification results obtained for Plantae, Chromalveolata, and Fungi were worse than for vertebrates. The latter have diverged in just two directions – terrestrial and aquatic – providing a good separation.

Within kingdom Plantae, members of Angiospermae were completely separated from liverworts (*Marchantiophyta* and *Bryophyta*) among land plants (Embryophyta), while land plants were separated from algae (Figure 2). Land plants and algae have thus evolved independently under natural selection in terrestrial and aquatic spheres, respectively, as observed in vertebrate evolution (Sorimachi & Okayasu, 2013). In addition, Magnoliopsida was completely separated from Liliopsida in Angiospermae, although amino acid compositions were similar between both groups (Figure 4). Although terrestrial and aquatic vertebrates were clearly separated in our previous study (Sorimachi & Okayasu, 2013), amino acid compositions were substantially similar between the two groups and among various vertebrates (Sorimachi & Okayasu, 2013). These clear separations may be due to significant changes in amino acid composition between the two groups or among many organisms. Characteristic differences in amino acid composition are indeed observed between terrestrial and aquatic vertebrates (Sorimachi & Okayasu, 2013). Similarly, bacteria are classified into two groups, "S-type" represented by *Staphylococcus aureus* and "E-type" represented by *Escherichia coli*, based on differences in amino acid compositions (Sorimachi & Okayasu, 2004b; Okayasu & Sorimachi, 2009). This phenomenon has been confirmed by other bioinformatics analyses (Qi X. Q., Won, & Qi Z. H., 2007).

Molecular phylogenetic trees are typically constructed from nucleotide or amino acid sequences of gene(s), such as cytochrome C (Dayhoff, Park, & McLaughlin, 1977), 18S rRNA (Anguinaldo et al., 1997), 16S rRNA (Sorimachi & Okayasu, 2013; Sorimachi et al., 2013; Weiseburg, Brns, Pelletier, & Lane, 1991; Puslednik & Serb, 2008; Poulakakis, Pakaki, Mylonas, & Lymberakis, 2008), 12S rRNA (Puslednik & Serb, 2008; Poulakakis, Pakaki, Mylonas, & Lymberakis, 2008), and tRNA (Maizels & Weiner, 1994; DePouplana, Turner, Steer, & Schimmel, 1998). Vertebrate mitochondrial genomes are of similar size among different species, and comprise 13 coding genes. This size appears to be too long, however, to use the entire nucleotide sequence for phylogenetic construction. In addition, plant mitochondrial genomes vary widely in size ($2.5 \times 10^4 – 7.0 \times 10^5$ bp). Based on these facts, methods based on nucleotide or amino acid sequences do not therefore seem to be applicable for phylogenetic tree construction. However, because the ratios of individual amino acids to total amino acids or nucleotides to total nucleotides are independent of genome size and species differences, these indices, which represent whole genome characteristics, are useful tools for genomic research. Consistent with this expectation, reasonable results based on amino acid composition and nucleotide content were obtained in this investigation and in our previous study (Sorimachi & Okayasu, 2013).

Choanoflagellates (e.g., *Monosiga brevicollis*), which have a unique cellular morphology consisting of a single flagellum surrounded by a "collar" of microvilli, are unicellular aquatic flagellates. This cellular morphology is very similar to that of the collared cells (choanocytes) of sponges. On this basis, colonial choanoflagellates are thought to be the closest living relatives of animals. Recent molecular phylogenetic studies have investigated the relationship between Choanozoa and Metazoa (King et al., 2008), but it is difficult to assert that the evolutionary process leading from a highly differentiated unicellular organism to an ancient multi-cellular organism with organized tissues has been elucidated because of the large difference between two organisms (Lavrov, Forget, Kelly, & Lang, 2005). Based on cluster analysis using amino acid composition in our current study, *Monosiga brevicollis* was found to be closely related to Excavata (*Malawimonas jakobiformis*), Fungi (*Smittium culisetae*, *Pleurotus ostreatus*, and *Epidermophyton floccosum*), and Plantae (*Cyanidioschyzon merolae*) (Figure 9) rather than to animals including sponges (Figure 8). Differing results between our study and those of others are due to differences in algorithms and analyzed traits, as phylogenetic trees are not universally representative (unpublished data).

In the present study, primitive organisms, i.e., water-mold (*Phytophthora sojae*, *Phytophthora ramorum*, *Phytophthora infestans*, and *Saprolegnia ferax*), were separated from both Plantae and Fungi (Figure 2), while other primitive organisms, i.e., Protozoa (Amoebozoa, Choanozoa, and Excavata: *Tetrahymena pyriformis*, *Monosiga brevicollis*, *Malawimonas jakobiformis*, *Physarum polycephalum*, *Reclinomonas americana*, *Polysphondylium pallidum*, *Dictyostelium discoideum*, and *Dictyostelium citrinum*), were separated from Animalia (Figure 8 and Supplemental Figure 1). Using a combined sample set of both primitive organisms, only a subset of Amoebozoa (*Polysphondylium pallidum*, *Dictyostelium discoideum*, and *Dictyostelium citrinum*) clustered together, whereas other Amoebozoa (*Physarum polycephalum*), Excavata (*Malawimonas jakobiformis* and *Reclinomonas americana*) and Prosista (*Tetrahymena pyriformis*) were grouped into the same clusters with different kingdoms (Chromalveolata, Fungi and Plantae) (Figure 9 and Supplemental Figure 2). The two primitive organismal groups could not evidently be separated into clusters independent of one another. These results thus suggest that the primitive organisms examined diverged from a common origin. The hypothesis that these primitive lineages may have a common origin is supported by genomic analyses using nucleotide content relationships (Sorimachi, 2010).

5. Conclusions

The ratios of amino acids to the total amino acids or of nucleotides to total nucleotides predicted from complete mitochondrial genomes consisting of huge number of nucleotides can characterize a whole organism. As these values are independent of species and genome size, these indexes are very useful for genome research, as well as single gene research. Indeed, Ward's clustering method using amino acid compositions or nucleotide contents predicted from complete mitochondrial genomes provided consistent phylogenetic trees.

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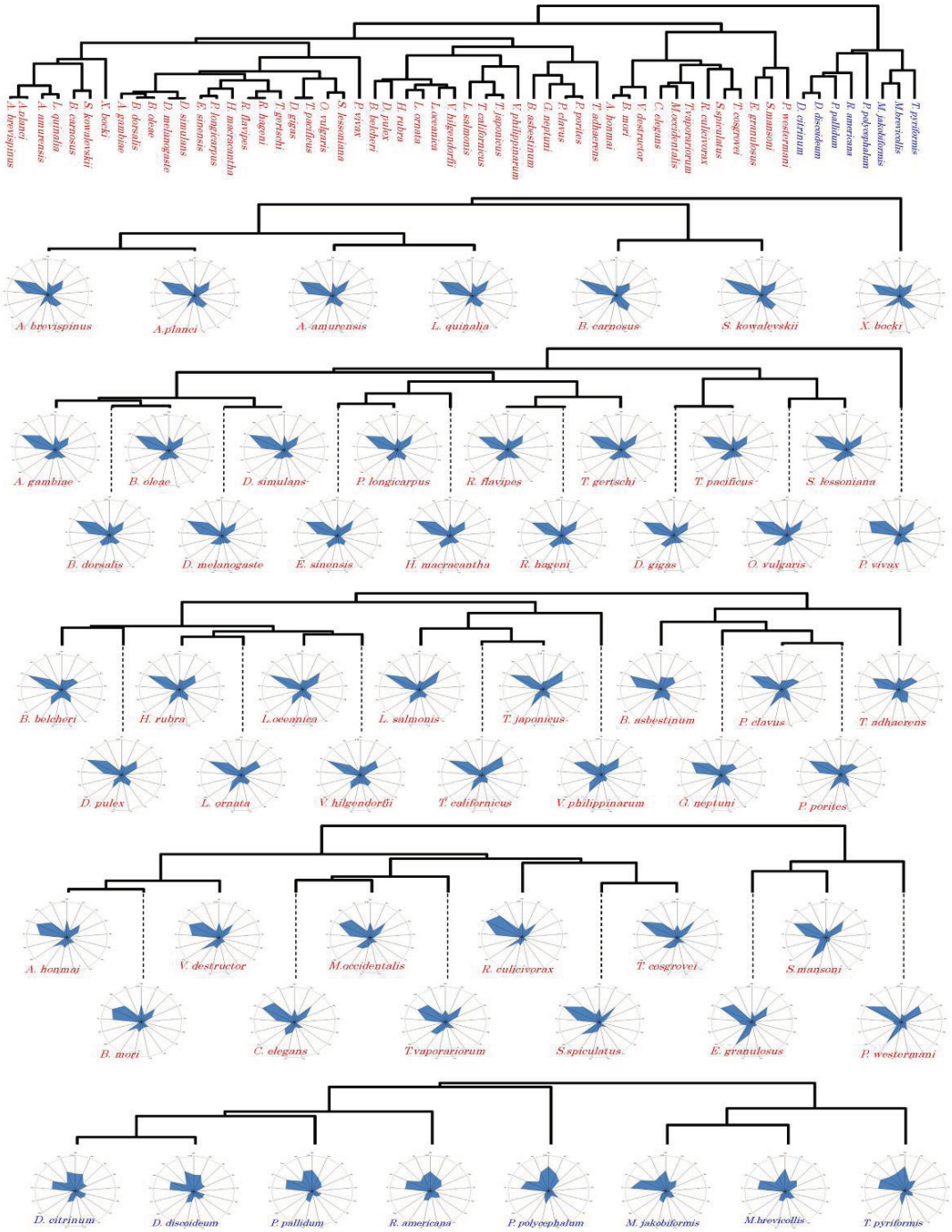
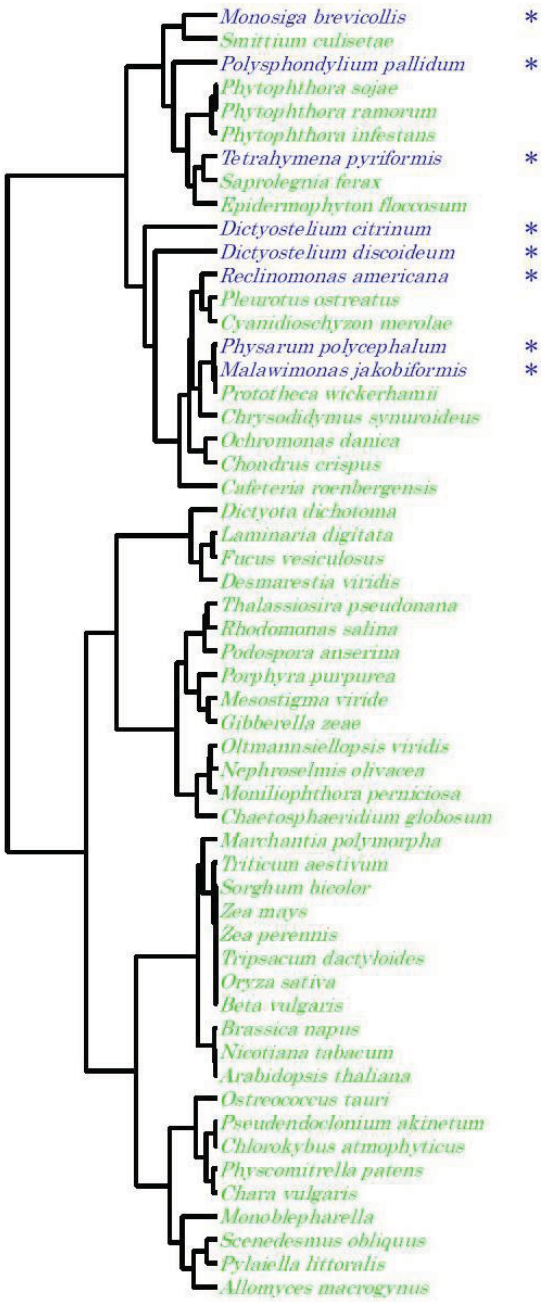
Supplemental Figures



Supplemental Figure 1. Phylogenetic tree based on Ward's clustering using amino acid composition, and radar charts of amino acid composition predicted form complete mitochondrial genomes. Blue characters (the right cluster in the highest phylogenetic tree and the lowest phylogenetic tree) correspond to Amoebazoa, Excarvata, and Choanozoa, and red characters (the other clusters and phylogenetic trees) indicate Animalia



Supplemental Figure 2. Phylogenetic tree based on Ward's clustering using amino acid composition. Blue (*) characters correspond to Amoebazoa, Excarvata, and Choanozoa, and green (null) characters indicate Non-Animalia

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Morphological Variation and Species Distribution of *Baccaurea dulcis* (Jack) Müll. Arg. in West Java, Indonesia

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Abstract

Baccaurea dulcis is an underutilized plant, primarily grown for its fruit, distributed and cultivated only in Sumatra, Borneo and western part of Java Island, and its population is under threat. On the other hand, very few studies have been carried out of this species. The objective of this study was to estimate population distribution and ecology of *Baccaurea dulcis* in West Java, characterize plant morphological characters and correlate plant habitat of *B. dulcis* and the plant and fruit. In West Java *B. dulcis* is only distributed in the sub district Taman Sari of Bogor and sub district Cijeruk of Sukabumi. Even though the species in West Java has a restricted distribution, its morphological characters is quite varied, including size and nature of tree; color and texture of the bark; size and shape of leaves and fruits; color of fruit peel and pulp; and the size, shape and color of seeds. The species grow well in the low land (250 m - 610 m above sea level) of tropical region at neutral soil pH of regosol or latosol soil type and smooth rather coarse soil texture at land slope from 0% until 45%. Using 32 variables of trunk, leaf, fruit and seed, all samples could be clustered into 6 groups with the proportion correct 0.903 and with specific fruit characters in each group. There were some significant positive and negative correlations found between habitat and fruit characters and among tree and fruit variables.

Keywords: *Baccaurea dulcis*, underutilized tropical fruit, West Java, characterization, variation, clustering, correlation

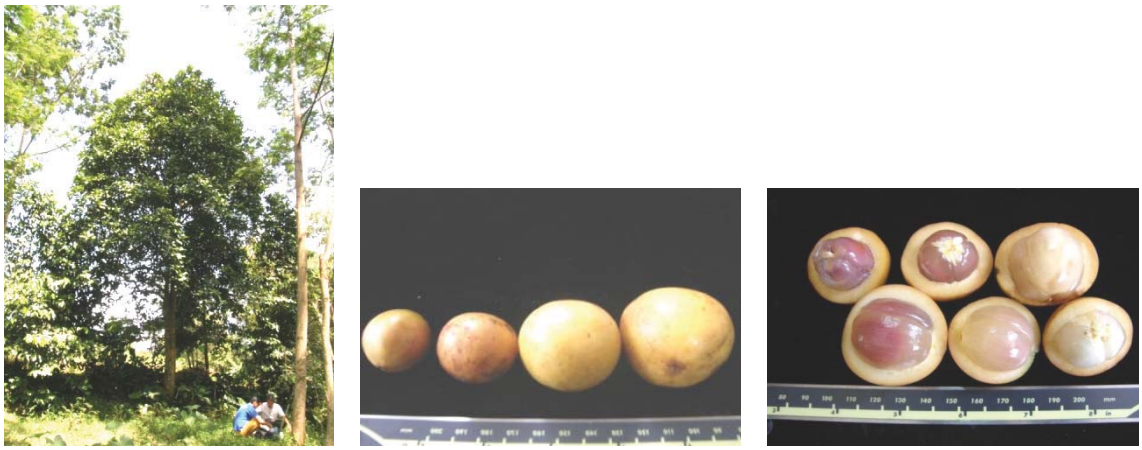
1. Introduction

Baccaurea dulcis (Jack) Müll.Arg. is a dioecious plant species belonging to the family Phyllantaceae (previously under Euphorbiaceae) (Haegens, 2000; Wurdack et al., 2004). Its common names are ketupa (English), cupa, tupa, kapul, menteng negri, menteng besar (Indonesian), tjupa, tupa (Malaysian). This species is distributed and cultivated only in Sumatra and western part of Java Island (Uji, 1992).

This species is primarily grown for its fruit. It is propagated mainly by seeds, but rarely by vegetative means. The tree produces fruits in high quantities and the fruits are usually taste sour or fairly sweet. The fruits can be pickled and used in stew or fermented to make wine (Uji, 1992; Munawaroh, 2001). The leaf of *B. dulcis* is boiled and the resulted decoction is used to treat stomachache during menstruation (Munawaroh, 2001). The tree trunk is used in construction (Heyne, 1987). The fruits of *B. dulcis* are rounded, with a diameter of 3.5 - 4 cm and brownish yellow in color. The edible arils are cream, white or reddish in color. Nutrition of fresh pulp of *B. dulcis* per 100 g were 82.3 g water, 0.4 g protein, 7.5 g saccharosa, 0.2 g fibers, 0.5 g ash, 5 mg vitamin C, 0 g vitamin B1 and B2 (Uji, 1992).

In general, the species is underutilized and used only locally. The species is usually grown in the home-yard and the fruits are usually self-consumed and rarely sold in local markets in West Java. Even though, the species is relatively known by the local people, but until now the distribution in West Java is only in very small areas. There is no information about the cultivation of the plant, reproductive period, growth pattern, pest and diseases and production and harvest of the species (Uji, 1992). Vegetative propagation of the species by shoot tip grafting could be applied, which almost 100 % success rate, whereas the airlayering propagation technique resulted only 27.5 % - 40 % of rooted shoot (Lestari, 2009, 2010). The risk of extinction of a fruit species is higher because there are not many people interested in the fruits and the fruits availability in the market is rare (Subekti et al., 2005). Field trips were conducted in West Java to study the species.

The aims of the study were (1) to find out the distribution of the species *Baccaurea dulcis* in West Java and to characterize the plants and fruits based on the morphology and qualitative parameter such as color and texture, (2) to find out the variation and clustering *B. dulcis* plants and fruits in West Java, and (3) to find out the correlation between the characters of the plants and fruits, and the plant's habitat.



A **B** **C**

Figure 1. A. *Baccaurea dulcis* plant; B. and C. *B. dulcis* fruits

2. Materials and Methods

2.1 Distribution, Characterization and Variation of Plant and Fruit

Field trips, and ecological observations of *B. dulcis* focused on trees, fruits and their habitat and interviews with the farmers were conducted two days a week during the harvest period, starting from February until April 2008 mostly in Bogor, Cianjur, Sukabumi, Tangerang, Depok, Bekasi and Purwakarta of West Java, Indonesia (Figure 2).

Figure 2. Study site in West Java of Indonesia

The distribution of a total of 103 fruiting plants was recorded. The habitat of the plants and the variables of the tree and fruit were documented. The observation of the plant habitat included location of plant growth, altitude, longitude and altitude, air temperature, relative humidity, slope, light intensity, soil pH, soil relative humidity, soil type and soil texture/drainage. The distribution and habitat of the plant was also examined from the herbaria at the Bogor Herbarium (BO). Detail character variables of tree and fruit observed could be seen in Table 1.

As many as 30 fruits randomly were harvested from every plant observed, the minimum and maximum length and width of fruit and seed were measured. The fruits were grouped into 3 and each group of fruits was weighed to calculate the average fruit weight. Using kitchen knife, all of those fruits were peeled and then observed for the easiness to peel, the minimum and maximum amount of the pulp segment and the seed weight. The average of each of those parameters was calculated. The thickness of fruit peel and pulp, and soluble solid content of pulp were measured from 3 samples of fruits randomly. Some qualitative parameters were observed during the field trips and the result of the observation was ranked for the data analysis (Table 2).

Table 1. The Variables observed of trees and fruits of *B. dulcis* in West Java

No	Variables	No	Variables
1	Tree height	17	Fruit peel color
2	Trunk diameter	18	Easiness to peel the fruit
3	Canopy width	19	Thickness of fruit peel
4	Canopy condition (sparse/dense)	20	Peel weight per fruit
5	Lowest branch height	21	Pulp color
6	Bark color	22	The number of fruit segment
7	Bark texture	23	Soluble solid content of pulp
8	Leaf shape	24	Seed shape
9	Leaf length	25	Seed length
10	Leaf width	26	Seed width
11	The Ratio of maximum leaf length/leaf width	27	Ratio of the longest seed length/ seed width
12	Fruit shape	28	Seed weight
13	Fruit length	29	Percentage of peel weight per fruit
14	Fruit width	30	Percentage of pulp weight per fruit
15	The ratio of maximum fruit length/ fruit width	31	Percentage of seed weight per fruit
16	Fruit weight	32	Seed color

Table 2. Qualitative parameters and rank of qualitative number of the observation of *B. dulcis* trees, fruits and seeds in West Java

No	Qualitative parameter	Rank of Qualitative Number and Information
1	Canopy condition	(1) Dense, (2) Medium dense, (3) Sparse
2	Bark color	(1) Light brown/cream, (2) Medium brown, (3) Grey - medium brown, (4) Grey, (5) Browned - grey, (6) Grey, yellowed - medium brown, (7) Grey - dark brown, (8) Dark brown, (9) Browned - black
3	Bark texture	(1) Smooth, (2) Flaky - smooth, (3) Lenticelate, (4) Flaky - lenticelate, (5) Lump - flaky, (6) Flaky, (7) Flaky - fissured, (8) Flaky - rectangular
4	Fruit peel color	(1)Yellow, (2) Light orange/Yellowish orange, (3) Orange/ dark orange, (4) Reddish yellowish - orange
5	Easiness to peel the fruit	(1) Easy, (2) Medium, (3) Difficult
6	Fruit pulp color	(1) White, (2) Transparent - white, (3) Cream, (4) Pink, transparent - cream, (5) Pink, white - transparent, (6) Cream - transparent, (7) Pink - transparent, (8) Pinked/purpled lines - cream/white
7	Seed color	(1) Light brown, (2) Pink - light brown, (3) Brown, (4) Pink - brown, (5) Purplish - brown, (6) Light brown - Pink, (7) Pink

2.2 Measurement Equipments

The equipment used during the observation were GPS Garmin (latitude), Termohygrometer Haar-Synth-Hygro, Germany (air temperature and relative humidity), Soil tester TEW Type 36, Demetra, Japan (soil pH and relative humidity), Clinometer, Suunto PM-5/360, Finland (slope), Light meter, LX-101 A, Lutron, Taiwan (light intensity), Altimeter (altitude). Other equipment used are to measure the tree height (BL 6, Carl Leiss, Berlin, Germany), the diameter of trunk, fruit and seed (diameter tape 20 m x 5 m, Tool No. D-5M, YAMAYO, Japan), canopy width (Tape measure, 50 m), the width of fruit and seed (Digital caliper, 200 mm, Mitutoyo CO., Japan), the fruit and seed weight (Balance, capacity 2 kg), Soluble solid content (Digital refractometer, Palette Series PR 101 α, ATAGO CO., LTD, Japan)

2.3 Statistical Analysis of the Data

As many as 32 tree and fruit variables observed from 103 numbers of *B. dulcis* in West Java were clustered to find out the groups based on the similarity characters using the MINITAB program version 14. The variables chosen were those that are not influenced by the age of the tree observed. Those variables were bark color and texture, maximum and minimum of leaf length and width, ratio of maximum leaf length and width, maximum and minimum of fruit length and width, ratio of maximum fruit length and width, fruit weight, peel color, easiness to peel the fruit, maximum and minimum of thickness of peel, peel weight, pulp color, maximum and minimum number of fruit segment, soluble solid content of pulp, maximum and minimum of seed length and width, ratio maximum seed length and width, seed weight, percentage of peel, pulp and seed weight per fruit and seed color. The qualitative parameters and detailed rank of qualitative number and information of the observation of *B. dulcis* trees, fruits and seeds for clustering are shown in Table 2.

The same variables were also tested by Linier Discriminant Analysis using the MINITAB program version 14 to find distinctive characteristic of each group. The correlations between the tree and fruit variables and its habitat as well as among the trees and fruits variables were tested using the statistical MINITAB program version 14.

3. Results and Discussion

3.1 Distribution, Characterization and Variation of Baccaurea dulcis in West Java

From the study of herbaria, it was known that *Baccaurea dulcis* is distributed in Sumatra, Borneo and Western part of Java. In Sumatra, the species is distributed in Palembang, Lampung, Riau, Payakumbu, Bangka, Siberut and Jambi, whereas in Borneo, the species was spread in Ketapang, Gunung Palung National Park, Sarawak, and West Samarinda. In West Java, the species was found in Batutulis and Kotabatu of Bogor. Most of the herbaria observed were collected by Dutch explorers long time ago, before or in the early to mid 1900's. Therefore, the distribution of the species at present including in Sumatra and Borneo may have changed. Indeed, increasing human population and land-use intensification resulted in the loss habitats and increasing species extinction rates. Several strong climate oscillation and disaster could affect vegetation shape and species distribution (Ounsavi & Sokpon, 2010). Whereas in West Java at the moment, there is no *B. dulcis* plant found in Batutulis and Kotabatu anymore.

From the field trips and observation in West Java, it is known that besides as collection in the botanical garden, the distribution of *B. dulcis* in West Java was only in sub districts Taman Sari of Bogor district and Cijeruk of Sukabumi district. The result showed that the occurrence of the species in West Java is only in very restricted areas. According to people in the local areas, the species used to abundant in the past, including in other villages of Bogor district. However, people usually cut the trees and used the trunk for many purposes such as material for building/house and equipments. On the other hand, the species is rarely planted. The plants usually grew from seeds that drop at the surrounding of the mother plants. The risk of extinction of the plant species is high because there were not many people interested in the fruits. Therefore, conservation and development of species to become more commercialized are needed.

The results of the characterization of trees and fruits of *B. dulcis* and plant habitat can be seen in Table 3. The plants were only found in the home-yard, small garden or botanical garden at an altitude range of 250 m - 610 m above sea level, this means that the species could grow well and be planted in relatively low lying areas. From Table 3, it is also known that the trees grow well in the tropical region at neutral soil pH of regosol or latosol soil type and smooth until rather coarse soil texture at land slope from 0% until 45%. From the study of herbaria, it is also known that the species grew well in gully river bank, in hillside of primary forest, swampy places, riverside and also cultivated at "kampoeng" or remote village. The plants could grow at the location until 1100 m above sea level at sandyloam soil and swampy places.

From the observation and measurement results, the morphological characters of *B. dulcis* were various (Table 3).

Those included nature of tree, color and texture of the bark, size and shape of leaves and fruits, color of fruit peel and pulp and the size, shape and color of seeds. It is clear from the study that there is considerable phenotypic variation in almost every parameter observed and measured. Similar to another study on the variation of fruit of *Irvingia gabonensis*, an indigenous fruit tree of west and central Africa, there were significant variation in fruit, nut and kernel size and weight (Leakey et al., 2000). Differences were also identified in shell weight and brittleness, fruit taste, fibrousity and pulp color (Leakey et al., 2000). Salisbury (1942) mentioned that seed size varies tremendously among plant species and was investigated early as a life-history trait of obvious importance. Variation in seed size and weight of *Desmodium paniculatum* (Leguminosae) was also reported in a population in two locations in North Carolina, USA, which caused by environmental conditions and nutrient supply (Wulff, 1986). The result of other study on population of a single seeded fruit *Ocota tenera* (Lauraceae) from Monteverde, Costa Rica showed that the fruits that vary from 1.4 to 2.4 cm and much variation occurred within individual trees (Wheelwright, 1993). The relative size of fruits produced by different trees remained generally constant over an 11-year period despite slight differences between years in the average size of fruits produced by a given tree (Wheelwright, 1993)

Table 3. Character and variation of *B.dulcis* trees and fruits in West Java and the plant habitat

No	Data Recorded	Measurement/Description
Habitat		
1	Location of the plant	Homeyard, small people's garden, Botanical garden
2	Altitude	320 - 610 m above sea level
3	Longitude and	06º 34'49.7"- 06º 40' 06.9" and
	Latitude	106º 43' 55,9"- 106º 49' 25,0"
4	Air temperature	18 °C - 33 °C
5	Relative humidity	59 % - 100 %
6	Slope	0 % - 45 %
7	Light intensity	341 - 299,000 lux
8	Soil pH	5.8 - 7
9	Soil relative humidity	17 % - 90 %
10	Soil type	Brown regosol and red-brown latosol
11	Soil texture	Smooth-rather coarse
Morphology of Tree and Fruit Measurement/Description		
12	Tree height	4.5 - 21 m
13	Trunk diameter	10.5 cm - 80.5 cm
14	Canopy width	3.2 m - 13.2 m
15	Canopy condition	Sparse - Medium - Dense
16	Lowest branch height	0.55 m - 7.0 m
17	Bark color	Light brown/cream, Medium brown, Grey-medium brown, Grey, Browned-grey, Grey, yellowed-medium brown, Grey-dark brown, Dark brown, Browned-black
18	Bark texture	Smooth, Flaky-smooth, Lenticelate, Flaky-lenticelate, Lump-flaky, Flaky, Flaky-fissured, Flaky-rectangular
19	Leaves shape	Obovate, lanceolate
20	leaf length	7.7 cm - 30 cm
21	Leaf width	3.1 cm - 14.9 cm
22	Ratio maximum leaf length/maximum leaf width	2.01

23	Fruit shape	Rounded, slightly oval, or truncate at one end
24	Fruit length	2.4 cm - 4.6 cm
25	Maximum fruit width	4.8 cm
26	Ratio of fruit length/ fruit width	2.2 cm
27	Length of main fruits stalk	0.65 cm - 10.67 cm
28	Length of branch fruit stalk	2.02 mm - 14.7 mm
29	Fruit weight	7.0 - 38.33 g
30	Fruit peel color	Yellow, Light orange/Yellowish orange, Orange/dark orange, Reddish yellowish-orange
31	Easiness to peel the fruit	Easy-difficult
32	Fruit peel thickness	1.65 mm - 9.72 mm
33	Peel weight per fruit	4.17 gr - 23.67 gr
34	Pulp weight	1.56 mg - 15.26 mg
35	Pulp color	White, Transparent-white, Cream, Pink, transparent-cream, Pink, white-transparent, Cream-transparent, Pink-transparent, Pinked/purpled lines-cream/white
36	Fruit pulp segment	1 - 6
37	Soluble solid content of pulp	11 Brix - 20 Brix
38	Seed shape	Ovate, thin, 1 - 6 curves
39	Seed length	0.75 cm - 2.31 cm
40	Seed width	0.52 - 1.95 cm
41	Seed thickness	1 - 4 mm
42	Ratio of longest seed length/ seed width	1.18
43	Seed weight	0.12 mg - 0.74 mg
44	Percentage of peel weight per fruit	36.64 % - 78.31 %
45	Percentage of pulp weight per fruit	26 % - 63 %
46	Percentage of seed weight per fruit	1.17 % - 2.96 %
47	Seed color	Light brown, Pink-light brown, Brown, Pink-brown, Purplish-brown, Light brown-Pink, Pink
48	Fruit production per tree	5 kg - 200 kg

3.2 Clustering of Baccaurea dulcis Variation in West Java

Using 32 variables of trunk, leaf, fruit and seed, all samples could be clustered into 6 groups with the proportion correct 0.903 (Figure 4, Table 5). As can be seen at Table 4, the characters that belong to group 1 were small and light fruit, light fruit peel, small seed and sweeter fruit taste, whereas those of group 2 were fruit pulp color white/transparent, small seed, low portion of fruit pulp and high portion of fruit peel. Group 3 belongs to the tree and fruit of *B. dulcis* with the characters of big and heavier fruit, high portion of fruit pulp, more heavy seed, more segment of fruit pulp (Table 4). The characters of group 4 were thin and light fruit peel, more segment of fruit pulp, high portion of fruit pulp, low portion of fruit peel, color of fruit pulp reddish/purple. Group 5 were characterized by big and more heavy fruit, thick and more heavy fruit peel, fruit pulp color white/transparent, big seed and high portion of fruit pulp, whereas group 6 were characterized by light fruit, sour taste, small seed, color of fruit pulp reddish or purple (Table 4). Another study on the variation of pomelo (*Citrus grandis*) in Nepal found that from the multivariate analysis of the data produced five discrete groups, which differed significantly in fruit shape and size, pulp, juice, total soluble solids and acid content, seed number, leaf shape and size (Paudyal & Haq, 2008).

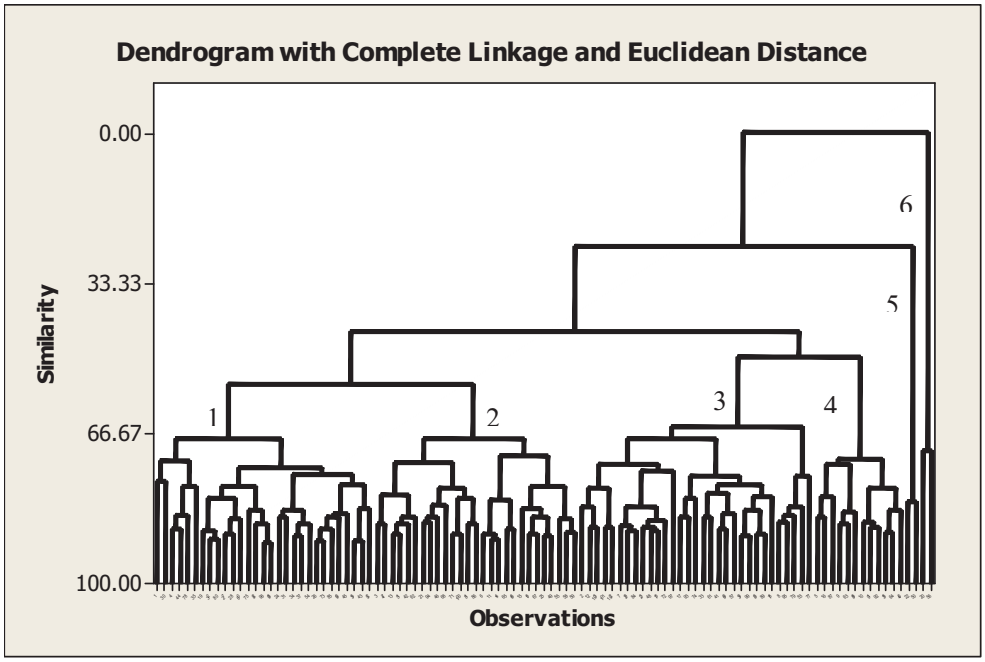


Figure 3. Dendrogram of *B. dulcis* in West Java based on the similarity variables

Table 4. Average value of characteristic component of 6 groups *B. dulcis* in West Java

Characteristic variable	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Population mean
Bark color	2.90	3.09	3.73	1.60	4.50	4.00	3.32
Bark texture	4.79	4.74	5.31	5.60	5.25	5.00	5.01
Leaf length	21.75	23.09	22.78	21.58	23.46	20.23	22.49
Leaf width	10.68	10.68	10.65	9.80	10.90	9.65	10.62
Fruit size	35.94	37.12	39.60	38.26	41.87	36.80	38.01
Fruit weight	18.44	21.18	26.44	22.95	31.96	20.01	23.03
Peel color	2.39	2.82	2.55	2.40	2.75	2.75	2.60
Peel thickness	6.50	6.94	6.27	5.29	7.87	6.39	6.65
Peel percentage	58.25	65.92	54.39	46.81	62.23	59.9	59.26
Pulp percentage	39.84	32.33	43.85	51.45	36.27	38.20	38.97
Minimum pulp segment	1.7	1.7	2.0	2.4	1.8	1.8	1.8
Maximum pulp segment	3.5	3.4	3.7	3.6	3.3	3.3	3.5
Pulp color	3.03	2.96	3.86	3.80	2.75	4.25	3.27
SSC*	16.51	15.55	15.82	15.72	15.69	14.69	15.88
Seed length	16.9	17.0	18.0	18.1	18.6	17.0	17.5
Seed width	12.6	13.0	14.1	13.9	14.1	13.6	13.4

*SCC = Soluble solid content of fruit pulp.

Table 5. Results of test of correctness based on discriminant analysis for summary of classification

	True Group					
Put into Group	1	2	3	4	5	6
1	28	2	2	0	0	0
2	0	23	0	0	0	1
3	0	0	23	0	1	0
4	0	0	1	5	0	0
5	0	0	0	0	11	0
6	1	2	0	0	0	3
Total N	29	27	26	5	12	4
N correct	28	23	23	5	11	3
Proportion	0.966	0.852	0.885	1.000	0.917	0.750

Note: N = 103, N Correct = 93 and Proportion Correct = 0.903.

3.3 Correlations Among Habitat, Plant and Fruit Variables

The result of the statistical data analysis for significant correlation between plant habitat and variables of tree and fruit could be seen in Table 6. There was one significant positive correlation and nine significant negative correlations known. The positive correlation was between slope and fruit pulp color, whereas significant negative correlation were between altitude and pulp weight; light intensity and pulp soluble solid content (SSC); soil pH and number of pulp segment; soil texture and trunk diameter, canopy width, pulp color, pulp weight, peel weight portion; drainage and number of pulp segment (Table 6).

This findings could indicate that higher slope the plant grow, the pulp color of the fruit tend to be pink or purple. On the other hand, higher the altitude of the plant position could correlate with less weight of the fruit pulp; more light intensity could correlate with less pulp SSC. Light intensity could have an effect on the pulp color and pulp SSC, while air temperature could affect the weight of fruit pulp.

The other significant negative correlation indicated that higher soil pH correlates with less pulp segment; more coarse the soil texture correlates with less trunk diameter, less canopy width, more white/transparent pulp color, less pulp weight, less peel portion; higher soil drainage correlates with less number of pulp segment. The soil texture and soil drainage could correlate with the soil fertility condition, which then could affect the size of the plant. Other study on pomelo (*Citrus grandis*) in Nepal found that yield related characters, such as fruit weight had positively correlated with tree size and soil fertility level, but none of these factors were correlated with fruit quality, such as percent of pulp and pulp SSC (Paudyal & Haq, 2008)

Table 6. Significant correlation between plant habitat and variables of *Baccaurea dulcis*

Plant/Habitat	Altitude	Slope	Light intensity	Soil pH	Soil Texture	Drainage
Trunk Diameter	-0.094	0.077	0.073	0.123	-0.199*	-0.098
Canopy width	0.011	0.029	-0.070	-0.081	-0.200*	-0.024
Pulp color	-0.213	0.226*	-0.056	-0.166	-0.205*	-0.137
Minimum number of pulp segment	-0.047	0.070	-0.133	-0.223*	0.180	0.006
Maximum number of pulp segment	0.120	-0.168	0.079	0.110	-0.004	-0.194*
Pulp soluble solid content	0.106	0.135	-0.208*	0.047	0.166	0.086
Pulp weight	-0.246*	0.007	0.058	-0.006	-0.261*	-0.154
Peel weight portion	-0.155	0.016	-0.071	0.093	-0.294*	-0.063

* Significant correlation.

It could be seen at Table 7 that there was significant correlation among the variable of *B. dulcis* trees and fruits in West Java. Significant positive correlations were found between (1) Canopy diameter and trunk width; (2) Maximum fruit width and fruit weight, pulp thickness, peel color, peel thickness, pulp soluble solid content (SSC), seed length, seed width, seed weight; (3) Maximum fruit length and seed weight per fruit; (4) Fruit weight and peel color, peel thickness, pulp SSC, seed length, seed width, seed weight; (5) Pulp thickness and peel weight, pulp SSC, seed width, seed weight; (6) Easiness to peel and peel color; (7) Peel color and peel thickness; (8) Peel thickness and pulp SSC, seed length, seed width, seed weight, pulp portion; (9) Pulp color and seed weight, pulp weight; (10) Pulp SSC and seed width; (11) Maximum seed length and maximum seed width, seed weight; (12) Maximum seed width and seed weight.

The results of positive significant correlation among the variable of *B. dulcis* trees and fruits in West Java (Table 7) indicate that the trunk width is in accordance with canopy diameter; the fruit size is in accordance with size of seed, peel and pulp; more difficult to peel the fruit correlates with more red/orange peel color and more thick the peel.

On the other hand, the significant negative correlation among the variable of *B. dulcis* trees and fruits in West Java (Table 7) were between (1) Peel weight and tree height, trunk diameter, peel color, pulp color; (2) Pulp thickness and easiness to peel, peel portion; (3) Easiness to peel and pulp color, maximum pulp segment, pulp SSC, maximum seed length, seed weight per fruit, pulp weight per fruit; (4) Peel color and peel weight, pulp weight per fruit, peel portion; (5) Peel thickness and pulp weight, peel portion; (6) Pulp color and pulp SSC; (7) Maximum pulp segment and easiness to peel, pulp SSC, maximum seed width; (8) Seed weight and pulp weight per fruit; (9) Pulp weight per fruit and leaf length, leaf width, easiness to peel, peel color, peel thickness, pulp portion; (10) Peel portion and leaf length, leaf width, fruit length, fruit width, fruit weight, pulp thickness, peel color, peel thickness, seed weight per fruit.

The result of significant negative correlation among the variable of *B. dulcis* trees and fruits in West Java (Table 7) indicates the composition of fruit parts is in accordance; more weight of fruit peel correlate with smaller tree and more dull peel and pulp color; more thick the pulp, more difficult to peel the fruit; more red/purple pulp color less sweet of the pulp; more weight of pulp and peel portion correlates with less size of leaf. Other study regarding fruit variation of *Irvingia gabonensis*, an indigenous fruit tree of West and Central Africa found that there were very weak relationship between fruit size and weight with nut and kernel size and weight (Leakey et al., 2000).

Table 7. Significant correlation among the variables of *Baccaurea dulcis* trees and fruits from West Java

CODE	X1	X2	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	X17	X18	X19	X20	X21
X3	0.54	**0.67***	0.135	0.149	0.082	-0.146	-0.042	-0.077	0.091	-0.082	0.049	**-0.24***	-0.094	-0.135	0.089	0.108	-0.106	0.009	-0.032	0.067
X9	0.01	-0.03	-0.18	0.18	0.08	0.72	**0.75***	1	0.243	**-0.19***	0.079	**0.22***	**0.90***	0.068	0.103	0.046	-0.109	**0.41***	**0.52***	**0.60***
X10	0.05	0.14	0.13	0.04	-0.07	0.24	**0.22***	0.24	1	**-0.30***	0.08	**0.23***	0.01	-0.01	-0.10	**0.32***	0.11	**0.11***	**0.18***	0.01
X11	-0.18	-0.21	-0.08	0.08	0.10	-0.13	-0.16	-0.19	**-0.30***	1	**0.20***	-0.09	0.11	**-0.21***	**-0.28***	**-0.19***	**-0.23***	-0.06	**-0.27***	**-0.20***
X12	-0.08	-0.14	-0.13	0.08	0.18	0.32	**0.31***	**0.22***	0.08	**0.20***	1	**0.39***	**-0.18***	-0.11	-0.03	0.07	0.07	0.06	-0.07	**-0.39***
X13	-0.01	-0.02	**-0.17***	0.25	0.18	0.69	**0.71***	**0.90***	**0.23***	-0.09	**0.39***	1	0.01	-0.03	-0.11	**0.33***	**0.41***	**0.48***	**0.48***	**-0.36***
X14	**-0.19***	**-0.22***	-0.09	-0.07	-0.10	0.08	-0.06	0.07	0.01	0.11	**-0.18***	0.01	1	**-0.23***	-0.15	-0.06	0.08	0.10	0.12	0.14
X15	0.06	0.02	0.06	0.11	0.10	-0.02	0.12	0.10	-0.01	**-0.21***	-0.11	-0.03	**-0.23***	1	0.02	0.02	0.15	-0.02	**0.25***	**0.28***
X16	-0.12	0.07	0.09	-0.09	-0.04	-0.04	-0.02	-0.11	-0.10	**-0.28***	-0.03	-0.11	-0.15	0.02	1	**-0.22***	-0.09	**-0.18***	-0.07	0.03
X17	0.18	0.13	-0.10	-0.03	-0.09	0.47	**0.33***	**0.41***	**0.32***	**-0.19***	0.07	**0.33***	-0.06	0.02	**-0.22***	1	0.11	**0.29***	0.38	0.10
X18	0.00	-0.09	-0.06	0.08	0.10	0.43	**0.61***	**0.52***	0.11	**-0.23***	0.07	**0.41***	0.08	0.15	-0.09	0.11	1	**0.53***	**0.49***	0.13
X19	0.07	-0.06	-0.07	0.00	-0.07	0.40	**0.55***	**0.60***	**0.11***	-0.06	0.06	**0.48***	0.10	-0.02	**-0.18***	**0.29***	**0.53***	1	0.13	-0.14
X20	0.03	-0.02	-0.13	0.04	-0.07	**0.54***	**0.55***	**0.82***	**0.18***	**-0.27***	-0.07	**0.48***	0.12	**0.25***	-0.07	0.38	**0.49***	**0.54***	1	0.62
X21	0.02	-0.01	0.01	**-0.18***	**-0.24***	-0.02	-0.04	0.08	0.01	**-0.20***	**-0.39***	**-0.36***	0.14	**0.28***	0.03	0.10	0.13	0.13	0.62	1
X22	-0.02	0.01	-0.02	0.19	0.24	0.04	0.06	-0.05	-0.01	0.19	0.40	**0.39***	-0.14	**-0.27***	-0.03	-0.09	-0.13	-0.14	-0.60	**-1.00***
X23	0.10	0.00	0.24	**-0.21***	**-0.13***	**-0.46***	**-0.33***	**-0.56***	**-0.12***	0.16	**-0.20***	**-0.55***	0.00	-0.14	-0.06	-0.19	-0.09	0.31	**-0.41***	0.03

Note: * Significant correlation.

X1 = Tree height, X2 = Trunk diameter, X3 = Canopy width, X4 = Lowest branch height, X5 = Maximum leaf length, X6 = Maximum leaf width, X7 = Maximum fruit length, X8 = Maximum fruit width, X9 = Fruit weight, X10 = Pulp thickness, X11 = Easiness to peel, X12 = Peel color, X13 = Peel thickness, X14 = Peel weight, X15 = Pulp color, X16 = Maximum pulp segment, X17 = Pulp SSC, X18 = Maximum seed length, X19 = Maximum seed width, X20 = Seed weight per fruit, X21 = Pulp weight per fruit, X22 = Pulp portion, X23 = Peel portion

4. Conclusion

This paper explored the distribution and variation of *Baccaurea dulcis* (Jack) Müll. Arg. in West Java, Indonesia. The population of the species is under threath. The species has restricted distribution, however the morphological characters were various. Therefore, its conservation is needed. Further study on the influence of genetic and environment aspects to the variation of the species is also important. Moreover, other interesting topic should be gaining high quality of fruits to become more commercialized one.

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Supplemental Tables

Supplemental Table 1. Linear Discriminant Function for Groups

	Groups					
Variables	1	2	3	4	5	6
Constant	-1.919	-2.066	-3.039	-8.559	-7.571	-6.580
C42	-0.701	-0.243	0.497	-0.123	1.011	0.612
C43	0.138	-0.279	0.046	1.101	-0.265	0.003
C44	-0.261	1.509	-0.645	-3.309	-0.209	0.669
C45	0.716	-3.269	1.334	**10.999**	1.320	**-9.505**
C46	-0.278	-0.887	0.650	1.976	0.651	-0.648
C47	-0.952	3.241	-0.800	**-11.732**	-1.707	**10.016**
C48	-1.293	2.705	-0.626	**-8.412**	-0.576	**7.426**
C49	-1.914	0.208	-0.190	2.227	3.198	1.330
C50	**-4.671**	**-3.609**	**6.154**	**8.508**	**4.450**	**-5.760**
C51	0.394	-1.632	2.082	0.206	-1.029	-2.545
C52	3.520	3.502	-5.073	-7.086	-4.258	5.448
C53	3.893	2.359	-3.890	-7.533	-4.531	4.152
C54	**-7.480**	**-4.515**	**18.165**	**23.060**	**-10.383**	**-31.038**
C55	-0.480	0.501	-0.218	-0.889	0.629	0.742
C56	0.151	0.421	-0.730	0.411	0.044	0.167
C57	0.271	-0.621	0.065	0.541	0.443	-0.203
C58	-0.446	0.468	-0.049	-1.910	0.835	0.274
C59	**5.645**	**6.563**	**-21.309**	**-26.441**	**16.976**	**35.403**
C60	-0.337	-0.352	0.742	1.158	-0.446	-0.112
C61	-0.100	0.609	-0.255	-0.398	-0.581	0.513
C62	0.223	-0.062	0.091	-0.813	0.007	-0.792
C63	0.362	-0.779	0.318	0.320	0.405	-1.047
C64	-0.398	-0.417	0.057	0.212	1.121	1.701
C65	**-0.587**	**1.870**	**-3.977**	**-3.173**	**5.852**	**3.893**
C66	-0.317	0.176	-0.681	-2.298	2.935	-0.393
C67	0.442	-1.600	2.808	3.369	-4.489	-1.398
C68	0.899	-1.967	4.179	3.417	-6.527	-5.095
C69	1.965	-1.866	1.869	-0.756	-3.015	-3.816
C70	40.719	-21.741	12.235	1.751	-60.747	-47.938
C71	38.601	-21.998	18.918	8.233	-67.798	-61.238
C73	-0.658	0.215	0.307	0.604	0.063	0.383

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Impact of Culture Filtrate of *Piriformospora indica* on Biomass and Biosynthesis of Active Ingredient Aristolochic Acid in *Aristolochia elegans* Mart

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Abstract

The mycorrhiza plant partnership is the basic, essential and integral part of plant survival and growth. In the present investigation we are reporting the effect of culture filtrate of *Piriformospora indica,* a growth promoter and bioprotector fungus on *Aristolochia elegans* Mart. The culture filtrate of the fungus increased overall growth, biomass, and active ingredient-aristolochic acid in the leaves of plants. In untreated control plants, the overall growth was reduced. *P. indica* culture filtrate application increased root number, root length, and root dry weightby 28%, 98%, and 123% respectivelyin plants of *Aristolochia.* Also stem height and shoot length was enhanced by 43% and 155% respectively.There was increase in number of leaves by 79% and length of leaves by 36%. The increase in total biomass was 136%. The improvement in content of aristolochic acid in leaves was between7.6% and 28.8%in treated plants as against untreated control plants

Keywords: *Aristolochia elegans*, aristolochic acid, *Piriformospora indica,* symbiotic fungus

1. Introduction

The fungus *Piriformospora indica*, is related to the Hymenomycetes of the Basidiomycota, which is a root endophyte that has capabilities of a typical, arbuscular mycorrhizal fungus, (Verma et al., 1998; Weiss et al., 2004; Prasad et al., 2008a; Bagde et al., 2010a, 2010b, 2010c, 2011), but unlike Arbuscular Mycorrhizal fungi it is cultivable in axenic conditionseasily. This mycorrhiza like fungus can form association with roots for enhanced growth and development of plants (Varma et al., 1999, 2001; Oelmüller et al., 2009; Sirrenberg et al., 2007; Prasad et al., 2008a; Bagde et al., 2011). *P. indica* interacted mutually with various plants including *Fabaceae* and *Rhamnaceae* species (Varma et al., 2001), *Arabidopsis* (Peškan-Berghöfer et al., 2004), tobacco (Barazani et al., 2005), *Poaceae* species (Waller et al., 2005).

P. indica enhanced nutrient uptake, helped plants to survive in extreme drought,temperature and salt conditions, exhibited systemic resistance to toxins, acted as biofertilizer, bioprotector, stimulator of growth, increased seed production, and played a key role in increasing the tolerance to insects (Varma et al., 1999; Waller et al., 2005, 2007; Serfling et al., 2007; Prasad et al., 2008a, 2008b). It helps in biological hardening to tissue culture raised plants, provides protection against ‘shock of transplantation ’and pathogens of roots (Sahay & Varma, 1999; Hazarika, 2003; Prasad et al., 2008a, 2008b).

Aristolochia elegans plant belongs to Aristolochiaceae family. It is generally called pine vines or Dutchman’s pipes. It is an annual plant with slender, woody stems, climber on support with heart shaped leaves and calico flower. It grows all over the world including in tropical climates such as India and other countries and is also called birthworts and commonly used to ease pain of childbirth, treat malaria and other diseases (Kimura & Kimura, 1981). Plant is cultivated and used in some medicinal preparations in China (Lopes et al., 2001). It contains important alkaloid aristolochic acid which is antimicrobial in nature (Imran & Bagde, 2007) and is useful for variety of ailments. The fruits and roots have been used by Chinese people in medicine as anodynes, antiphlogistics, expectorants and anti-asthmatic agents and is also used in treatment of snakebite, anti-tumor, anti-platelet aggregator agent and lung inflammation (Vila et al., 1997; Wu et al., 1999; Tian-Shung et al., 2000).

However, its certain harmful activities such as mutagenicity and carcinogenicity have also been reported (Arlt et al., 2002).

So far all the accounts are on the interaction of fungus propgules but in this communication we document that *P. indica* culture filtrate also enhanced the overall growth parameters of *Aristolochia elegans* and contents of aristolochic acid in leaves.

2. Materials and Methods

2.1 Mycobiont

P. indica culture for this study was procured from Amity University'sAmity Institute of Microbial Technology, India.

2.2 Photosymbiont

Aristolochia elegans Mart. (Aristolochiaceae) is the perennial shrub cultivated as ornamental plant in India. Species of *Aristolochia* are cultivated and used in medicinal preparations (Lopes et al., 2001). The plantlets were procured from Jijamata Udyan Byculla, Mumbai, India and were multiplied in environmentally controlled green house. Sterile substratum was used to conduct the experiments.

2.2.1 Culturing the Fungus *P. indica*

A. elegans was cultivated and maintained on modified synthetic media fortified with 1.2% agar (w/v) in dark at 28 ± 2 °C (Hill & Käfer, 2001; Prasad et al., 2005). pH of medium was kept at 6.5. For mass propagation, the fungus was also cultivated in liquid broth medium under constant shaking at 120 rpm in dark (GFL 3019, Germany). Media were sterilized in autoclave at 15 psi pressure for 15 minutes.

2.2.2 Separation of Culture Filtrate

The fungus was grown in liquid medium for 15days and was first filtered through sterile muslin cloth followed by bacterial filter (Millex-GV, 0.22 μm Filter Unit, Millipore) and kept at 4 °C if not used afresh.

2.2.3 Co-Cultivation Experiments

Plantlets grown for Fifteen days were transferred to sterile 10″ diameter plastic pots containing sterile unfertilized garden soil autoclaved on three consecutive days. Initially two plantlets were planted in each pot. Once they got acclimatized then one of the plantlet was removed, finally retaining only one plantlet in each pot. To each pot containing 1 kg of soil 15ml of freshly eluted culture filtrate to experimental pots and an equal volume of sterile nutrient medium were added to control pots one day before transfer of the plantlet into the pots. Again after a period of one month this treatment was repeated.

2.2.4 Growth Conditions

Pots were kept in green house at temperature of 26 ± 2 °C and 16 h light/8h dark and 60%-70% relative humidity and a light intensity of 20,000 lux. Growth of plants was measured after 90 days by use of centimeter scale. For estimation of dry biomass, plant was chopped and dried at 80 °C for 12 h in a Memmert oven and dry biomass was estimated after cooling at room temperature and weighing on electric- mono-pan balance.

2.3 Aristolochic Acid Analysis

Leaves of *Aristolochia* were used to extract and estimate aristolochic acid. For preparation of extract, leaf material was ground to fine powder by mechanical grinding using HPLC grade methanol and formic acid. 2 gm. of ground sample was taken in a bottle, thoroughly mixed with a mixture of 50 ml of methanol (80%) and 20 ml of 10% formic acid in water. The contents were stirred for 30 minutes at 500 rpm (Innova Model 2001 bench top platform shaker, New Brunswick, USA) and then centrifuged for 4 minutes at 4000 rpm. The supernatant was taken for determination of aristolochic acid (Gaudreault et al., 2001; Flurer et al., 2001). Estimation of aristolochic acid in the leaves extract of *A. elegans* was carried out by HPTLC using standard aristolochic acid as reference (Sigma, USA) in the range of 0-200 μg/ml. Stationary phase used for HPTLC contained Silica gel 60 (Merck) plates of 10x10 cm size. The mobile phase used for the chromatogram consisted of toluene, ethyl acetate, water and formic acid in the ratio of 20:10:1:1. The sample used was 10 μg. For developing the plate twin trough chamber was saturated for 20 minutes and the plate was dried with hair drier (cold air) for 5 minutes. The plate was evenly sprayed with tin (II) chloride reagent and further dried at 100 °C for a minute. Plates were observed under UV light at 366 nm and acid content was measured. This was determinedafter 15, 30, 45, 60, 75 and 90 days.

3. Results and Discussion

When morphological appearance of *P. indica* was observed on Käfer agar medium the pattern of growth of the fungus was marked by uniform rhythmic zonation (Figure 1a). The rapid growth on Käfer nutrient broth was observed after 15 days incubation at temperature of 28 ± 2 °C (Figure 1b). The colonies showed prominent crowded balls of coral morphology in conformity with previous studies by various workers (Varma et al., 2001; Singh et al., 2003). The important characteristics of this organism have been described earlier (Varma et al., 2001).

Figure 1(a). Growth of *P. indica* on solidified agar medium

Figure 1(b). Cultivation of *P. indica* in aspergillus broth medium

The observations made in this study indicated that culture filtrate of fungus exerted positive impact on various parameters of the plant as depicted in Figures 2 & 3 and Tables 1 & 2. When *A. elegans* plant was treated with culture filtrate *P. indica*, it enhanced the number, length and biomass of the root (Table 1). Pretreatment also resulted in an increase in root number, root length and root dry weight by 28%, 98%, and 123% respectively in *Aristolochia.* Increased root length and number can enhance absorption of more nutrients due to increased absorbing area resulting in improved plant growth (Marschner & Dell, 1994). Similar observations were made by other workers (Mugnier & Mosse, 1987; Varma et al., 2001). Inoculation of culture filtrate in case of grasses, trees and herbaceous sp. also showed enhancement of plant growth (Varma et al., 2001).

Figure 2. Effects of *P. indica* culture filtrate inoculation on *Aristolochia elegans* (A treated and B untreated)

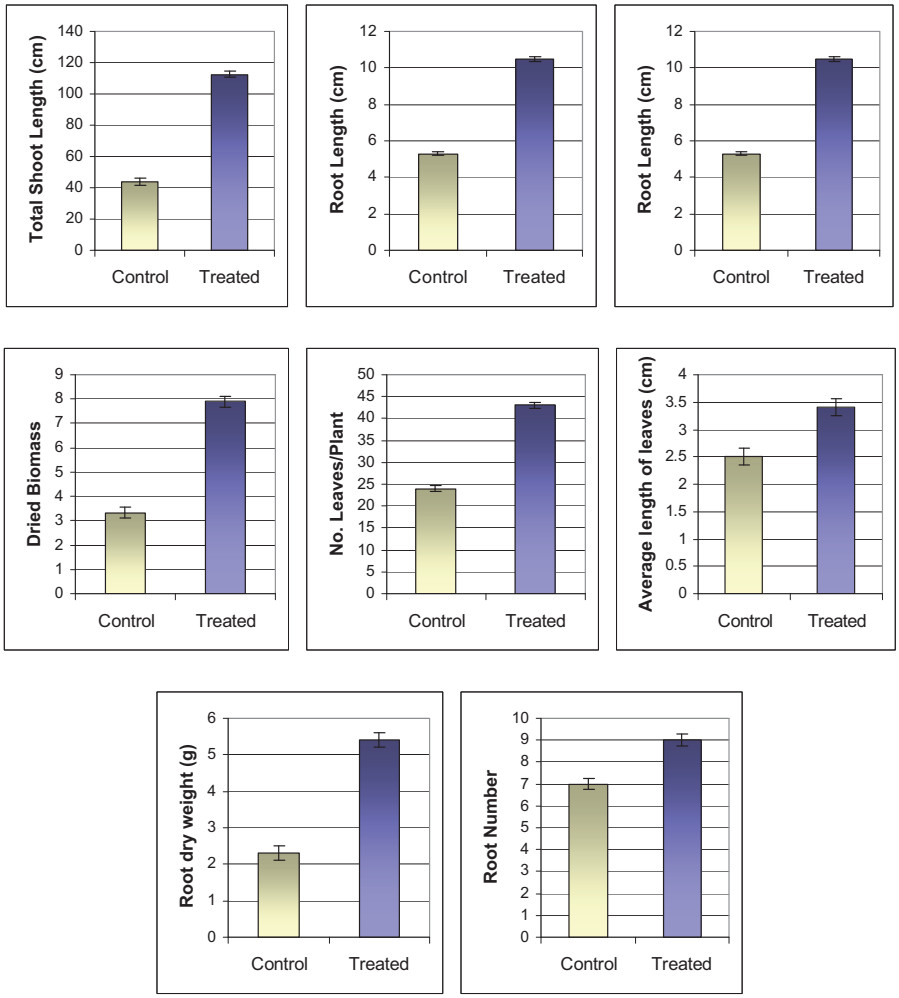

Figure 3. Effect of *P. indica* culture filtrate on *Aristolochia elegans*

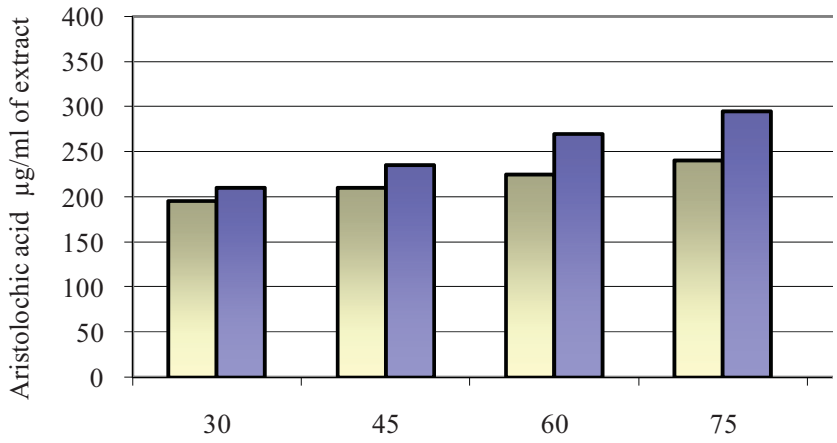

Figure 4. Effects of *P. indica* inoculation on concentrations of aristolochic acid in leaves of *Aristolochia elegans*

Table 1. Effect of *P. indica* culture filtrate on growth performance of *Aristolochia elegans.* Average values are for five replicates

	Characteristics	Control (untreated)	Experimental (*P. indica* treated)	S. E.	S. D.	Percent increase over control
Root	Number	7.00	9.00	± 0.26	0.2	28
	Length(cm)	5.30	10.5	± 0.12	0.12	98
	Dry weight (g)	2.30	5.4	± 0.20	0.20	123
Stem	Height(cm)	7.2 0	10.5	± 0.02	0.02	43
	Shoot length(cm)	44.00	112.5	± 2.17	2.17	155
Leaves	Number	24.00	43.0	± 0.68	0.68	79
	Length(cm)	2.50	3.4	± 0.15	0.15	36
Total Biomass (g)	Roots, Stems, Leaves,	3.32	7.9	± 0.22	0.22	136

S. E = Standard Error; S. D. = Standard Deviation.

Table 2. Effects of *P. indica* inoculation on concentrations of aristolochic acid in leaves of *Aristolochia elegans*

Days After planting	Aristolochic acid µg/g of extract		S. D.	Percent increase over the Control
	Un-treated (Control)	Treatment with *P. indica*		
30	195 ± 1.76	210 ± 1.76	1.76	7.6
45	210 ± 2.10	235 ± 2.10	2.10	11.9
60	225 ± 5.64	270 ± 5.64	5.64	20.0
75	240 ± 4.85	295 ± 4.85	4.85	22.8
90	260 ± 6.47	335 ± 6.47	6.47	28.8

Enhanced root growth, and root length was observed after application of the fungus in several plant species studied earlier (Varma et al., 2001). Not only the mycelium but even culture filtrate enhanced growth of the plants. Earlier it was reported that plant root cells can be killed by colonization of this fungus (Deshmukh et al., 2006), however it also increased root growth, weight and branching (Varma et al., 1999; Waller et al., 2005).

Increased rooting of calli of *N. tabacum* and cuttings of other plants was also noticed (Varma et al., 1999; Drudge et al., 2007). When culture filtrate of *P. indica* was applied, a diffusible factor from it enhanced root growth of *Arabidopsis*. There was stunted but highly branched roots in treated plants (Sirrenberg, 2007). The overall increment in the plant growth reported in this study may be due to increased nutrients uptake by the roots. This may also be due to application of culture filtrate that contained many growth promoters that exerted desirable effect on plant.

When culture filtrate of *P. indica* was applied, Increments in stem height and shoot length of plants were observed in plants in the present study (Table 1). Stem height increased by 43% and shoot length by 155%.This is in conformity to observations made in earlier investigations (Varma et al., 2001; Nautiyal et al., 2010; Bagde et al., 2010a, 2011).

The number of leaves increased by 79% and length by 36% in *P. indica* culture filtrate treated plants in comparison to untreated plants (Table 1) Increase in number and length of leaves was also reported in other plants using fungal mass or fungal culture filtrate (Varma et al., 2001; Fakhro et al., 2010; Bagde et al., 2011). A greater number of leaves, with increased length produced in treated plants could have contributed to increased rate of photosynthesis (Kungu, 2004).

P. indica culture filtrate treatment enhanced growth as well as total biomass of plants in comparison to untreated control plants in present study (Table 1). Similarly there was reported increase in total biomass by 136% as against treated control plants in herbaceous species (Varma et al., 2001) and *Helianthus annus* (Bagde et al., 2011), winter wheat plants (Serfling et al., 2007).

When six strains of *Sebacina vermifera* were tested on *Panicum virgatum* roots, it was noticed that there was positive effects on plant height and biomass production. It was also observed that culture filtrates from some strains of *S. vermifera* increased seed germination in *P. virgatum* by 52% over the control. In spring barley *P. indica* increased plant biomass and grain yield by 11% (Waller et al., 2005). Serfling et al. (2007) observed that fungus *P. Indica* colonization increased plant biomass in winter wheat plant.

When fungal culture filtrate was applied to the soil before planting, it increased total content of aristolochic acid in leaves between 7.6% to 28.8% (Table 2). The quantity of leaves and content therein were augmented as compared to control plants when treated plantlets were transferred to the pots. This positive influence in promoting the plant growth and yield in terms of biomass and medicinal ingredients may be due to positive effect of stimulatory factors or components present in the culture filtrate.

Besides several reports pertaining to the association of cells of *P. indica* with plants that enhanced growth, present study reports positive effect of even culture filtrate of fungus on plant growth. This is due to special characteristics of culture filtrate that was used. Culture filtrate is a complex growth enhancer of which all ingredients are not known (Bagde et al., 2010b). Culture filtrate contains fungal exudates, hormones, enzymes, proteins etc. that increased root number, length, root dry weight, stem height, shoot length, number and length of leaves, total biomass and aristolochic acid content of leaves in culture filtrate treated plants. Similar observations were made in case of maize, *Bacopa monniera,* and tobacco (Varma et al., 2001), neem and maize (Kumari, 2002; Singh et al., 2003). In *Helianthus annus*, treatment with *P. indica* culture filtrate promoted overall growth of the plant in terms of increased, root collar diameter, number of secondary roots, root length, root weight, stem diameter, stem height, number of leaves, length and width of leaf, flower number, flower diameter, flower dry weight, number of seeds, weight of seeds and total biomass as compared to untreated control plants. Seed oil content considerably increased in treated plants. Seed oil content increased by 51.13 per cent in sun gold variety and 70.33 per cent in treated Japanese gold variety of *H. annus* plants (Bagde et al., 2011).

Varma et al. (2001) also reported that application of culture filtrate of *P. indica* led to increase in root length, shoot length and plant biomass in treated plants. In present study treatment of *Aristolochia elegans* increased growth of roots, stems, leaves, total biomass as well as aristolochic acid over untreated plants (Table 1). These observations are in conformity to observations of Singh et al. (2003) wherein treatment resulted in considerable increase in growth and development in *Azadiracta indica* and *Zea mays* plants. Similarly when *Helianthus annus* plants were treated with culture filtrate of *P. indica,* root number, length, root collar diameter and dry weight of root increased considerably (Bagde et al., 2011). Observations like these were also made by other investigators, who reported luxurious and elaborate root growth and biomass when treated with mycelia of fungus (Varma et al., 2001; Kungu, 2004).

According to Sirrenberg et al. (2007) actual mode of action of *P. indica* in enhancing the growth of plants was not yet clear. But it is suggested that effect was due to diffusible factor that could be IAA, as *P. indica* was found to produce IAA in culture filtrate in sufficient quantities and hence it must have contributed to the beneficial

effect on its host plants.The fungus may in addition induce auxin production in the plant (Peškan-Berghöfer et al., 2004). *P*lants colonized with *P. indica* can tolerate physical stress, nutrient deficiency, biotic and abiotic stresses and can fight pathogens including invaders of insects and facilitated increase in seeds and early flowering in medicinal plants (Oelmüller et al., 2009).

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Studies on The Allelopathic Effects of *Tithonia rotundifolia* on the Germination and Seedling Growth of Some Legumes and Cereals

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Abstract

The study investigated the allelopathic effects of *Tithonia rotundifolia* on the germination and growth of two legumes (*Vigna unguiculata* and *Glycine max*) and two cereals (*Zea mays* and *Sorghum bicolor*). This was with a view to determining the susceptibility of these test crops to allelochemicals. The germination studies were carried out by raising seedlings in Petri-dishes which had been lined with Whatman No. 1 filter paper. Ten millilitres of 100%, 75%, 50% and 25% concentrations of the methanolic or water extract solutions were used for the treatments while distilled water served as control. Germination and growth analyses were carried out according to standard methods. The data obtained were analysed by Factorial Analysis of Variance (ANOVA) to determine significant ($P < 0.05$) effects. The germination and growth of the juvenile seedlings of all the test crops were significantly inhibited by the methanolic and water extracts dose dependently. However, the methanolic extracts had a more pronounced inhibitory effect on these parameters. The study concluded that the methanolic extracts were more phytotoxic and had higher inhibitory effects on the parameters than the water extracts. Also, it was observed that the response of plants to allelochemical toxicity was dependent on plant species.

Keywords: allelopathic, methanolic extract, water extract, *Tithonia rotundifolia*, legumes, cereals

1. Introduction

The phenomenon of allelopathy has received increasing attention as a means of explaining vegetation patterns in plant communities (Miller, 1996). According to Inderjit et al. (1999), allelopathy may occur in all environments and should be considered as a part of community interaction. Kohli et al. (1998) and Singh et al. (2001) opined that allelopathy plays an important role in many agro-ecosystems. A large number of plants impose inhibitory effects on the germination and growth of neighbouring or successional plants by releasing allelopathic chemicals into the soil, either as exudates from living tissues or by decomposition of plant residues (Rice, 1984; Narwal, 1999; Alam & Islam, 2002; Khan et al., 2009). Bendall (1975) studied water and ethanol extracts and residues in soil and concluded that an allelopathic mechanism might be involved in the exclusion of some annual thistle (*Carduus crispus* L.), pasture and crop species in areas infested with *Cirsium arvense* (L) Scop. According to Stachon and Zimdal (1980), *C. arvense* litter reduced the growth of *Amaranthus retroflexus* L. and *Setaria viridus* L. more than that of cucumber (*Cucumis sativus* L.) or barley (*Hordeum vulgare* L.) in greenhouse experiments. They also observed that high densities of *C. arvense* reduced the incidence of annual weeds growing in the vicinity of *C. arvense*. Khan et al. (2009) stated that aqueous extracts of *Eucalyptus camaldulensis* L. inhibited seed germination, fresh and dry weight of wheat seedlings. Rawat et al. (2002) reported that aqueous extract of the root of *Helianthus annus* delayed and inhibited the germination and seedling growth of linseed (*Linum usitatissium* L.) and mustard (*Brassica juncea* L.) Aqueous extracts from the leaves of *Helianthus tuberosus L. Xanthium occidentale*, *Lactuca sativa* and *Cirsum japonica* all in the Asteraceae family inhibited the root growth of Lucerne (Chon et al., 2003). Ilori et al. (2007) observed that the radical growth of *Oryza sativa* was inhibited by aqueous extract of *T. diversifolia.* Otusanya et al. (2007) reported that the growth of *Amaranthus cruentus* was inhibited by aqueous extract of *T. diversifolia.* Javed and Asghari (2008) reported that the leaf extract of *Helianthus annus* inhibited the rate of germination of wheat seedlings.

In Nigeria, *Tithonia rotundifolia* is a widespread species having colonized roadsides, waste places, fallow land

and disturbed open spaces like abandoned construction sites etc. and displacing traditional weedy species like *Chromolaena odorata and Panicum maximum* (Adebowale & Olorode, 2005). The plant associates with common crops like vegetables, cassava, yam, rice, sorghum, soyabean e.t.c. and becomes a dominant plant where it is present (Tongma et al., 1998). Cowpea (*Vigna unguiculata* (L.) Walpers) and Soybean (*Glycine max* (L.) Merr.) which belong to the family *Fabaceae* are economically significant legumes in the tropics. Maize (*Zea mays* L.) and Sorghum (*Sorghum bicolor* (L.) Moench) are annual grasses belonging to the family Poacea. *Z. mays* L. is one of the most important cereal crops growing in the world. It is used as food for human consumption as well as food grain for animals (Moussa, 2001). *S. bicolor* (L.) Moench is a drought resistant cereal important for grain, forage and bioethanol production (Aishah et al., 2011). Considering the effects of *Tithonia* species on associated crops, the objectives of this work was to determine the effects of water and methanolic extracts of fresh shoots of *T. rotundifolia* on the germination, growth parameters (plumule and radicle lengths) and yield parameters (fresh and dry weights of plumule and radicle) of juvenile seedlings of *V. unguiculata*, *G. max*, *Z. mays* and *S. bicolor*.

2. Materials and Methods

2.1 Study Area

This study was conducted at the Botany Department of the Obafemi Awolowo University (O. A. U.), Ile-Ife, Osun State, Nigeria, Latitude 07°30'N - 07°35'N and Longitude 04 °30' - 04°40'E.

2.2 Plant Materials

The plant materials that were utilized in this study are the seeds of the following plants *Tithonia rotundifolia* (Miller) S. F. Blake, *Vigna unguiculata* L.Walp, *Glycine max* L. Merr., *Zea mays* L. and *Sorghum bicolor* (L.) Moench. The seeds of the test crops (*Vigna unguiculata*, *Glycine max*, *Zea mays*, and *Sorghum bicolor*) were collected from IITA (International Institute of Tropical Agriculture) Ibadan. *T. rotundifolia* seeds were collected along Road 20 at the Senior Staff Quarters of O. A. U., Ile Ife.

2.3 Germination Experiment

Preparation of extracts for the different treatments was carried out according to the modified method of Qasem and Abu - Irmaileh (1985). The extract solution (100%) was diluted appropriately with water to give 75%, 50%, and 25% concentrations of the aqueous extracts while distilled water served as control. Petri-dishes were thoroughly washed and oven dried**.** The seeds of the different test plants were selected randomly on the basis of uniformity of size and the seeds were then soaked for five minutes separately in 5% sodium hypochlorite to prevent fungal infection. Thereafter they were rinsed for about five minutes in running tap water. Ten of the seeds were placed in each of the clean oven dried Petri-dish which had been lined with a Whatman No. 1 filter paper. The filter paper in each of the Petri-dishes allocated to the control was moistened with ten millilitres of distilled water while that of the Petri-dishes allocated to the other treatments were moistened with ten millilitres of the appropriate concentration of the extracts. The Petri-dishes were incubated at room temperature for two weeks. Emergence of one millimetre of the radicle was used as the criterion for germination. Measurements of germination, plumule and radicle lengths, fresh and dry weights were carried out using standard methods.

2.4 Statistical Analysis

The data obtained were analysed by factorial Analysis of Variance (ANOVA) to determine significant ($P < 0.05$) effects.

3. Results

The percentage germination of the control seeds of the test crops was higher than that of the seeds treated with the different extracts (Figure 1). In most cases the percentage germination increased as extracts concentration decreased. There was significant reduction of the germination of the seeds by all the concentrations of FME and FWE at $P < 0.05$. Seedlings of the test crops in the control had plumule and radicle lengths that were significantly higher than those of the seedlings in all the extract regimes and these plumule and radicle lengths reduced with increase in the concentration of the methanolic extracts and water extracts (Figures 2 & 3). The control seedlings of all the test crops had plumule fresh weight that was significantly higher than that of the seedlings in both the FME and FWE regimes (Figure 4). The plumule fresh weights of the seedlings in all the extract regimes increased with decrease in the concentration of the extracts. The radicle fresh weight of *V. unguiculata* and *S. bicolor* seedlings in the 25% and 50% FWE was almost equivalent or slightly higher than that of the control seedlings while the other extracts inhibited the radicle fresh weight of these seedlings. In the case of the *G. max* and *Z. mays* seedlings, the methanolic extract was more phytotoxic than the water extracts and the radicle fresh weight was inhibited by all the extracts (Figure 5). The plumule dry weight of the control *V. unguiculata* seedlings was higher

than that of the seedlings in all the extract regimes while that of the seedlings in the 75% and100% extract regimes were almost equivalent and lower than that of the 25% extracts regime. Also, the 25% FWE seedlings of *Z. mays* had the same plumule dry weight with that of the control while that of the *S. bicolor* seedlings in the 25% FME was lower than that of the seedlings in the 50% FME regime. In the case of the *G. max*, the plumule dry weight of the seedlings in the 100% FME and FWE regimes was much lower than that of the control, FME and FWE seedlings (Figure 6). *V. unguiculata*, *G. max* and *Z. mays* seedlings in the control regime had radicle dry weight that were higher than those of the seedlings in all the extract regimes. However, the radicle dry weight of the control *S. bicolor* seedlings was equivalent to that of the 25% FWE seedlings (Figure 7). Interactions of extracts x crops, crop x extract concentrations were found significant for all the parameters except plumule dry weight (Table 1).

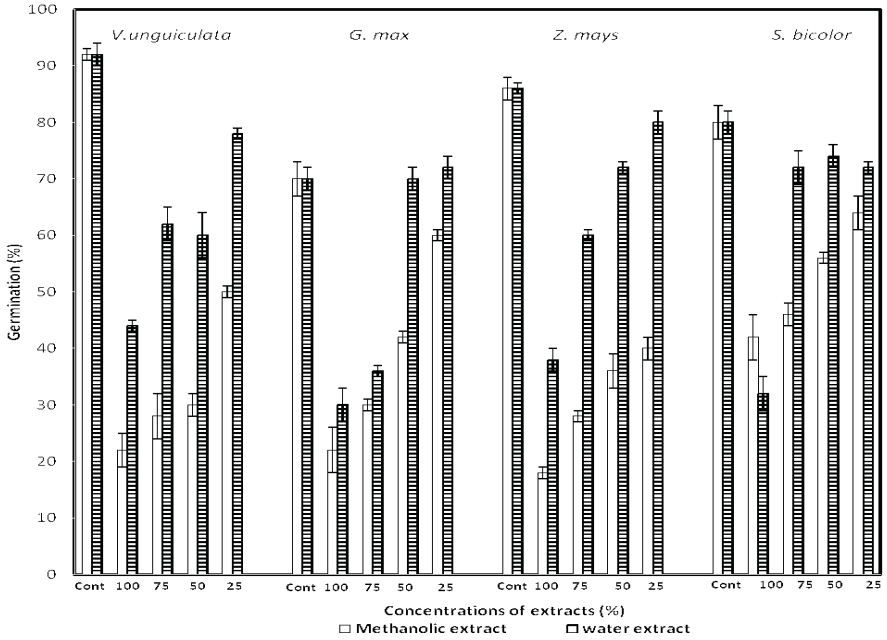

Figure 1. Effect of the methanolic extracts and water extracts of the fresh shoots of *T. rotundifolia* on the germination of the test crops. Capped bars indicate standard errors

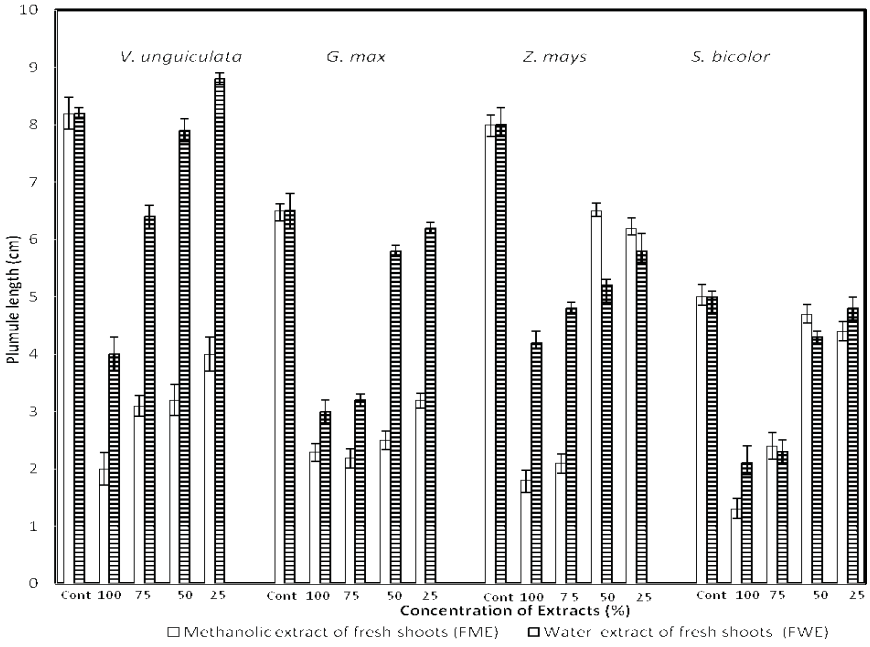

Figure 2. Effect of the methanolic and water extracts of the fresh shoots of *T. rotundifolia* on the plumule length of the test crops. Capped bars indicate standard errors

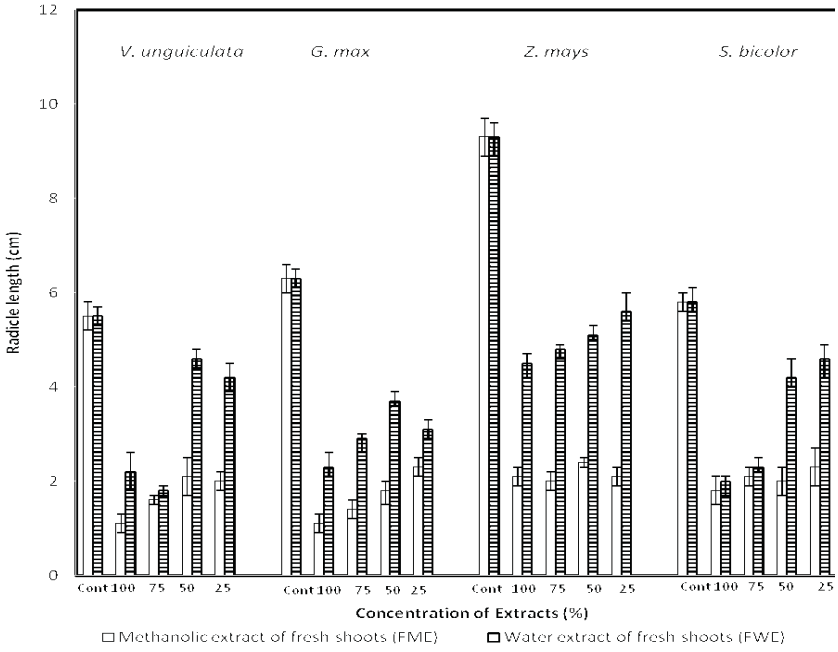

Figure 3. Variation in the radicle length of the test crops treated with the methanolic extracts and water extracts of the fresh shoots of *T. rotundifolia*. Capped bars indicate standard errors

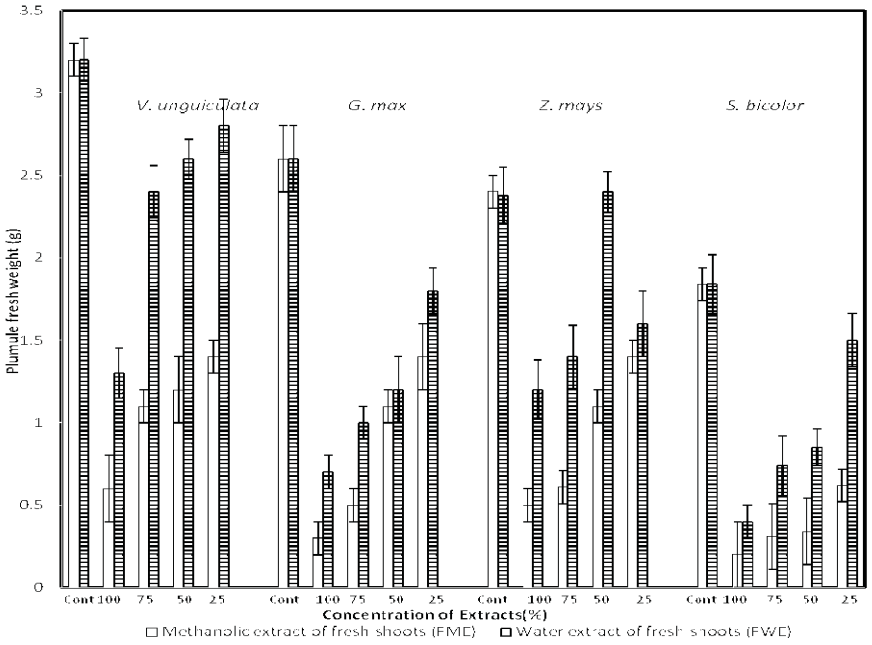

Figure 4. Variation in the plumule fresh weight of the test crops treated with the methanolic extracts and water extracts of the fresh shoots of *T. rotundifolia*. Capped bars indicate standard errors

4. Discussion

According to Leu et al. (2002) and Inderjit and Duke (2003), allelopathy in natural and agricultural ecosystems is receiving increasing attention because allelochemicals significantly reduce the growth of other plants and the yields of crop plants. Allelochemicals are secondary plant products or waste products generated by the plant's main metabolic pathways which are released into the environment in appreciable quantities via root exudates, leaf leachates, roots and other degrading plant residues (Putnam, 1988). These chemicals have harmful effects on crops in the ecosystem resulting in the reduction and delayed germination, seedling mortality and reduction in growth and yield (Herro & Callaway, 2003). The process of seed germination is a crucial stage in plant growth. During germination, biochemical changes take place, which provides the basic framework for subsequent growth and development (Khan et al., 2009). According to Bhownmik and Inderjit (2003), allelochemicals can affect the establishment or regeneration of population by affecting seed germination. These authors were of the opinion that increasing germination can enhance the competitive ability of a plant species for both above-ground and underground resources.

The water and methanolic extracts from *T. rotundifolia* had significant inhibitory effect on the germination of the seeds of all the test crops in this study.This observation agreed with the findings of Inderjit and Dakshini (1994) who reported that the water extracts from the roots of *Pluchea lanceolata* in the family Asteraceae inhibited the germination of tomato and mustard. The water extracts from tissues of *Helianthus annus* were also observed to inhibit germination of *Solanum nigrum* (Sedigheh et al., 2010). Rawat et al. (2002) found that the aqueous extract of the root of *Helianthus annus* delayed and inhibited the germination and seedling growth of linseed (*Linuna usitatissium* L.) and mustard (*Brassia Juncia* L.). Nandal and Dhillon (2005) reported that the aqueous extracts of poplar leaves adversely affected the germination and seedling growth of some wheat varieties at high extract concentrations. Mulatu et al. (2006) reported that aqueous extract of *Parthenium hysterophorus* leaves and flower inhibited seed germination of lettuce. Preliminary investigations have revealed that the aqueous extract from the leaves of *T. diversifolia* retarded the germination and the radicle growth of *Oryza sativa*, *Amaranthus cruentus*, *Capsicum annum and Lycopersicon esculentum* (Ilori et al., 2007; Otusanya et al., 2007; Otusanya et al., 2008). Khan et al. (2009) reported that the reduction in germination counts of wheat became more pronounced with increasing levels of *Eucalyptus camaldulensis* aqueous extract concentration. Javed and Asghari (2008) also found that the leaf extract of *Helianthus annus* inhibited the rate of germination of wheat seedlings. A related work by Arshad (2011) showed that the water and methanolic extracts of *Withania somnifera*

markedly suppressed the germination, root and shoot growth of *Parthenium hysterophorus*.

The growth of the plumule and radicle of the water and methanolic extracts treated seedlings of the test crops were significantly inhibited at $P < 0.05$. The inhibition of the growth of the radicle of *G. max* and *Z. mays* were more pronounced than that of the plumule growth. These results corroborates the earlier findings of several other workers such as Chou and Kuo (1986), Alam (1990), Zackrisson and Nilsson (1992) and Munir and Tawaha (2002) who all asserted that root growth was more sensitive to the increasing concentration of plant aqueous extracts in comparison to the shoot growth. The more accentuated effect of the allelochemical on the roots might be due to their closer contact with the leachates or extracts especially when maintained on filter/germination paper (Chung et al., 2001). Rahman (1998) reported that aqueous extract derived from the inflorescence, stem, and leaves of *Parthenium hysterophorus* L. inhibited the growth of radicle and plumule of *Cassia sophera* Linn. Florentine et al. (2006) observed that allelopathy is characterized by reduction in plants emergence or growth, reducing their performance in the association. A similar result was reported by Kushima (1998) who stated that there was an inhibition of the growth of the plumule length of tomato seedlings by the application of leachate from water melon seeds. James and Bala (2003) found that dried mango leaf powder significantly inhibited the sprouting of purple nutsedge tubers while Yang et al. (2006) reported that its aqueous extract inhibited the germination and growth of some crops. Also, the results in this study was consistent with the finding of Ilori et al. (2007) who stated that the radicle growth of *Oryza sativa* was inhibited by the aqueous extract of *T. diversifolia* (a close relative of the donor plant). This retardation of the juvenile seedling growth of the target crops was observed to increase significantly with increasing extract concentrations. This was consistent with the work of Khan et al. (2009) who reported that the inhibitory effects of aqueous extracts of *Eucalyptus camaldulensis L.* on germination and seedling growth (fresh and dry weight) of wheat were increased as the extract concentration increased. A similar result was obtained by Swapnal and Badruzzaman (2010) on the allelopathic effect of *Croton bonplandianum* Baill. weed on seed germination and seedling growth of crop plants. They reported that the root length, shoot length of *Melilotus alba* Medik., *Vicia sativa* L. and *Medicago hispida* Gaertn. decreased progressively when the plants were exposed to increasing concentration of the extract of *Croton bonplandianum* Baill. Khan et al. (2009) from their study of the effect of Eucalyptus extracts on twelve varieties of wheat concluded that the variation in germination of different varieties might be due to variation of the genetics of these twelve varieties. Likewise, it was observed in this study that the response of plants to allelochemicals toxicity was found to be dependent on plant species. The most affected crops were *G. max* and *Z. mays* for radicle fresh and dry weights and *V. unguiculata* for germination.

5. Conclusion

It can be summarized from the results of this study that both the water and methanolic extracts at any concentration inhibited the germination, growth and ultimately the yield of the test crops. The methanolic extract was more phytotoxic than the water extracts. The extent of the inhibition by the water and methanolic extracts followed this order: 100% > 75% > 50% > 25%. This affirmed the fact that the response of the target crops was extract concentration dependent. In conclusion, the effectiveness of these extracts on the germination and growth of the crops in this study showed that the presence of *T. rotundifolia* would negatively affect the neighboring or successional crop plants.

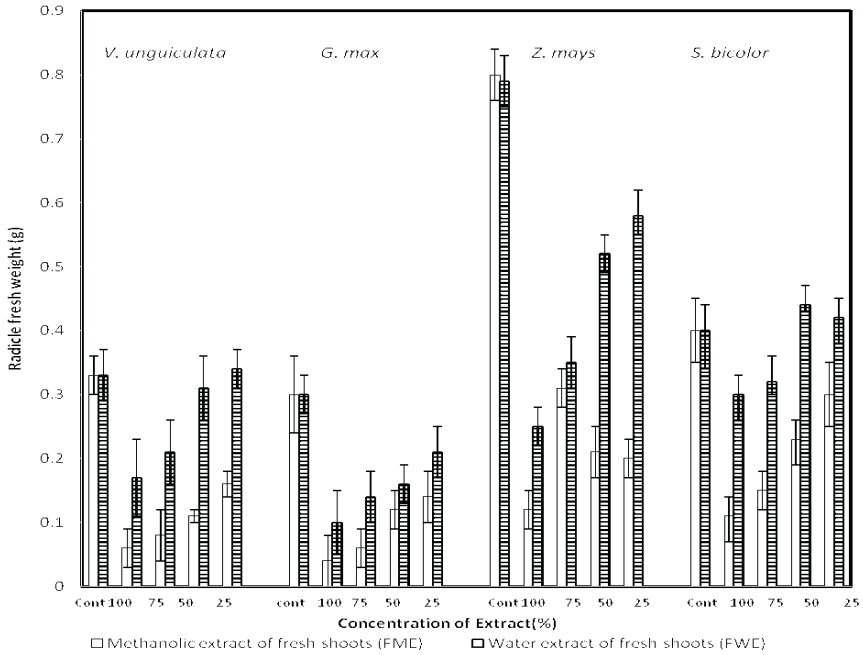

Figure 5. Radicle fresh weight of the test crops as affected by the methanolic and water extracts of the fresh shoots of *T. rotundifolia*. Capped bars indicate standard errors

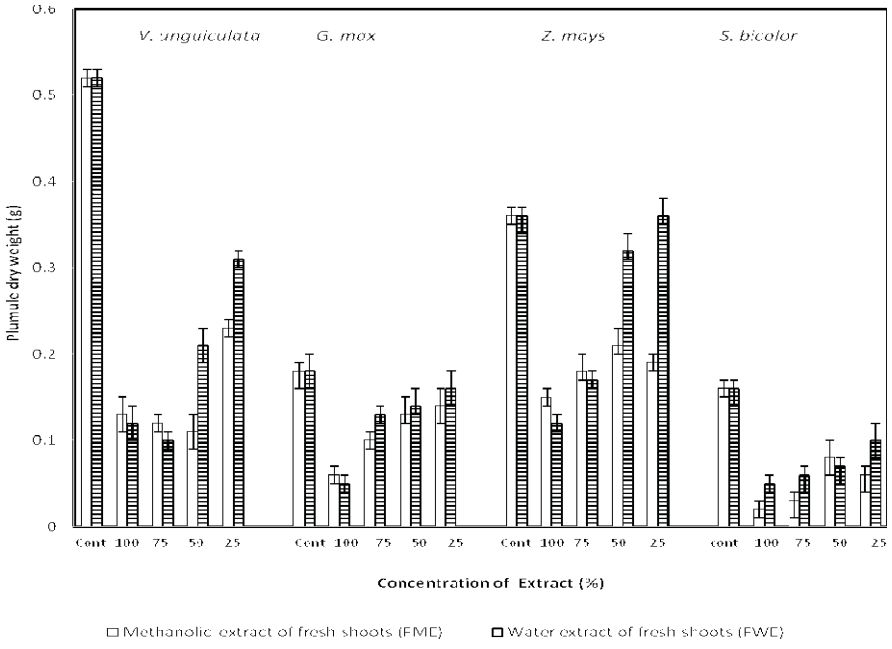

Figure 6. Plumule dry weight of the test crops as affected by the methanolic and water extracts of the fresh shoots of *T. rotundifolia*. Capped bars indicate standard errors

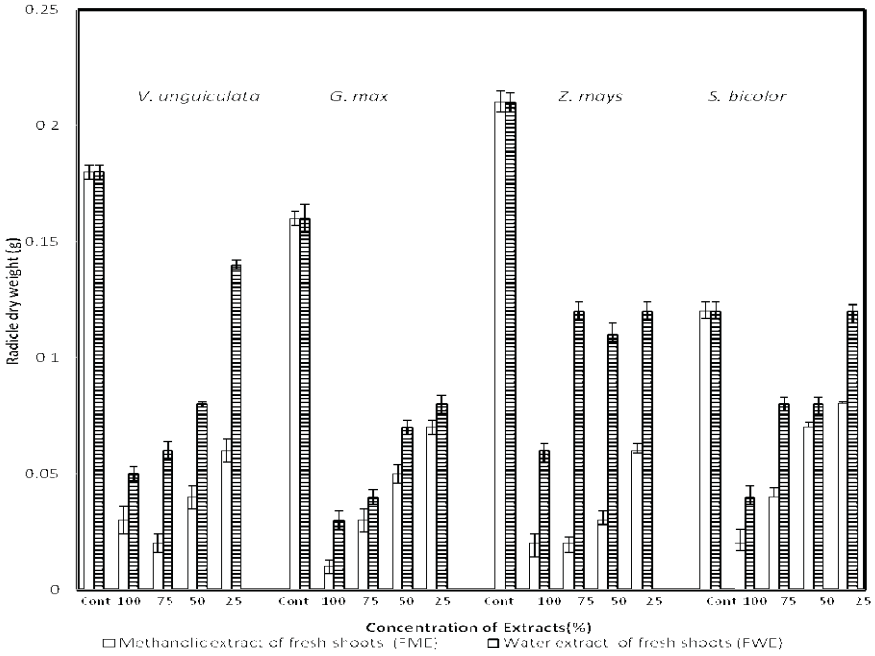

Figure 7. Radicle dry weight of the test crops as affected by the application of the methanolic extracts and water extracts of the fresh shoots of *T. rotundifolia*. Capped bars indicate standard errors

Table 1. Results of analysis of variance of the traits determined

Source	df	Germination percentage	Plumule length	Radicle length	Plumule fresh weight	Radicle fresh weight	Plumule dry weight	Radicle dry weight
Crops(C)	3	**	**	**	**	**	**	**
Extracts(E)	1	**	**	**	**	**	ns	**
Conc.	4	**	**	**	**	**	**	**
C x E	3	**	**	**	**	**	ns	**
C x Conc.	12	**	**	**	**	**	**	**
E x Conc.	4	**	**	**	**	**	ns	**
C x E x Conc	12	**	**	**	**	**	ns	**

** $P < 0.05$, df, degrees of freedom. Conc., concentration.

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Impact of New Natural Biostimulants on Increasing Synthesis in Plant Cells of Small Regulatory si/miRNA With High Anti-Nematodic Activity

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Abstract

Plant endoparasitic cyst nematode *Heterodera schachtii* Schmidt, gallic nematode *Meloidogyne incognita* and stem nematode *Ditylenchus destructor* damage various agricultural crops. The application of ecologically safe natural biostimulants with bioprotective properties is a newer approach for increasing plant resistance to parasitic nematodes. The molecular-genetic analysis of biostimulants action on plant genome is necessary for creation of new effective bioregulators for plant protection against phytopathogenic organisms. In our field and greenhouse experiments, we investigated the influence of new natural biostimulants Avercom and its derivatives on plant protection against nematodes *Meloidogyne incognita* and *Ditylenchus destructor*. Considerable increase of resistance to nematodes and productivity of cucumber and potato were observed for plants treated by biostimulant Avercom and its derivatives. Impact of biostimulants Radostim-super and Avercom on increase of resistance of sugar beet and cucumber sprouts to nematodes *Heterodera schachtii* and *Meloidogyne incognita* was studied in the laboratory conditions. Comparative analysis of morpho-physiological signs of control and experimental plants showed that plants treated by Radostim-super and Avercom were more viable and resistant to these nematodes as compared to control sprouts. In the molecular-genetic experiments, we studied the impact of these biostimulants on inducing synthesis of small regulatory si/miRNA, which plays key role in plant immune protection. Using method Dot-blot hybridization we studied degree of homology between si/miRNA with mRNA populations, isolated from plants untreated and treated with new natural biostimulants. We found considerable difference in the degree of homology (6-28%) between populations of mRNA and si/miRNA from nematode-infected plants that were either untreated or treated with biostimulants. We have also investigated silencing of translation of mRNA activity of si/miRNA in the wheat embryo cell-free system of protein synthesis. In these experiments, we found high inhibitory activity (38-65%) of si/miRNA from plants treated by biostimulants as compared to low inhibitory activity (15-20%) of si/miRNA from untreated plants. Obtained differences in the degree of homology between populations of mRNA and si/miRNA from untreated and treated with biostimulants plants, which were infected by nematode, and also the high inhibitory activity of si/miRNA from plants treated by biostimulants confirm that these biostimulants induce synthesis of anti-nematodic si/miRNA in plants, resulting in considerable increase of their resistance to these phytopathogens.

Keywords: *Heterodera schachtii*, *Meloidogyne incognita*, *Ditylenchus destructor*, natural biostimulants, anti-nematodic si/miRNA, the degree of homology between mRNA and si/miRNA, silencing activity of si/miRNA in the wheat embryo cell-free system of protein synthesis, plant resistance to nematodes

1. Introduction

Over the last ten years a key role of short interfering RNA (siRNA) and microRNA (miRNA) in the TGS and PTGS - the basic processes of plant development and adaptation to stress-factors of environment, is disclosed (Angaji et al., 2010; Chen, 2009; Filipowicz et al., 2005; Hamilton et al., 2002; Luna et al., 2012; Mirouze et al., 2011; Park et al., 2002; Rasmann et al., 2012; Vaucheret et al., 2001; Zhang et al., 2007). The miRNA is generated from pre-miRNA precursor of ~70 nucleotides (nt) derived from one strand of distinct genomic loci by two rounds of endoribonuclease cleavage by RNase III-like enzymes named Drosha and Dicer (Lee et al., 2003; Mourelatos et al., 2002). The siRNA of ~22-24-nt is generated from longer double-stranded RNA (dsRNA) molecules (derived from repetitive sequences such as transposons and transgenes) through their cleavage by RNase III endoribonuclease named Dicer (Hamilton et al., 2002).

In a process of PTGS also called RNA interference (RNAi) si/miRNA with antisense structure to mRNA functions in a dual role: 1) together with site-specific multi-subunit RNase, referred to as RNA-induced silencing complex (RISC), and with AGO (Argonaute) proteins si/miRNA determines an age period of endogenous mRNA molecule in each eukaryotic cell and 2) together with RISC and AGO proteins si/miRNA participates in enzymatic cleavage or in silencing of translation of homologous mRNA of pathogenic organisms providing protection against pathogens and parasites (Bakhetia et al., 2005; Chen, 2009; Fabian et al., 2010; Filipowicz et al., 2005; Hamilton et al., 2002; Park et al., 2002; Vaucheret et al., 2006; Zhang et al., 2007) Taking part in DNA methylation and histone modification during TGS, and silencing of translation of mRNA of various pathogenic organisms during PTGS, si/miRNA contributes to epigenetic inheritance of plant resistance to diseases (Calarco et al., 2012; Luna et al., 2012; Mirouze et al., 2011; Rasmann et al., 2012; Tsygankova, 2012).

Numerous studies have shown that during infection of plants by pathogenic organisms the changes in small RNA populations (the main components of plant immune system) occur (Hewezi et al., 2008; Katiyar-Agarwal et al., 2006; Padmanabhan et al., 2009; Patel et al., 2010). Targets for si/miRNA are mRNA transcripts of plant genes which expression is induced during infection (therefore damage of plants by phytopathogens raises), or highly homologous mRNA of pathogenic organisms (Baum et al., 2007; Hewezi et al., 2008; Katiyar-Agarwal et al., 2006; Li et al., 2012; Padmanabhan et al., 2009; Patel et al., 2010).

In the case of plant protection against pathogens and parasites, the number of si/miRNA molecules produced in plant cells in response to a mass infection is not sufficient to provide effective protection. There are two approaches to increase synthesis of si/miRNA in response to pathogen or parasite attacks; these are either to insert additional genes of si/miRNA in the cells using genetic transformation or to activate the synthesis of endogenous si/miRNA in plant cells by specific inductors, for example by phytohormones (Bakhetia et al., 2005; Gheysen et al., 2006; Padmanabhan et al., 2009; Spoel et al., 2012; Zhang et al., 2011).

In our previous investigations we have elaborated and proposed the new strategy of nematode disease management: increase of plant resistance to nematodes by the way of inducing of RNA-interference process (RNAi or PTGS) in plant cells, i.e. inducing synthesis of si/miRNA using new ecologically safe polycomponent biostimulants with bioprotective and immune-modulating effects. In our laboratory and field experiments we found that biostimulants significantly increased plant resistance to viral pathogens, nematodes and insect herbivores through stimulation of synthesis in plant cells of immune-protective small regulatory si/miRNA (Tsygankova, Andrusevich et al., 2011; Tsygankova, Galkin et al., 2011; Tsygankova, Andrusevich, Beljavskaja et al., 2012). In these works we used Dot-blot hybridization for the study of changes in the degree of homology between populations of cytoplasmic RNA and small regulatory si/miRNA, isolated from rape, sugar beet, wheat and cucumber plants of the first generation, infected by parasitic nematodes *Heterodera schachtii* and *Meloidogyne incognita*, as well as wheat and nut plants of the second generation, infected by pathogenic micromycetes *Fusarium graminearum* and *Fusarium oxysporum f. ciceris* (Tsygankova, 2012; Tsygankova, Andrusevich et al., 2012; Tsygankova, Ponomarenko et al., 2012; Tsygankova, Stefanovska, Andrusevich et al., 2012; Tsygankova, Stefanovska, Galkin et al., 2012; Tsygankova, Andrusevich, Beljavskaja et al., 2012). Obtained differences in the degree of homology between mRNA and si/miRNA populations we used as genetic markers of increase of plant resistance to phytopathogens**.** Silencing of translation of mRNA activity of si/miRNA was also verified in the experiments with wheat embryo cell-free system of protein synthesis, which is widely used along with other cell-free systems (the rabbit reticulocyte lysate system and cell-free system from syncytial blastoderm Drosophila embryos) for in vitro study of mRNA translation and for investigation of silencing activity of si/miRNA on in vitro inhibition of mRNA translation (Maniatis et al., 1982; Promega, 1991; Tang et al., 2003; Tuschl et al., 1999).

To induce the synthesis of endogenous si/miRNA we treated plant seeds by new natural polycomponent

biostimulants with bioprotective effect - Avercom and its derivatives, created at Zabolotny Institute of Microbiology and Virology, NAS of Ukraine and biostimulants Biogene, Stimpo, and Regoplant, Radostim-super created at the Institute of Bioorganic Chemistry and Petrochemistry, NAS of Ukraine in association with National Enterprise Interdepartmental Science and Technology Center "Agrobiotech" of the NAS and the Ministry of Education, Science and Sport of Ukraine (Iutynska, 2012; Tsygankova, Ponomarenko et al., 2012; Tsygankova, Stefanovska, Galkin et al., 2012; Tsygankova, Andrusevich, Beljavskaja et al., 2012; Tsygankova, Stefanovska, Andrusevich et al., 2012).

Aims of the present work are: a) study of impact of new natural biostimulants on morpho-physiological signs of resistance of sugar beet, cucumber and potato plants to parasitic nematodes: *Heterodera schachtii*, *Meloidogyne incognita* and *Ditylenchus destructor* in the field, greenhouse and laboratory conditions, b) determination of degree of homology between mRNA and si/miRNA populations, isolated from sugar beet, cucumber and potato plants, which were untreated and treated with biostimulants and infected by parasitic phytonematodes *Heterodera schachtii*, *Meloidogyne incognita* and *Ditylenchus destructor*, and c) investigation of silencing activity of si/miRNA populations, isolated from untreated and treated with biostimulants plants, on translation mRNA, isolated from infected plants and from parasitic nematodes, in the wheat embryo cell-free system of protein synthesis.

2. Materials and Methods

2.1 Plant Growing and Treatment

In our greenhouse, field and laboratory molecular-genetic experiments the sugar beet *Beta vulgaris L*., cucumber *Cucumis sativus* of cultivar Gravina and potato *Solanum tuberosum* of cultivar Bellarosa plants infected by the parasitic nematode *Heterodera schachtii* Shmidt, gallic nematode *Meloidogyne incognita* Chitwood and stem nematode of potato *Ditylenchus destructor* Thome respectively were used.

We investigated bioprotective anti-nematodic effects of new polycomponent biostimulants Avercom and its derivatives (contain metabolites of the soil streptomycete *Streptomyces avermitilis* UCM Ac-2179, i.e. antiparasitic antibiotic avermectine, aminoacids, free fatty acids, vitamins of the B group, and phytohormones: indole-3-acetic acid, isopentenyl adenine, zeatin, zeatin riboside, brassinosteroids) (Iutynska, 2012; Tsygankova, Andrusevich, Beljavskaja et al., 2012); Radostim-super (contains antiparasitic antibiotic aversectine C - metabolites of the soil streptomycete *S. avermitilis* and metabolites, i.e. aminoacids, fatty acids, polysaccharides, phytohormones, and microelements, of cultivated *in vitro* micromycete *Cylindrocarpon obtusiuscuilum* 680, isolated out of Panax ginseng root system) (Tsygankova, Stefanovska, Galkin et al., 2012; Tsygankova, Stefanovska, Andrusevich et al., 2012).

Experimental plants growing at laboratory, greenhouse and field conditions were treated by biostimulants Avercom and Radostim-super. Avercom was obtained by ethanol extraction from of 7-days biomass of *Streptomyces avermitilis* UCM Ac-2179, the concentration of avermectine is 100 μg/ml, its derivates: Avercom nova-1 contains 50 ml of Avercom with antibiotic avermectine at concentration 100 μg/ml with adding 50 ml of supernatant of liquid culture *Streptomyces avermitilis* UCM Ac-2179 and 0.05 mM of salicylic acid; total content of avermectine is 50 μg/ml; and Avercom nova-2 contains 50 ml of Avercom with antibiotic avermectine in concentration 100 μg/ml and 50 ml of supernatant of liquid culture *Streptomyces avermitilis* UCM Ac-2179 and 0.01 mM of water-soluble chitosan of "Sigma" company; the total concentration of avermectine is 50 μg/ml.

Sugar beet and cucumber seeds were sprouted in Petri dishes (9.5 cm in diameter) in nematode-free aqueous medium (control) or with a suspension of nematodes *H. schachtii* and *M. incognita* eggs (at the concentration of 20-50 nematode eggs/ 20 seeds). The seeds were incubated at 23 °C and the nematode larvae hatched in 5-7 days later in average. Each experiment performed in three replicates. In experiments with sugar beet and cucumber seeds we used 4 variants: 1) seeds incubated on aqueous medium (control), 2) seeds incubated on aqueous medium with biostimulant Radostim-super (at the concentration of 25 μl/ml distilled water with final content of aversectine C - 0.025 μg/ml) and with biostimulant Avercom (at the concentration of 25 μl/ml distilled water with final content of avermectine - 0.05 μg/ml), 3) seeds incubated on aqueous medium with a suspension of nematode eggs, 4) seeds incubated on aqueous medium with biostimulant Radostim-super (at the concentration of 25 μl/ml distilled water with final content of aversectine C - 0.025 μg/ml) and with biostimulant Avercom (at the concentration of 25 μl/ml distilled water with final content of avermectine - 0.05 μg/ml) and a suspension of nematode eggs.

Cucumber plants of cultivar Gravina were also growing at greenhouse conditions at a background of artificial contamination with nematode *M. incognita* in a quantity of 700 larvae and eggs in 100 cm^3 of soil sample. 7 days after the contamination hollows were made in the substratum, in which 100 ml of 2% solutions of each

biostimulants were added; after 2 days the seedlings were planted, roots of seedlings were immersed into solutions of biostimulants for 5 minutes before planting. Damage caused by nematodes was scored in points according to methodical guidelines (Sigareva, 1986).

Investigations with potato of cultivar Bellarosa were carried out in field conditions as little-strip experiments with natural and artificial invasive background, created by planting into hollows 50 g of potato tubers infectected previously by nematode *D. destructor* larvae. Potato plants were treated by biostimulants in the next concentrations: for cultivation of 1 tone of planting material 0.4 l of Avercom or its derivates were dissolved in 20 l of water.

All the experiments were performed in four replicates.

2.2 Identification of Degree of Homology Between si/miRNA and mRNA Populations

Degree of homology between cytoplasmic mRNA and small regulatory si/miRNA populations, isolated from control and experimental plants was determined in the molecular-genetic experiments using Dot-blot hybridization method (Maniatis et al., 1982). Isolation of total RNA from plant cells and separation of high-purity si/miRNA preparations were carried out by our earlier published method (Tsygankova, Andrusevich et al., 2011; Tsygankova, Stefanovska, Andrusevich et al., 2012), the size (of 21-25 nt) of isolated si/miRNA preparations was verified by electrophoresis in a 15% polyacrylamide gel (Maniatis et al., 1982).

Plant si/miRNA labeled *in vivo* with ^{33}P using $Na_2HP^{33}O_4$ before isolation was later used for Dot-blot hybridization with own plant mRNA and with nematode mRNA (Tsygankova, Andrusevich, Beljavskaja et al., 2012; Tsygankova, Stefanovska, Andrusevich et al., 2012). Hybridization was conducted on modified and activated cellulose filters (Whatman 50, 2-aminophenylthioether paper of Company Amersham-Pharmacia Biotech, UK) that form covalent linkages with deposited DNA or RNA unlike the cellulose and nitrocellulose filters that form hydrogen bonds with DNA or RNA enabling to avoid losses of the nucleic acids in the course of the filter washing out (Maniatis et al., 1982).

Radioactivity of hybrid molecules was detected (imp/count per min/20 μg ± SE of mRNA) on glass Millipore AP-15 filter in toluene scintillator using Beckman LS 100C scintillation counter. Degree of homology (%) was determined according to the difference of hybridization between mRNA and si/miRNA from experimental plants and control plants (Tsygankova et al., 2010; Tsygankova, Stefanovska, Andrusevich et al., 2012).

2.3 Determination of Silencing Activity of si/miRNA Populations in the Cell-Free System of Protein Synthesis

Investigation of silencing activity of si/miRNA, isolated from untreated and treated by biostimulants plants, on translation of own plant mRNA or nematode mRNA, was conducted in the wheat embryo cell-free system of protein synthesis, which preparation is described in details elsewhere (Marcus et al., 1974; Tsygankova et al., 2010). Reagents of different companies, namely Amersham-Pharmacia Biotech, UK; New England Biolab, USA; Promega Corporation Inc, USA and Boehringer, Dupont, NEN, USA and Mannheim GmbH, Germany were used for preparation of cell free-system. Determination of inhibition by si/miRNA of protein synthesis on the templates own plant mRNA or nematode mRNA in the cell-free system was carried out according to index of decreasing of incorporation [^{35}S] methionine into proteins and was accounted (in imp./count per min/1mg of protein) on glass filter Millipore AP-15 in toluene scintillator in the scintillation counter LS 100C. Unlabelled si/miRNA were used for testing of inhibitory activity of si/miRNA in the cell-free system of protein synthesis. The silencing activity of si/miRNA (%) was determined as a difference of radioactivity of polypeptides (count per min/1mg of protein) synthesized on template mRNA by experimental plants compared to control plants.

The statistical analysis of the data was carried out by dispersive (Student) method (Bang et al., 2010). The least substantial difference ($LSD_{0.05}$) was also calculated for the field experiments (Dospechov, 1985).

3. Results

3.1 Investigation of Morpho-Physiological Signs of Plant Resistance to Parasitic Nematodes Under Impact of Natural Biostimulants

Biometric researches showed that in the experiments with cucumber plants, which were grown at greenhouse conditions on artificial infectious background created by nematode *M. incognita*, at the end of vegetation period the height of cucumber plants, treated (pre-sowing treatment of seeds and crop spraying) with biostimulant Avercom and its derivatives, exceeds the height of control plants by 10-24% (Table 1). The highest height of the plants was obtained at their treatment by biostimulant Avercom nova-1. The analysis of plant damage by nematodes showed that the plant damage by nematode *M. incognita* reached up to 3.4 points on the control plot. At the same time for cucumber plants treated by biostimulants, considerable decrease of contamination by

nematode *M. incognita* (to 29.4% on an artificial background) was observed. In the case of Avercom plant damage by nematodes was not observed, in the case of Avercom derivatives the plant damage was less than 0, 2 - 1, 0 points.

Table 1. Biometric characteristics and degree of damage by nematodes of cucumber plants treated with biostimulants

Experience variant	Height of plants		Damage of plants by nematode *M.incognita*, (in point)
	cm	% in relation to control	
Control (without use of biostimulants)	168±5.9	100	3.4
Avercom	197±6.9	117	0
Avercom nova-1	208±7.3	124	0.2
Avercom nova-2	184±6.4	110	1.0

We have also studied the impact of biostimulants on plant productivity. The yield of cucumbers treated by Avercom and its derivatives was 16-26% higher compared to control. The highest yield was obtained when the plants were treated with Avercom (Table 2).

Table 2. The yield of cucumber plants treated with biostimulants

Experience variant	Yield, kg/m^2	Yield increase	
		kg/m^2	% in relation to control
Control (without use of biostimulants)	6.8	0	100
Avercom	8.6	1.8	126
Avercom nova-1	8.3	1.5	122
Avercom nova-2	7.9	1.1	116
LSD $_{0.05}$	0.2		

In the experiments with potato plants, which were carried out on a natural invasive background and artificial background, created by potato stem nematode *D. destructor*, it was shown that pre-sowing treatment of potato tubers and crop spraying with biostimulants Avercom and its derivatives decreased damage of potato tubers by dytylenchosis and increased crops productivity (Table 3).

Table 3. Damage of potato by ditylenchosis and productivity of potato plants under various conditions of growing

Experiment	Damage of plants by ditylenchosis		Productivity of crops	
	Quantity of sick potato tubers,%	Biological efficiency of biostimulants	Yield, pounds/ha	Yield increase (%) in relation to control
Natural background				
Control (without use of biostimulants)	40.1	-	36375.9	0
Avercom	11.8	70.5	40785.1	12.1
Avercom nova-1	24.7	38.4	37478.2	3.0
Avercom nova-2	34.4	14.2	38580.5	6.1
$LSD_{0.05}$	5.1	-	1763.7	
Artificial invasion background				
Control (without use of biostimulants)	48.0	-	33950.8	100
Avercom	21.5	55.2	37478.2	10.4
Avercom nova-1	34.0	29.2	36596.4	7.8
Avercom nova-2	44.0	8.3	35494.1	4.5
$LSD_{0.05}$	5.1	-	1543.2	

We have obtained the considerable decrease of contamination (to 26.5% on an artificial background) of potato plants by nematode *D. destructor*. Biological efficiency of Avercom reached more than 70% in experiments on a natural nematodic background and 55% on artificial invasion background created by nematodes, the less biological efficacy showed by Avercom nova-1 - 38.4% and 29.2%.

Impact of the biostimulants on potato productivity was also studied. Biostimulants promoted increase in potato productivity up to 12.1% - on natural nematode infection and up to 10.4% - in conditions of artificial infection.

On natural invasive background the most statistically significant increase in productivity (up to 12.1% compared to the control) of potato was achieved at pre-sowing planting material treatment with Avercom. On artificially created invasive background Avercom promoted the increasing of yield of potato by 10.4%.

Similar experiments devoted to impact of biostimulant Radostim-super on morpho-physiological signs and productivity of winter wheat plants have been conducted by us early in field conditions (Figure 1) (Sweere et al., 2011). Results obtained in these experiments testify about positive influence of biostimulant Radostim-super on dynamics of growth and increase of productivity of winter wheat. We conducted similar experiments on other agricultural plants; the considerable increase of photosynthetic activity (i.e. assimilation of CO_2 by plants) was obtained when plants were treated by biostimulant Radostim-super: rye (up to 12.5%), barley (up to 10.3%), oats (up to 13.7%), millet (up to 11.4%) corn (up to 12.9%), rice (up to 13.2%), buckwheat (up to 10.7%) (Sweere et al., 2011). Considerable increase of productivity of these crops from 20% to 65% under the impact of biostimulant Radostim-super was found in these investigations.

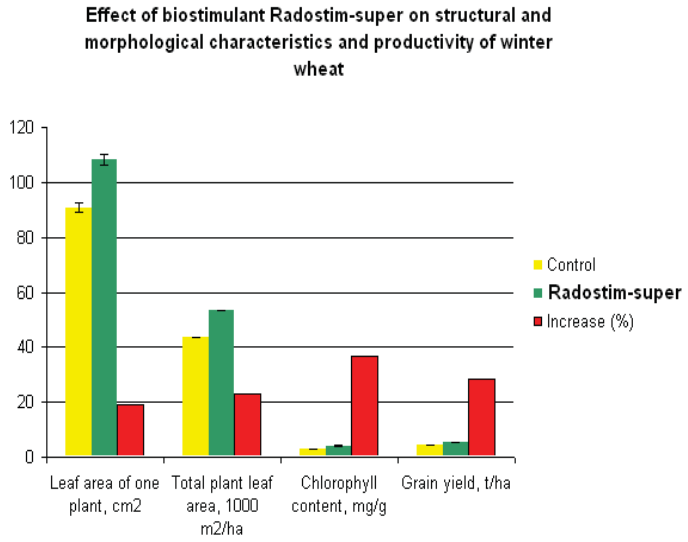

Figure 1. Impact of biostimulant Radostim-super on morpho-physiological signs and productivity of winter wheat plants grown under field conditions (seeds of winter wheat were treated before sowing with biostimulant Radostim-super at the concentration of 25 ml/t of seeds)

We have also tested in the field and greenhouse conditions the bioprotective effects of biostimulant Radostim-super against sugar beet nematode *H. schachtii* (Tsygankova, Stefanovska, Galkin et al., 2012). We have shown that treatment of sugar beet seeds and spraying of crops in vegetation period by Radostim-super considerably decreases the sugar nematode population density in the soil by 74.2%, whereas in control experiments (seeds treated by water) the beet nematode number in soil increased by 22%. In addition to the reduction in nematode numbers, the application of Radostim-super increased the sugar beet yield and sugar yield which were significantly higher than in the control by 40.0 and 6.2 tons/ha, respectively (Tsygankova, Stefanovska, Andrusevich et al., 2012; Tsygankova, Stefanovska, Galkin et al., 2012).

It was found in the laboratory experiments (Figure 2 and Figure 3) that 5-day sugar beet sprouts (obtained from seeds infected by nematode *H. schachtii* and treated with biostimulant Radostim-super) and 5-day cucumber sprouts (obtained from seeds infected by nematode *M. incognita* and treated with biostimulant Avercom) were more viable and resistant to these nematodes as compared to control sprouts (infected by nematodes and untreated with biostimulants).

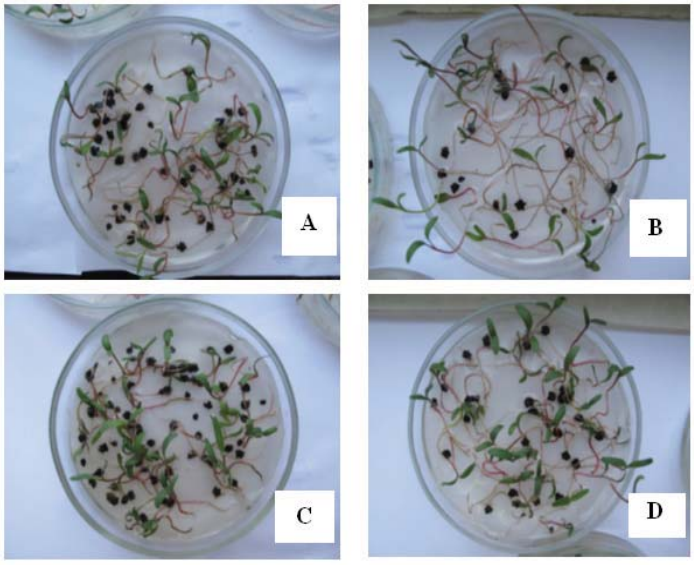

Figure 2. Impact of biostimulant Radostim-super on germination of sugar beet *Beta vulgaris L.* seeds and development of sprouts

A) 5-day sprouts grown on distilled water (control); B) 5-day sprouts grown on infectious background, created by parasitic nematode *H. schachtii*; C) 5-day sprouts treated with Radostim-super and grown on infectious background, created by parasitic nematode *H. schachtii*; D) 5-day sprouts treated with Radostim-super and grown without infectious background.

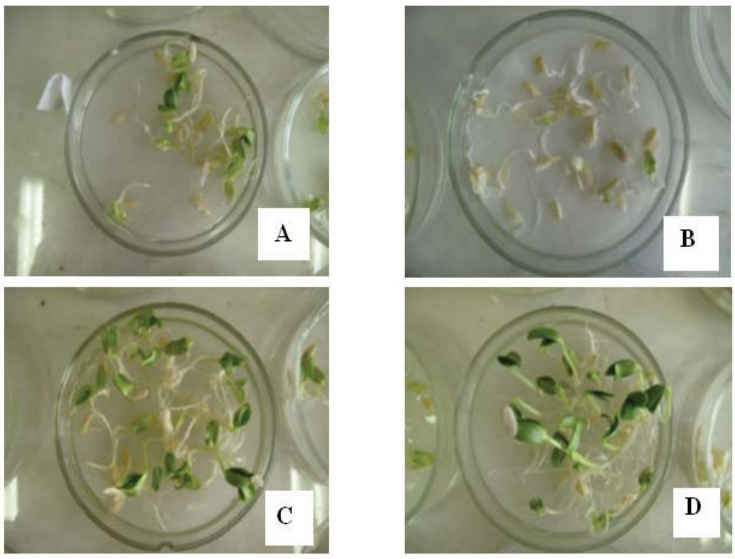

Figure 3. Impact of biostimulant Avercom on germination of cucumber seeds of cultivar Gravina and development of sprouts

A) 5-day sprouts grown on distilled water (control); B) 5-day sprouts grown on infectious background created by parasitic nematode *M. incognita*; C) 5-day sprouts treated with Avercom and grown on infectious background, created by parasitic nematode *M. incognita*; D) 5-day sprouts treated with Avercom and grown without infectious background.

3.2 Impact of Biostimulant Radostim-Super on Changes in the Degree of Homology Between si/miRNA and mRNA, and on Silencing Activity of si/miRNA

Changes in the level of si/miRNA synthesis (according to degree of homology between si/miRNA and mRNA)

in the control plants, in the plants treated by Radostim-super, in the plants incubated with nematode *H. schachtii* and in the plants infected by nematode *H. schachtii* and treated with Radostim-super were determined using Dot-blot hybridization method.

Radioautographs on cellulose filters of probes which are hybrid molecules of mRNA isolated from control plants with [P^{33}]-si/miRNA isolated from experimental plants treated by biostimulants and grown without infectious background are presented in Figure 4.

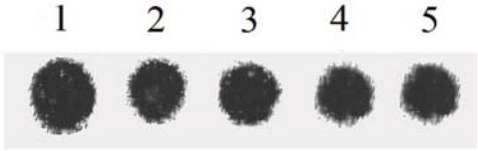

Figure 4. Radioautographs on cellulose filters of probes (hybrid molecules of mRNA from control plants with [P^{33}]-si/miRNA from experimental plants)

1) mRNA and si/miRNA from control plants; 2) mRNA from control plants and si/miRNA from sugar beet plants, treated with biostimulant Radostim-super; 3) mRNA from control plants and si/miRNA from cucumber plants, treated with biostimulant Avercom; 4) mRNA from control plants and si/miRNA from cucumber plants, treated with biostimulant Avercom nova-1; 5) mRNA from control plants and si/miRNA from cucumber plants, treated with biostimulant Avercom nova-2.

It is shown in Figure 5 that according to degree of homology between populations of mRNA and si/miRNA Radostim-super considerably increased the synthesis of si/miRNA in plants not infected by nematodes, but on the contrary in plants infected by nematodes and untreated with this biostimulant the synthesis of si/miRNA is sharply reduced. Biostimulant Radostim-super increases si/miRNA synthesis in infected plants, but in these plants the level of synthesis is lower compared with level of synthesis in plants not infected by nematodes and not treated with this biostimulant.

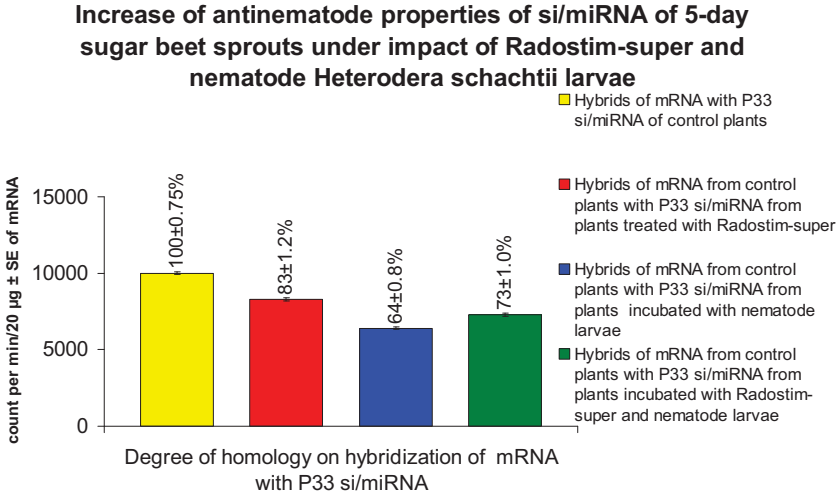

Figure 5. The degree of homology (%) between populations of mRNA and si/miRNA from control and experimental sugar beet plants infected by nematode larvae and treated with biostimulant Radostim-super

Investigation of inhibitory (silencing) activity of si/miRNA on the template of mRNA from plants in the wheat embryo cell-free system of protein synthesis showed (Figure 6) that si/miRNA, isolated from plants not infected by nematodes and treated with Radostim-super, showed high inhibitory activity (82%), close to those of control plants (100%). Obtained results testify that this biostimulant changes si/miRNA population in plant cells.

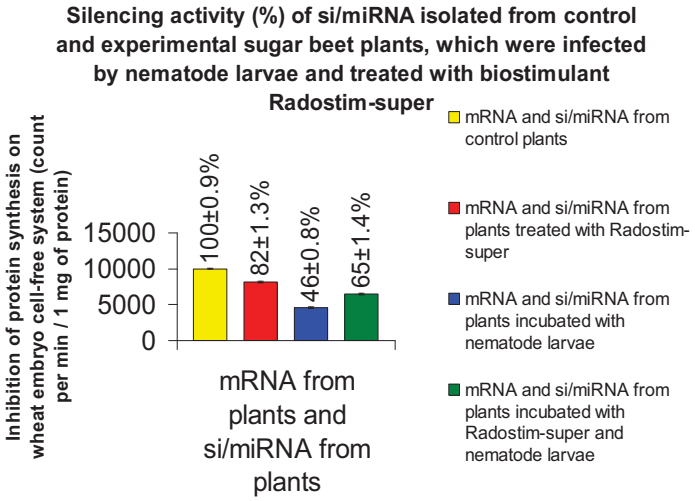

Figure 6. Inhibition of protein synthesis in the wheat embryo cell-free system on the template of mRNA from control and experimental plants by si/miRNA from control and experimental sugar beet plants infected by nematode larvae and treated with biostimulant Radostim-super

The inhibitory activity of si/miRNA, isolated from the same plants, treated by biostimulant, on the template mRNA from nematode larvae (Figure 7) was slightly higher (15%) than that of control si/miRNA, isolated from untreated plants (10%). This shows insignificant homology between plant si/miRNA and nematode mRNA.

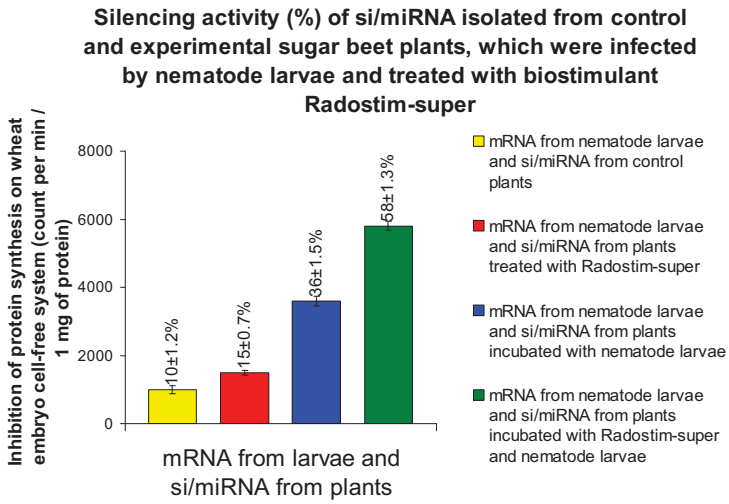

Figure 7. Inhibition of protein synthesis in the wheat embryo cell-free system on the template of mRNA from nematode larvae by si/miRNA from control and experimental sugar beet plants infected by nematode larvae and treated with biostimulant Radostim-super

At the same time (Figure 6 and Figure 7) the inhibitory activity of si/miRNA, isolated from plants, infected by nematode larvae and treated by Radostim-super, considerably increased both on the template of mRNA from the same plants (65%) and on template of mRNA from nematode larvae (58%).

At the same time inhibitory activity of si/miRNA, isolated from plants infected by nematode larvae and untreated by biostimulant, was lower both on the template of mRNA from plants (46%) and on the template of mRNA from nematode larvae (36%).

Obtained results confirm that biostimulant Radostim-super causes reprogramming of plant genome to induce synthesis of si/miRNA specific (antisence) both to own plant mRNA (which expression promotes infection) and to homologous mRNA of nematodes.

3.3 Impact of Biostimulant Avercom and Its Derivatives on Homology Between si/miRNA and mRNA, and on Silencing Activity of si/miRNA

Radioautographs on cellulose filters of probes which are hybrid molecules of mRNA isolated from control plants with [P^{33}]-si/miRNA isolated from experimental plants treated by biostimulants and grown without infectious background are presented above in Figure 4.

Data for the analysis of degree of homology between populations of cytoplasmic mRNA and si/miRNA, isolated from control and experimental potato plants and cucumber, which were grown in greenhouse and field conditions on an artificial infectious background and treated with biostimulant Avercom and its modifications, are presented in Figure 8.

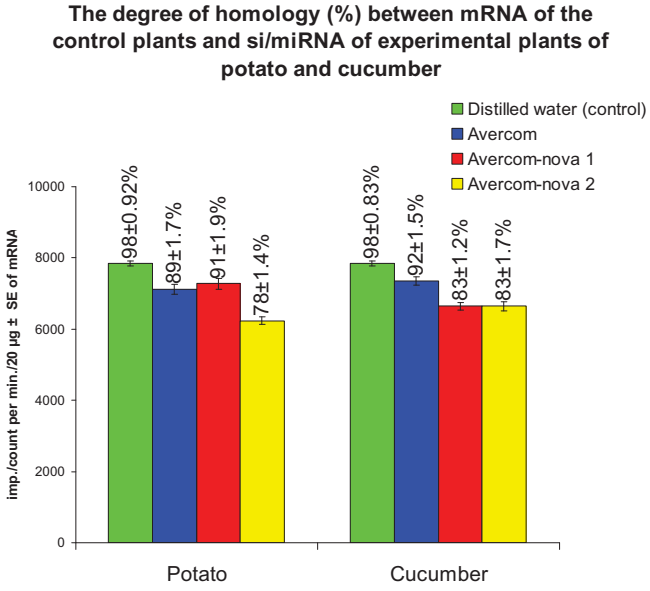

Figure 8. Degree of homology (%) between mRNA of the control plants and si/miRNA of the control and experimental potato and cucumber plants treated with biostimulants and infected by parasitic gallic nematode *M. incognita* and stem nematode of potato *D. destructor*

Comparative analysis of degree of homology (%) between si/miRNA and mRNA (Figure 8) obtained in the experimental plants compared to the same values in the control plants, showed that the largest difference in the degree of homology regarding to control plants was observed in experimental plants treated with biostimulants Avercom nova-2 (up to 20% - in potato and up to 15% - in cucumber plants) and Avercom nova-1 (up to 15% - in cucumber and up to 7% - in potato plants), smaller difference in the degree of homology was found in the experimental plants treated with biostimulant Avercom (up to 6% - in cucumber and 9% - in potato plants).

According to experiments in the wheat embryo cell-free system of protein synthesis, results of inhibition of the translation of mRNA from the cucumber and potato plants infected with nematodes *M. incognita* and *D. destructor* and treated with biostimulant Avercom and its derivates show significant increase of silencing activity of si/miRNA (similar to activity of si/miRNA from not infected plants - control N1) isolated from cucumber and potato plants infected by nematodes *M. incognita* and *D. destructor* and treated with these biostimulants (Figure 9).

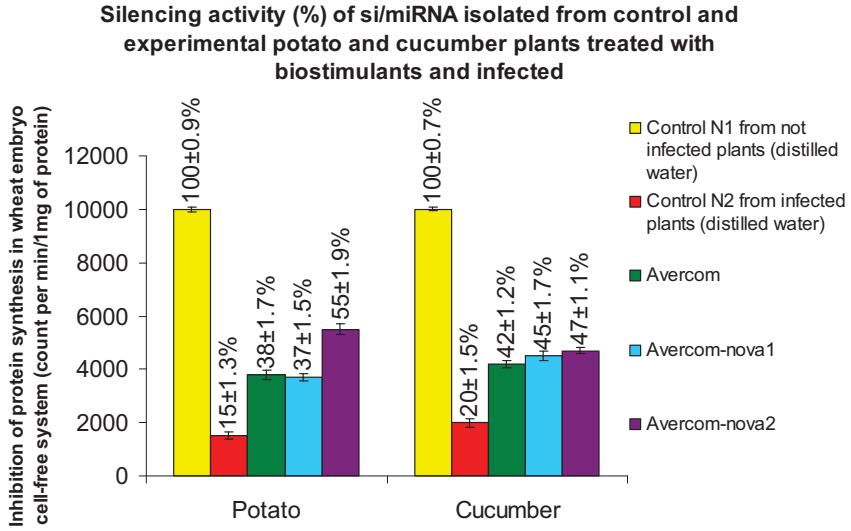

Figure 9. Inhibition of protein synthesis in the wheat embryo cell-free system on the template of mRNA from control and experimental plants by si/miRNA from control and experimental potato and cucumber plants treated with biostimulants and infected by parasitic gallic nematode *M. incognita* and stem nematode of potato *D. destructor*

According to results of inhibition of the translation of mRNA from cucumber and potato plants infected with nematodes *M. incognita* and *D. destructor* the highest silencing activity (compared to control N 1) was shown by si/miRNA isolated from the same plants treated with biostimulants (Figure 9) Avercom nova-2 (up to 55% - in potato and up to 47% - in cucumber plants) and Avercom nova-1 (up to 45% - in cucumber and up to 37% - in potato plants), the lower silencing activity was shown by si/miRNA isolated from experimental plants treated with Avercom (up to 42% - in cucumber and up to 38% - in potato plants). Significantly lower silensing activity (up to 15% - in potato, up to 20% - in cucumber plants) was shown by si/miRNA, isolated from infected plants, which were untreated with biostimulants (control N 2).

4. Discussion

Existing methods for controlling the distribution of nematodes and the reduction in the yield of important crops caused by them are chemically synthesized soil fumigants, nematicides (belonging to the classes of organophosphates and carbamates), and various types of insecticides of natural origin, for example, phytoinsecticide pyrethryn and its synthetic analogs, i.e., pyrethroids (Mitkowski et al., 2003; Oka, 2010; Winter et al., 2006). In most countries worldwide, however, a trend is observed towards practically restricting their use because of their high toxicity to humans and contamination of the environment. Traditional methods to regulate the amount of parasitic nematodes also include various biocontrol technologies, i.e., application of various organic soil fertilisers and industrial waste of vegetable or animal origin, compost, and changes in soil pH (acidification of up to pH 4 or alcalinization of up to pH 8); introduction of antagonistic and competitive microorganisms (bacteria of the strains *Burkholderia cepacia* and *Bacillus chitinosporus* and the fungi micromycetes *Myrothecium verrucaria* and *Paecilomyces lilacinus*) to soil; crop rotation with the development of cultures resistant to nematodes, using biopreparations that contain essential oils of various herbs with an anti-nematodic effect (for example, the oil of sesame, garlic, rosemary, or white pepper); etc. (Oka, 2010). Unfortunately, a combination of the above listed methods can only depress the high viability of this pest class.

Now the success in increasing of plant resistance to nematodes has been reached by genetic engineering and breeding methods (Bleve-Zacheo et al., 2007; Fairbairn et al., 2007; Fuller et al., 2008; Gheysen et al., 2006; Liao et al., 2003; Tsygankova, Andrusevich, Ya, Ponomarenko et al., 2013; Tsygankova, Yemets et al., 2013).

The newer approach for nematode disease management is to increase plant resistance against agricultural pests by new ecologically safe plant growth regulators of natural or synthetic origin, phythohormones, seaweed and plant extracts, and organic compounds (such as sugar or ascorbic acid). In favor of this new approach testify numerous studies (Acquaah, 2007; Aktaruzzaman et al., 2012; Arrigoni et al., 1979; Bleve-Zacheo et al., 2007; Dias-Arieira et al., 2013; Fortum et al., 1983; Khan et al., 2009; Mitkowski et al., 2003; Moghaddam et al., 2012;

Olaiya et al., 2013; Tsygankova, Andrusevich et al., 2012; Tsygankova, Andrusevich et al., 2013; Verhage et al., 2010).

For example, the impact of five commercial products marketed as systemic resistance (SR) and plant growth promotion (PGP) inducers on increase of tomato plant (*Lycopersicon esculentum* Mill.) resistance to pathogenic bacteria or nematodes in greenhouse conditions has been studied by Vavrina et al., 2004.

These SR/PGP inducers included a bacterial suspension [Companion (*Bacillus subtilis* GB03)], two plant defense elicitors with nutrients (Keyplex 35ODP plus Nutri-Phite, and Rezist with Cab'y), natural plant extracts (Liquid Seaweed Concentrate and Stimplex), and synthetic growth regulator (Actigard 50W). Comparative analysis of growth stimulating and bioprotective effects of SR/PGP inducers have shown that highest suppression of bacterial spot [*Xanthomonas campestris* pv. *vesicatoria* (Xcv)] is caused by synthetic regulator Actigard. Other SR/PGP inducers: Companion, Keyplex 35ODP plus Nutri-Phite, Rezist, Cab'y, Liquid Seaweed Concentrate and Stimplex induced only partial suppression of bacterial spot in inoculated tomato plants. The alpha-keto acids plus nutrients (Keyplex 35ODP plus Nutri-Phite) increased plant growth by 14.3% and improved root condition compared to untreated control following exposure to nematodes.

In the present work, we investigated the impact of biostimulants on inducing of RNA-interference process (RNAi or PTGS) in plant cells, i.e. increasing synthesis of si/miRNA with immune-protective anti-nematodic properties. We studied genetic mechanisms of increase of sugar beet, cucumber and potato plant resistance to parasitic nematodes *H. schachtii*, *M. incognita* and *D. destructor* under impact of biostimulants: Radostim-super, Avercom and its derivatives. The experiments we based on the assumption that plants, infected with different types of pathogenic or parasitic organisms, increased the synthesis of si/miRNA specific both to own plant mRNA (which expression rises at the specialized infected plant cells and involves plant developmental processes) and to pathogenic or parasitic highly homologous mRNA (Hewezi et al., 2008; Ithal et al., 2007; Katiyar-Agarwal et al., 2006; Klink et al., 2009; Li et al., 2012; Padmanabhan et al., 2009; Patel et al., 2010). We also assumed that the biostimulants induce synthesis of si/miRNA which improves plant immunity through the specified mechanism of si/miRNA action.

We need to be sure that our assumptions are correct, so that a new generation of biostimulants with the properties of selective activation of synthesis of si/miRNA, which is specific to own plant cell mRNA or to pathogenic or parasitic highly homologous mRNA can be created. Thus we verified the changes in the populations of si/miRNA (according to degree of homology between si/miRNA and mRNA) and compared silencing activity of si/miRNA from biostimulant-treated and untreated plants. The conciderable differences obtained in these experiments in the degree of homology between si/miRNA and mRNA from control and experimental plants and high silencing activity of si/miRNA from plants, which were treated with biostimulants, testify about impact of these biostimulants on reprogramming of plant genome to induce synthesis of immune-protective si/miRNA in plant cells. As a result the resistance of plants to parasitic nematodes considerably rises.

The results of this work correlate and supplement the data of our previous experiments, in which we have conducted numerous investigations on various agricultural plants like rape, wheat, chickpea, corn and soybean, which were grown on invasion background created by pests: ground beetle, *Zabrus tenebrioides*, turnip moth *Scotia segetum* and *Chloropidae spp.*; pathogenic micromycetes: *Mucor spp.*, *Rhizopus spp.*, *Aspergillus spp.*, *Penicillium spp.*, *Trichothecium roseum*, *Fusarium graminearum*, *Fusarium oxysporum f. ciceris*, *Alternaria alternata*; phytonematode *Anguina tritici* (Tsygankova, 2012; Tsygankova, Ponomarenko et al., 2012; Tsygankova, Stefanovska, Andrusevich et al., 2012; Tsygankova, Andrusevich et al., 2011; Tsygankova et al., 2013). In these field and greenhouse experiments we have shown that treatment of plant seeds and spraying of crops in vegetation period by biostimulants of natural origin: Regoplant, Stimpo, Radostim and Radostim-super considerably increased (up to 74-98%) plant resistance to above mentioned phytopathogens.

Biological efficiency of biostimulant of microbiological origin Avercom and its composition with elicitors was also tested on early wheat, cucumber and tomato crops in field and greenhouse experiments with natural and artificial invasive backgrounds, created by phytonematodes: *Tylenchorbynchus dubius*, *Pratylenchus pratensis*, *Meloidogyne incognita* and by phytopathogenic micromycete *Fusarium oxysporum* (Iutynsk*a* et al., 2011; Tsygankova, Galkin et al., 2011; Tsygankova, Andrusevich, Beljavskaja et al., 2012). High antagonistic activity of biostimulants against all specified phytopathogens was found in the experiments. The plants treated with these biostimulants showed considerably increased resistance (up to 85-100%) to specified phytopathogens.

The molecular-genetic experiments, which were conducted in all these works, showed that increased plant resistance to phytopathogens is caused by inducing effect of these biostimulants on synthesis in plants of si/miRNA immune-protective against nematodes.

5. Conclusion

It was found that in the field, greenhouse and laboratory experiments according to morpho-physiological signs of plants the application of new natural biostimulants: Radostim-super, Avercom and its derivates (containing bioprotective substances - aversectine and avermectine) leads to considerable increase of resistance of sugar beet, cucumber and potato plants to nematodes *H. schachtii*, *M. incognita* and *D. destructor*.

In the molecular-biological investigations we found conciderable lowering of homology (from 6 to 28%) between si/miRNA and mRNA populations from experimental (infected by these nematodes and treated with biostimulants) plants and control plants.

These differences in degree of homology may be the result of activation of synthesis of small regulatory si/miRNA with high anti-nematodic activity by biostimulants in plants. Increase of silencing activity of si/miRNA (up to 38-65%) of the plants infected by nematodes conforms to this assumption. This effect under the impact of biostimulant Avercom and its derivatives significantly increases plant resistance to parasitic nematodes. Obtained changes in degree of homology between mRNA and si/miRNA populations can be used as genetic markers of increase of plant resistance to phytopathogens.

Acknowledgements

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Cadmium-Induced Changes in Germination, Seedlings Growth, and DNA Fingerprinting of *in vitro* Grown *Cichorium pumilum* Jacq.

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Abstract

The aim of this study was to assess the effect of Cd^{2+} on germination, growth, proline content, lipid peroxidation, and DNA fingerprinting of *in vitro* grown *Cichorium pumilum.* Results showed that seed germination was highly inhibited by cadmium (down to 47% at 1600 µM $CdCl_2$). In addition, root and shoot growth showed significant decreases in response to $CdCl_2$ level. Analysis of proline content and lipid peroxidation showed that with increasing $CdCl_2$ levels in the growing medium, the amount of proline accumulation and lipid peroxidation increased gradually. Total chlorophyll content was found to increase only at higher tested levels of Cd^{2+} (800 and 1600 µM). The results also show that Cd^{2+} inhibits callus growth at different levels starting from 50 µM $CdCl_2$ compared with the control in the callus growth experiment. Callus growth ceased completely at 200 µM $CdCl_2$ and above. Random amplified polymorphic DNA (RAPD) analysis showed DNA alterations in Cd^{2+}-treated *C. pumilum* microshoots compared with the control. The results of this experiment showed that Cd^{2+} stress affects several physiological, biochemical, and molecular processes in *C. pumilum.*

Keywords: Cadmium, proline, lipid peroxidation, fingerprinting, *Cichorium pumilum*

1. Introduction

Chicory (*Cichorium pumilum* Jacq., Asteraceae), is a bushy perennial herb with blue or lavender flowers which grows as a wild plant on roadsides. Chicory is also known as blue sailors, endive, radicchio, French endive, red endive, sugarloaf, witloof, elit, and coffeeweed. It is a culinary and medicinal plant grown worldwide. In the Middle East, its leaves are widely used in salads after being blanched, as the unblanched leaves taste bitter. In Europe, the root is eaten like a vegetable after being boiled, or it can be roasted then ground for use as a coffee substitute (Robert et al., 2008). Al Khateeb et al. (2012) showed that *C. pumilum* methanolic extracts have high levels of phenolic compounds and showed very strong antioxidant properties. Moreover, they found that methanol and ethanol extracts obtained from *C. pumilum* have antimicrobial effects on 10 different bacterial species.

In the last few decades, a dramatic increase in the contamination of the environment, (including soil, air, and water) has been observed. It appears that anthropogenic activities are the main source of the pollution that is causing the environment contamination (Gratao et al., 2005). Recently, it has been shown that large areas of land have been contaminated with heavy metals as a result of urban activities, agricultural practices, and industry.

Heavy metals are defined as the group of elements that have specific weights higher than about 5 $g \times cm^{-3}$. A number of them (Co, Fe, Mn, Mo, Ni, Zn, Cu) are essential micronutrients which are required for normal growth and for many metabolic processes in plants. Metals which are considered nonessential (Pb, Cd, Cr, Hg, etc.) are potentially highly toxic for plants (Sebastiani et al., 2004). Contamination of soil by heavy metals is a global ecological problem because heavy metals are included in the main category of environmental pollutants which can remain in the environment for long periods. Their accumulation is potentially hazardous to humans, animals, and plants (Benavides et al., 2005).

Agricultural soil contamination can severely affect humans, both directly (through the food web) and indirectly (by damaging environmental health) (Nriagu, 1990). For plants, heavy metals are phytotoxic, causing growth inhibition and eventually plant death through mechanisms that are still not completely understood (Romero-Puertas et al., 1999). The toxic effect of increasing cadmium (Cd) concentration in the environment has

become a major environmental concern (Shriarastava & Singh, 1989). Cadmium accumulation in soils may come from various sources: from air pollutants or through applications of commercial fertilizers, sewage sludge, manure, and lime (Kidd et al., 2007). Also, industrial effluents may contain a wide variety of pollutants depending on the industries involved (Iribar et al., 2000). Cd is generally present in soil as free ions or in different soluble forms, and its mobility is affected by pH and the presence of chelating substances and other cations (Hardiman & Jacoby, 1984). In plants, Cd is accumulated mainly in the edible parts, thus making crop yield a potential hazard for human and animal health. It has been suggested that Cadmium may cause damage even at very low concentrations, and healthy plants may contain Cd levels that are toxic for mammals (Chen et al., 2007). Pinot et al. (2000) showed that the main source of Cd accumulation in food is the Cd uptake by plants.

It has been shown that Cadmium can inhibit plant growth and photosynthesis, reduce chlorophyll content, and induce oxidative stress (Schill et al., 2003). In addition, the genotoxicity of Cd is directly related to its effect on the structure and function of DNA. Therefore, Cd toxicity not increases the mortality rate in the exposed organisms, but may also result in the modification of the population dynamics and the species biological diversity (Theodorakis et al., 2006). Furthermore, it has been shown that cadmium generates oxidative stress through the formation of reactive oxygen species (ROS). The formation of ROS by cadmium suggests that DNA can also be taken into account as a potential target of this metal (Błasiak, 2001).

It has been suggested that proline plays a role in protecting plants from heavy metal toxicity. Siripornadulsil et al. (2002) reported that proline reduces cadmium stress not by sequestering cadmium but by reducing cadmium-induced free radical damage, thus maintaining a more reducing environment in the cell. Xu et al. (2009) found that proline pre-treatment of *Solanum nigrum* reduces the reactive oxygen species levels and protects the plasma membrane integrity of callus under cadmium stress.

The objectives of this study were to study the effects of Cd on germination, seedling and callus growth, biochemical properties and DNA fingerprint of *in vitro* grown *C. pumilum*.

2. Materials and Methods

2.1 Plant Material, Seed Germination and Proliferation

Ripe fruits of *Cichorium pumilum* Jacq were collected from Irbid/Jordan during the summer of 2011. Seeds were surface sterilized with 2% sodium hypochlorite solution for 10 min, then washed with 70% ethanol for 30 s, followed by three rinses in sterile distilled water. Seeds were inoculated into Petri dishes containing germination medium of half strength Murashige and Skoog (MS) salts, 2% sucrose, and 0.8% Difco-Bacto agar in addition to different levels of $CdC1_2$. Plates were incubated in the dark at 24 ± 2 °C for 7 days, and then germination percentages, hypocotyl and root lengths were recorded.

Shoot tips were excised from in vitro grown seedlings and cultured on MS medium supplemented with 1 µM *Benzyl adenine* (BA) and 0.5 µM naphthaleneacetic acid (NAA) for shoot proliferation (Al Khateeb et al., 2012). The new microshoots were subcultured after 6 weeks on fresh medium containing different levels of $CdC1_2$ (50, 100, 200, 400, 800, and 1600 µM). Cultures were placed in a growth chamber (24 ± 2 °C and 16 h light in cool white fluorescent light) for further growth. After 6 weeks, the fresh weights of the shoots were recorded.

2.2 Callus Growth

Al Khateeb et al. (2012) protocol was used for callus induction. Three-week-old callus was divided into parts of 0.5 g. Then, these parts were subcultured onto the same medium supplemented with different $CdC1_2$ levels. Cultures were maintained at 24 ± 2 °C and 16 h light in cool white fluorescent light. Fresh weights were taken every week for a period of 6 weeks.

2.3 Chlorophyll Analysis

The effect of different concentrations of Cd^{2+} on chlorophyll content was tested. Microshoots grown on MS medium supplemented with different levels of $CdC1_2$ were extracted with 80% acetone overnight, the A_{645} and A_{663} were determined using spectrophotometer and chlorophyll content was calculated according to the method of Mackinney (1941).

2.4 Proline Analysis

500 mg of plant tissues from microshoots grown on MS medium supplemented with different levels of $CdC1_2$ were homogenized in 10 mL of aqueous solution of sulfosalicylic acid. The solution was then filtered rapidly through a Buchner funnel using Whatman filter paper Nº 2. 2 mL of the filtrate was transferred to a test tube in addition to 2 mL of ninhydric acid and 2 mL of glacial acetic acid, followed by one hour incubation at 100 °C. The reaction was then stopped in an ice bath. Afterwards, 4 mL of toluene was added and the contents of the tube

were inverted for 20 seconds. After this, the toluene phase was separated by centrifugation at 13,000 g for 10 minutes. Finally, the absorbance was measured at 520 nm with a visible light spectrophotometer. The concentration of proline was determined from the calibration curve.

2.5 Lipid Peroxidation

Lipid peroxidation was estimated based on measuring the malondialdehyde (MDA) content. The MDA content in microshoots grown on MS medium supplemented with different levels of $CdC1_2$ was analyzed following Heath and Packer (1968). This assay is based on the reaction with thiobarbituric acid. Fresh microshoots (0.5 g) were ground in 20 mL of 0.1% tri-chloroacetic acid (w/v) then centrifuged for 15 min at 13,000 *g*. One mL of the supernatant was reacted with 5 mL of 20% TCA solution containing 0.5% thiobarbituric acid (w/v). After that, the mixture was heated for 45 min. at 95 °C and then cooled immediately in an ice bath. Next, the mixture was centrifuged for 5 min at 13,000 *g*, and finally the absorbance of the supernatant was measured using spectrophotometer at 532 and 600 nm. MDA content was calculated using the extinction coefficient of 155/(mM/cm) (Soltani et al., 2006).

2.6 DNA Extraction and RAPD Analysis

DNA was extracted from *C. pumilum* microshoots using modified CTAB (cetyltrimethylammonium bromide) method (Porebski et al., 1997). DNA concentration was measured spectrophotometrically at 260 nm. The RAPD reaction was performed in a total volume of 50 µL containing 5 µL template DNA, 10 × PCR buffer, 5 mM $MgCl_2$, 250 µM deoxynucleoside triphosphates, 1.5 U of Taq DNA polymerase and 1 µM of each primer. Primers were obtained from commercially available kits (OPA, OPC, and OPG) (Operon Technologies, CA and USA) (Table 1). DNA amplification program in the thermal cycler was as follows; 40 cycles of 94 °C for 1 min, 52 °C for 45 sec and 72 °C for 30 s. A final extension step was also used at 72 °C for 5 min. PCR products were loaded on agarose gel (1.5% agarose) and run with Tris-borate-EDTA (TBE) buffer for 90 min.

Table 1. Sequence information of RAPD primers used for *C. pumilum* fingerprinting

RAPD Primers	Sequence Information
OPC	GTCCCGACGA
OPG03	GAGCCCTCCA
OPG05	CTGAGACGGA
PM5	CGACGCCCTG
PM6	GCGTCGAGGG
OPAB 14	AAGTGCGACC
OPO 08	GCTCCAGTGT
OPAH 15	CTACAGCGAG
OPAO 01	AAGACGACGG
OPAP 20	CCCGGATACA
CRC	GCGAACCTCG
CRA22	CCGCAGCCAA

2.7 Statistical Analysis

Statistical significance was confirmed by analysis of variance (ANOVA) using SPSS for Windows (version 16.0). Results were expressed as mean ± standard error. Means were separated by using Tukey t-test at 0.05 level of probability. All experiments were repeated at least three times.

3. Results

Analysis of variance (ANOVA) showed that cadmium affects germination, hypocotyl and root length, fresh weights of shoots, chlorophyll and proline content, lipid peroxidation level, and callus growth of *Cichorium pumilum* significantly at 0.05 level of probability.

3.1 Effect of Cadmium on Germination Percentage

In general, the germination percentage of *Cichorium pumilum* decreased as Cd^{2+} level increased (Figure 1. A). The highest germination percentage (100%) was found in the control. A sharp reduction in germination percentage was observed when the seeds were treated with 50 µM $CdCl_2$ (80%). No significant differences were observed between 100, 200 and 400 µM $CdCl_2$ levels. The lowest germination percentage was observed when *C. pumilum* seeds were treated with the highest $CdCl_2$ concentration (1600 µM) which resulted in only 50% germination.

3.2 Effect of Cadmium on Hypocotyl and Root Length

Results show that the hypocotyl length of *C. pumilum* decreased with increasing $CdCl_2$ concentration (Figure 1. B). No significant difference was observed for hypocotyls length between control and the lowest levels of $CdCl_2$. No significant differences in hypocotyls length was observed between 100, 200, 400 and 800 µM $CdCl_2$ levels. Treating *C. pumilum* seedlings with 1600 µM $CdCl_2$ resulted in the highest reduction of the hypocotyls length.

A clear trend for the effect of $CdCl_2$ on root length was observed: root length of *C. pumilum* decreased when increasing $CdCl_2$ concentration (Figure 1. C&D). Root length was moderately affected by 50 µM $CdCl_2$, while higher levels (100 and 200 µM) decreased root length significantly to 50% of the control. Furthermore, seedlings grown on 1600 µM $CdCl_2$ had the shortest roots.

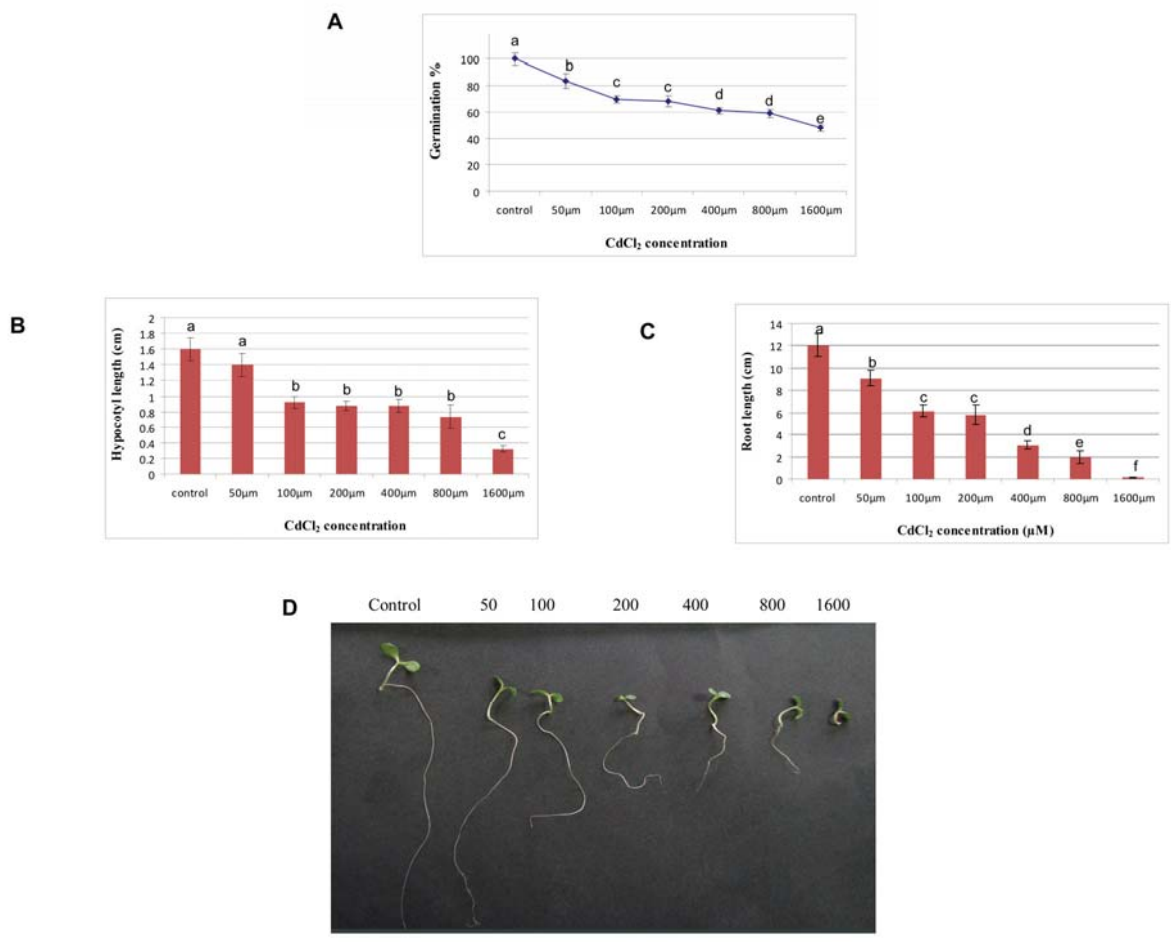

Figure 1. Germination percentage, hypocotyls length and root length of *C. pumilum* grown under different levels of $CdCl_2$. Data represents mean values ± standard error of ten replicates and the whole experiment was repeated three times. Means followed by the same letter are not statistically different at $p \leq 0.05$

3.3 Effect of Cadmium on Shoot Fresh Weight

Shoot fresh weight of *C. pumilum* was measured after 6 weeks of *in vitro* growth under different levels of $CdCl_2$.

Shoot fresh weight was affected adversely with increasing $CdCl_2$ concentration (Figure 2. A). Microshoots grown on MS medium supplemented with 50 µM $CdCl_2$ resulted in a significant reduction in shoot fresh weight with 7.6 g compared to 8.9 g in the control. Higher levels of $CdCl_2$ reduced shoot fresh weight severely. No significant difference for shoot fresh weight was observed between 100 and 200 µM $CdCl_2$. Sharp significant decreases in shoot fresh weights were observed for *C. pumilum* exposed to 800 and 1600 µM $CdCl_2$ which resulted in more than tenfold reduction compared to the control.

3.4 Effect of Cadmium on Chlorophyll Content

Chlorophyll content for *C. pumilum* microshoots at 50 and 100 µM $CdCl_2$ was found to be similar to control microshoots (Figure 2. B). In contrast, microshoots grown in MS medium supplemented with 200, 400, 800 and 1600 µM $CdCl_2$ showed higher levels of chlorophyll content than those grown in the control medium and lower levels of $CdCl_2$. No significant differences in chlorophyll content were observed between shoots grown on medium supplemented with 400, 800 and 1600 µM $CdCl_2$.

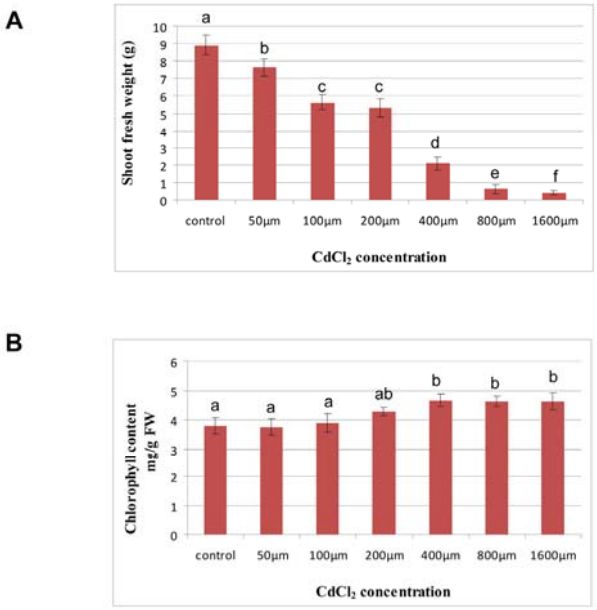

Figure 2. Shoot fresh weight and chlorophyll content of *in vitro* grown *C. pumilum* under different levels of $CdCl_2$. Data represents mean values ± standard error of eight replicates and the whole experiment was repeated three times. Means followed by the same letter are not statistically different at $p \leq 0.05$

3.5 Effect of Cadmium on Callus Growth

Different callus growth rates were observed based on fresh weight gain between the different levels of $CdCl_2$ (Figure 3). Calli grown on control medium showed the highest growth rate during the six weeks reaching a final fresh weight of 11.3 g. On the other hand, calli grown on MS medium supplemented with 50 µM $CdCl_2$ showed growth inhibition compared with the control and resulted in a final fresh weight of only 3.2 g. Calli grown on medium supplemented with 100 µM $CdCl_2$ showed more inhibition than that grown on 50 µM $CdCl_2$. Higher levels of $CdCl_2$ appear to be lethal for callus growth.

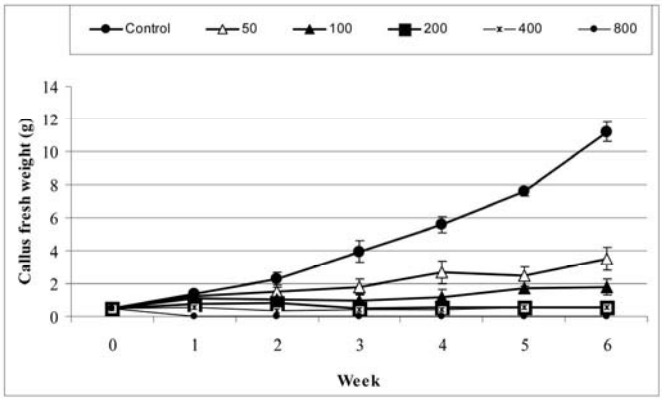

Figure 3. Callus growth curve of of *C. pumilum* grown under different levels of $CdCl_2$. Data represents mean values ± standard error of ten replicates and the whole experiment was repeated three times

3.6 Effect of Cadmium on Proline and Lipid Peroxidation Level

Proline content for *C. pumilum* microshoots was examined under different levels of $CdCl_2$. Results showed that proline content increased gradually and significantly as $CdCl_2$ level increased in the growth medium (Figure 4. A). The highest proline content was obtained from microshoots grown on the MS medium supplemented with the highest level of $CdCl_2$ (400 µM). Results showed that growing microshoots on 400 µM $CdCl_2$ increased proline content by more than tenfold.

The influence of Cd^{2+} on the lipid peroxidation rate of *C. pumilum* shoots was estimated by measuring MDA content, which is the product of lipid peroxidation. Lipid peroxidation rate in *C. pumilum* microshoots increased with increasing $CdCl_2$ level (Figure 4. B). Growing microshoots on MS medium supplemented with 50 µM $CdCl_2$ enhanced lipid peroxidation rate by more than twofold (compared with control). The highest lipid peroxidation rate was achieved in microshoots grown in MS medium supplemented with 400 µM $CdCl_2$.

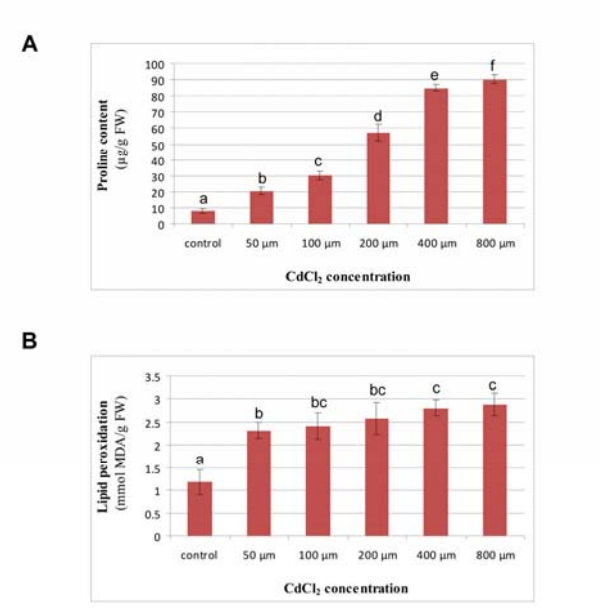

Figure 4. Proline content and lipid peroxidation rate of *C. pumilum* microshoots grown under different levels of $CdCl_2$ Data represents mean values ± standard error of eight replicates and the whole experiment was repeated three times. Means followed by the same letter are not statistically different at $p \leq 0.05$

3.7 DNA Fingerprinting Using RAPD Analysis

Genomic DNA was extracted from plants grown for 4 weeks on MS medium supplemented with different levels

of Cd^{2+}. Twelve primers were used in this experiment (Table 1). Amplified profiles resulting from these primers showed variation between untreated and treated plants in terms of number and size of DNA bands. Figureure 5 shows RAPD profiles of treated and untreated samples of *C. pumilum* microshoots obtained from primer OPAP 20 as a representative for the other primers. The RAPD profiles obtained showed bands between 300 and 1800 bp in length. A total of 184 bands scored, only 36 were found to be polymorphic. Figure 5 shows the appearance or absence of bands at 200 and 400 μM $CdCl_2$.

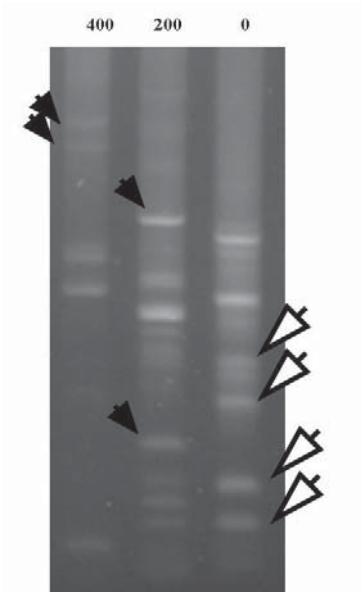

Figure 5. RAPD profiles (using primer OPAP 20) of genomic DNA extracted from *C. pumilum* microshoots grown under 0, 200 and 400 μM $CdCl_2$. Black arrows represent appearance of new bands and white arrows represent absence of bands relative to control (0)

4. Discussion

Plant growth and development under stress conditions are generally negatively affected. One of these stress conditions that affect plants is heavy metals. Recently, heavy metals have become a hot topic of research for many researchers around the world, mostly due to their detrimental effects on many organisms including plants.

Much research has been conducted on the effect of Cd^{2+} on crops and other agricultural plants. However, little information is available on the toxicity of Cd^{2+} on medicinal plants. Thus, the aim of this study was to assess the effect of cadmium on germination, growth, proline content, lipid peroxidation, and DNA fingerprinting of *in vitro* grown *C. pumilum*. Here, *in vitro* culture was used which is a convenient system for the study of mechanism of metal toxicity, as it eliminates the interfering processes of translocation and organ-specific trapping of metal ions.

The results of this study showed that Cd^{2+} levels affected all of the studied parameters in *C. pumilum* by different magnitudes. A significant reduction in percentages of seed germination, hypocotyl and root lengths of *C. pumilum* was observed here. This is in agreement with many published reports that studied the effects of Cd^{2+} on other plant species. Mathur et al. (1987) found that higher levels of cadmium inhibited germination percentage and the growth of the early seedlings of *Allium cepa.* Similarly, He et al. (2008) found that cadmium stress significantly inhibits germination index and shoot and root growth of rice. Pasquale et al. (1995) studied the influence of cadmium on the growth and biological activity of the medicinal plant *Coriandrum sativum* L. They found that growing plants under cadmium stress significantly reduced shoot and root lengths, resulting in leaf yellowing and in a major alteration in the essential oil quality and quantity. It has been shown that cadmium stress causes many problems in plants including growth and photosynthesis inhibition, alteration in nutrients and formation of free radicals (Sahu et al., 2007). Seed germination reduction due to heavy metals stress could be attributed to higher levels of seeds stored nutrients breakdown and/or change in permeability characteristics of the cell membrane (Shafiq et al., 2008). In peanuts, it has been shown that root and shoot growth and the initiation of lateral roots decreased with the increase in cadmium levels (Renjini & Janardhanan, 1989). The

reason for reduced seedling length in metal treatments could be due to the reduction in meristematic cells present in this region and of some enzymes contained in the cotyledon and endosperms.

It has been shown that another species of Chicory (*Cichorium intybus* L.) showed a potential to be used as heavy metal bioindicator, *C. intybus* plants grown in nutrient solution supplemented with 0.5-50 μM cadmium showed high levels of Cd, in their shoots and roots (Simon et al., 1996). Another study (Kostantinos et al., 2008) showed that the fresh and dry weights of *Cichorium endivia* L. were not affected when grown on soil supplemented with different levels of Cd. Furthermore, they found that no toxicity symptoms were observed on *Cichorium endivia* plants.

Moreover, the result of this study showed that Cd^{2+} treatment significantly increased proline accumulation in *C. pumilum*. Proline accumulation is used as an indicator of stress conditions, including heavy metals. It has been shown that proline acts as a Cd^{2+} chelator in plants and forms a non-toxic complex with Cd^{2+} (Sharma et al., 1998). Similarly, Dinakar et al. (2009) found that proline content increased under cadmium stress in *Arachis hypogaea* L. It has been shown that plants subjected to $CdSO_4$ stress in the presence of proline showed a lower amount of reactive oxygen species compared to plants without proline. (Xu et al., 2009).

In addition to proline accumulation, the amount of lipid peroxidation also increased in response to Cd^{2+} stress. This is in agreement with Shah et al. (2001) who found an increase in malondialdehyde (MDA) levels (enhancement of lipid peroxidation) in rice seedlings after $Cd(NO_3)_2$ exposure. Similarly, Soltani et al. (2006) found that cadmium stress increased lipid peroxidation levels in *Brassica napus* plants. Lipid peroxidation is the main sign of free radical elevation. Plants may have two classes of antioxidative systems against the perceived oxidative stress: enzymatic antioxidants (such as superoxide dismutase (SOD)) and non-enzymatic low molecular weight antioxidants (such as proline, ascorbic acid, and glutathione) that can directly detoxify free oxygen radicals.

Different classical genotoxic assays have been used to examine the effect of heavy metals on plants including the comet assay and the micronucleus assay (Cambier et al., 2010). Recently, DNA fingerprinting has been successfully applied to test the effect of such stresses at the molecular level in different species (Korpe & Aras, 2011; Liu et al., 2012). The ability of cadmium to induce DNA mutations and/or damage has been shown previously (Gichner et al., 2004; Liu et al., 2012). Insertions and deletions, point mutations, base substitutions, single/double-strand breaks are examples of the effect of cadmium stress on DNA (Castano & Becerril, 2004). Here, RAPD profile shows different changes in the DNA fingerprint indicating that Cd^{2+} affects the genome integrity. Not all primers showed variations in the RAPD profile between treated and non-treated plants, which could be explained by the variation in genome sensitivity to heavy metals stress between different regions. Also, some genome areas could be protected from external damage (Liu et al., 2012).

In conclusion, the results of this study showed that Cd^{2+} had a toxic effect on germination, growth, proline content, lipid peroxidation, and DNA fingerprinting of *in vitro* grown *C. pumilum*. A reduction in hypocotyl and root length and shoot fresh weight was observed in seedlings grown under Cd^{2+} stress. A gradual increase in proline content and lipid peroxidation along with increasing Cd^{2+} concentration was also observed. The variation that occurred in the RAPD profiles of microshoots following Cd^{2+} treatment can be efficiently used as a sensitive tool to detect DNA damage and genotoxicity.

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Sperm DNA Fragmentation as a Factor of Male Low Reproductive Function in IVF Practice

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Abstract

The correlation between semen DNA fragmentation level and the male age was investigated. The dependence for the blastocyst formation rates on the sperm DNA fragmentation has been examined in patients with low reproductive function. The increase of DNA fragmentation level correlates with the decrease of the blastocyst formation rates ($p < 0.05$). The significant negative relationship between sperm DNA fragmentation and the male age is proved ($p < 0.05$). The age of 35 years old could be discussed as clinically critical male age for the process of chromatin compaction during the process of spermatogenesis.

Keywords: sperm DNA fragmentation, blastocyst formation rates, IVF

1. Introduction

1.1 Sperm DNA Fragmentation as a Male Infertility Factor

Recently high attention in the reproductive medicine is paid to the paternal genome failures. Assisted reproductive techniques (ART) such as conventional *in vitro* fertilization (IVF), and especially intracytoplasmic sperm injection (ICSI), allow couples whose sperm characteristics are impaired to obtain a pregnancy, whereas a few years ago, these couples would have had to use sperm donation in order to obtain their child. One can nevertheless wonder about the capacity of poor quality sperm samples to generate embryos having normal capacities of development. Among the factors involved in the failure of obtaining embryos and/or pregnancies, the impaired sperm genome is frequently incriminated (Ahmadi, 1999). Sperm DNA fragmentation is rather new discussible reason of male infertility. There are contradictory data about the possible influence of sperm DNA fragmentation on the sperm fertilization ability and the process of embryo development, particularly on the process of the blastocyst formation (Agarwal, 2003; Findikli, 2004; Borini, 2006). There are data about the correlation of the sperm DNA fragmentation level and high percentage of the spontaneous miscarriages for patients included in the IVF treatment (Benchaib, 2003; Seli, 2005; Oleszczuk, 2011). Sperm DNA fragmentation consists of single and doublestranded DNA breaks, frequently occurring in semen of subfertile patients (Lopes et al., 1998; Irvine et al., 2000; Muratori et al., 2000). Despite the origin and the mechanisms responsible for such genomic anomaly are not yet clarified, it has been proposed that sperm DNA fragmentation could be a good parameter to predict the male fertility status as an alternative or in addition to poorly predictive standard parameters presently determined in routine semen analysis (Lewis, 2007; Erenpreiss et al., 2006). Spermatogenesis is a complicated process that includes spermatozoa development and maturation. It depends on such factors as genetics, hormonal statement, environmental conditions etc. Spermatogenesis failures could lead to the formation of aneuploid sperm or sperm with the DNA damage (Cayli, 2004; Tesarik, 2002).

1.2 The Etiology of Chromatin Compactization Failures

The level of sperm DNA fragmentation reflects the integrity of genetic material of the gamete. This parameter is important since DNA lesions of many types induce mutations commonly observed in mutated oncogenes and tumour suppressor genes (Marnett, 2000). The possible reason of sperm DNA fragmentation are changing of chromatin structure during the process of spermatogenesis and apoptosis (Brugnon, 2006; Schlegel, 2005; Calle, 2008). Defects of the process of spermatozoa maturation should also be discussed. In average about 20% of spermatozoa in ejaculate could be found as apoptotic in men with different deviations in the semen analysis

(Mehdi, 2003; Muratori, 2008). It is also found out that the sperm DNA fragmentation level is statistically higher for patients with spermatogenesis failures comparing with men with normal semen parameters (Speyer, 2012). On the next side there are data that DNA fragmentation level has no correlation with such semen parameters as motility, concentration or sperm morphology (Luke, 2010). There is still discussible question about the dependence of sperm DNA fragmentation level on the male age (Bronet, 2012). Results of studies aimed to establish whether the amount of sperm DNA fragmentation could predict the outcome of Assisted Reproduction Techniques (ARTs) are conflicting.

1.3 The Research Design

The aim of the presence work was to investigate the correlation for sperm DNA fragmentation level on the male age and to examine if there is any dependence in the process of the early embryo development on the sperm DNA fragmentation in patients with low reproductive function during the infertility treatment using the methods of ART. Taking into account that the most important criteria of the normal embryo development is the blastocyst formation, the affect on sperm DNA fragmentation level on the blastocyst formation rates were examined for infertile patients during the infertility treatment using the IVF procedure including the manipulation of ICSI. It was also very important to compare sperm DNA fragmentation level in the group of infertile patients and in men with normal reproductive function.

2. Method

2.1 Sperm DNA Fragmentation Level Examination

Sperm DNA fragmentation level was examined using the method of the sperm chromatin dispersion (SCD) (HaloSperm, Halotech, Spain). The method is based on the chromatin dispersion around the spermatozoa nuclei that allows finding the spermatozoa with fragmented DNA. The SCD test is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops that is observed in sperm with non-fragmented DNA, following acid denaturation and removal of nuclear proteins. The SCD test is a simple, accurate, highly reproducible, and inexpensive method for the analysis of sperm DNA fragmentation in semen and processed sperm. It could potentially be used as a routine test for the screening of sperm DNA fragmentation in the andrology laboratory (Fernández, 2003). The examination was carried out using the fluorescent microscope Nikon Eclipse 80i. The obtained photos were documented with the cytogenetic program Lucia FISH (LIM, Czech Republic). Normally the sperm DNA fragmentation level should not be higher then 20.0%.

2.2 Participants Characteristics

During the investigation the group consisted of 33 infertile patients with the male low reproductive function were formatted. The middle male age in the group was 38.0 ± 5.7 years old. The IVF procedure and the examination of the sperm DNA fragmentation level were carried out for all the participants of the mentioned group. Simultaniously the control experiment with a normal group consisted of 20 fertile men was carried out to compare the value of DNA fragmentation level. The middle male age in the control group was 34.0 ± 6.9 years old. Basic sperm parameters (concentration, motility and morphology) showed a high variability among individual patients, ranging between normal values and severe oligoasthenoteratozoospermia. Blastocyst formation rates were variable for the patients (Table 1).

Table 1. Basic sperm parameters of examined group of infertile patients

Patient	Basic sperm parameters			Sperm DNA fragmentation, %	BFR, %
	Concentration ($\times 10^6$/ml)	Motility, %	Normal forms, %		
1	15	52	22	38.0	18.1
2	34	26	31	3.0	12.5
3	23	17	12	14.5	56.2
4	54	70	37	27.5	0
5	72	31	41	14.5	69.5
6	12	34	39	3.8	50.0
7	43	63	19	5.2	100.0
8	7	13	15	20.0	0
9	32	49	34	10.0	50.0
10	8	35	21	33.4	62.5
11	19	41	36	11.7	25.0
12	21	15	45	13.2	78.2
13	59	48	42	22.3	42.1
14	37	52	39	42.2	44.4
15	29	24	17	12.3	33.3
16	3	23	5	10.5	47.1
17	51	63	39	21.3	33.3
18	26	17	14	61.5	14.3
19	38	46	34	18.3	54.5
20	6	10	11	37.5	14.2
21	42	23	13	12.4	77.8
22	40	39	25	9.8	37.5
23	74	57	57	12.0	61.5
24	67	68	54	2.0	80.0
25	31	20	35	3.5	58.3
26	18	23	13	60.0	20.0
27	4	16	10	5.5	60.0
28	22	46	24	1.5	61.5
29	65	58	51	7.5	28.5
30	11	29	9	14.5	50.0
31	9	14	35	5.2	50.0
32	20	36	21	46.7	38.4
33	38	48	18	38.0	55.5

2.3 IVF Procedure

During the IVF programs the protocols with a-GnRH (antagonist-Gonadotropin Releasing Hormone) were used for controlled ovary stimulation for oocytes retrieval. Ovary stimulation took not less then 10 days in every case. In the day of the transvaginal puncture the size of follicles achieved 18 mm. To maintain the luteal phase the progesterone consisted medicines were used. Oocyte retrieval was carried out under general anaesthesia by a vaginal ultrasonography-guided aspiration. At 16-18 h after insemination or microinjection, as previously

described (Borini et al., 2004a, 2004b), oocytes were assessed for two PN presence.The fertilization of oocytes was carried out using the procedure of ICSI taking into account the male factor of infertility. ICSI manipulation was done on the inverted microscope Nikon Eclise TI-u with the Eppendorf micromanipulators. Spermatozoa were immobilezed using the laser system OCTAX (MTG, Germany). The obtained oocyted and embryos were cultured in the Universal IVF Medium and ISM-1 Medium (Medicult) during the first three days and in the Blast Assist (Medicult) till the fifth day of culture. The gametes and embryos were cultured in temperature 36.8 ºC - 37.1 ºC and in 5.5% - 5.8% CO_2 level.

2.4 Statistics

The obtained data for abnormal distribution were statistically checked using nonparametric methods of statistic analysis. The correlation was examined by the method of analysis with the Spearman coefficient. Calculation was done using the program module Statistica-6.

3. Results

The positive correlation between the sperm DNA fragmentation level and male age was proved for the examined group. Total Spearman coefficient is 0.35 ($p < 0.05$). The critical male age for the sperm DNA fragmentation level growth is 35 years old. Spearman coefficient for patients elder then 35 years old is 0.45 ($p < 0.05$). There is no any correlation for DNA fragmentation and male age in the group of patients younger then 35 years old. Spearman coefficient for the group of patients younger then 35 years old is -0.04 ($p > 0.05$). The obtained results are mentioned in Table 2.

Table 2. Correlation between the sperm DNA fragmentation level and male age

Age group	N	Age, years	DNA fragmentation, %	r_s	$r_{critical}$	p
≤ 35 years	11	30.7±2.7	13.5±6.6	-0.04	0.58	$p > 0.05$
> 35 years	22	41.6±4.4	21.2±15.0	0.45	0.43	$p < 0.05$
Total group	33	38.0±5.7	18.6±12.5	0.35	0.34	$p < 0.05$

The significant negative correlation between the semen DNA fragmentation level and blastocyst formation rates is proved. However, the DNA fragmentation less then 5.0% did not impair the blastocysts formation rates. The critical DNA fragmentation level for blastocysts formation rates is 5.0%. The Spearmen coefficient for the group of patients with the DNA fragmentation level higher then 5.0% is -0.44 ($p < 0.05$). The Spearmen coefficient for the total examined group is -0.41 ($p < 0.05$). The obtained results are mentioned in Table 3.

Table 3. Correlation between the semen DNA fragmentation level and blastocyst formation rates

Critical DNA fragmentation level	N	DNA fragmentation, %	BFR, %	r_s	$r_{critical}$	p
> 5.0%	28	21.4±12.6	43.2±18.6	-0.44	0.38	$p < 0.05$
< 5.0%	5	2.8±0.8	52.5±17.0	-0.60	0.94	$p > 0.05$
Total group	33	18.6±12.5	44.9±18.9	-0.41	0.34	$p < 0.05$

Photo of spermatozoa with the fragmented DNA and with normal compactization of chromatin is present on Figure 1.

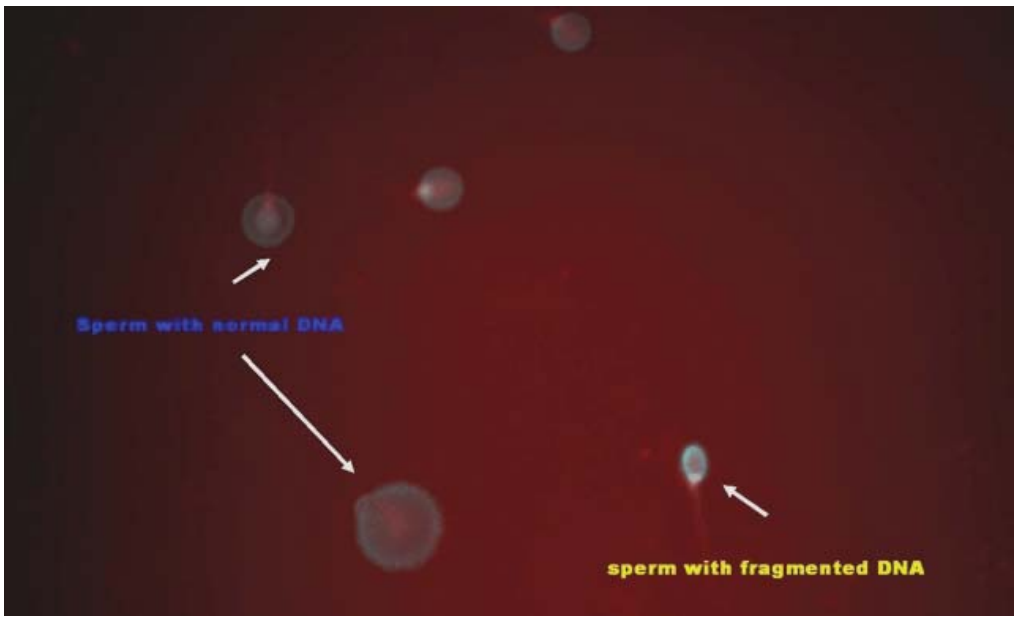

Figure 1. Sperm DNA fragmentation by the method of SCD

The average semen DNA fragmentation level in the control group of fertile men was 5.48 ± 2.03. The level of DNA fragmentation level was significantaly higher in infertile patients comparing with the control ($p < 0.05$). Such fact could prove the influence of the sperm chromatin compactization failures on the semen parameters and male reproductive function. Further investigations should be performed.

4. Discussion

In studies investigating the impact of sperm DNA fragmentation on reproduction, the prevailing idea is that sperm with damaged DNA, even if retaining the ability to fertilize the oocyte (Ahmadi, 1999), affect the subsequent steps resulting in increased failure of embryo development and miscarriage (Agarwal, 2004; Lewis, 2005; Li, 2006). However, data on the relationship between DNA damage and ART outcome are very conflicting (O'Brien, 2005; Li, 2006). The obtained data confirmed that DNA fragmentation could be one of the male infertility factor that effects the embryo development. The increase of DNA fragmentation showed negative correlation with the blastocyst formation rates (BFR) ($p < 0.05$). The level of sperm DNA fragmentation 5.0% could be reported as a critical one for the process of blastocyst formation in ART (assisted reproductive techniques). Such conclusion is practically important because of an absence of the strict recommendations about normal or abnormal level of semen DNA damage levels. The discussible range of acceptable level of semen DNA fragmentation varies in diapasone 10%-40% in different studies (Benchaib, 2003). The studies by previous workers reported above, supported by the present study, indicate clearly that strand breaks in the sperm DNAhave little or no effect on fertilization and early embryo growth but begin to have an effect at the stage of blastocyst development, and then have a very marked effect on implantation of the embryo, but the exact clinically important DNA fragmentation level that could impair the blastocyst development is still discussible (Ahmadi, 1999; Fatehi, 2006). There was no significant effect on fertilization rate or early cleavage, but the effects on blastocyst development and implantation rate were very marked. The negative correlation between the sperm DNA fragmentation on the male age is proved ($p < 0.05$). The patients' age 35 years old could be discussed as clinically critical male age for the process of chromatin compaction during the spermatogenesis. Such conclusion is clinically important, taking into account that nowadays more attention to the female age is paid and there is a little information about the age of male fertility. As both males and females decide to conceive later, the question of whether this may impact their fertility individually and as a couple becomes even more crucial. A paternal age of over 40 years at the time of conception is a frequently quoted male age threshold, however, currently there is no clearly accepted definition of advanced paternal age or even a consensus on the implications of advancing male age (Humm, 2013). Further study is also needed to determine if there is any dependence of sperm DNA damage on the basic sperm parameters. Several studies have stressed the importance of traditional sperm parameters as predictors of fertility potential (Nallella et al., 2006). Because of the evidence of correlations between sperm DNA fragmentation and clinical pregnancy and pregnancy loss rates, it would be

practically important to find a relationship between traditional sperm evaluation parameters (concentration, motility and morphology) and pregnancy and pregnancy loss rates in IVF groups.

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Phylogenetic Position of *Xenoturbella bocki* and Hemichordates *Balanoglossus carnosus* and *Saccoglossus kowalevskii* Based on Amino Acid Composition or Nucleotide Content of Complete Mitochondrial Genomes

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Abstract

To clarify the phylogenetic position of *Xenoturbella bocki* and hemichordates (*Balanoglossus carnosus* and *Saccoglossus kowalevskii*), which occupy an ambiguous evolutionary position between vertebrates and invertebrates, we expanded a pre-existing baseline vertebrate data set by incorporating randomly selected invertebrates. Based on nucleotide content calculated from complete mitochondrial genomes, invertebrates were classified into two groups, high C/G and low C/G, with vertebrates placed within the high-C/G invertebrate group. *X. bocki* and hemichordates (*B. carnosus* and *S. kowalevskii*) also fell into the high-C/G invertebrate group. We also analyzed amino acid composition and nucleotide content of complete mitochondrial genomes or 16S rRNA genes using Ward's clustering method and neighbor-joining. In the resulting phylogenetic trees, invertebrates are classified into high C/G and low C/G categories, and vertebrates are divided into terrestrial and aquatic groups. In addition, *X. bocki* and representative hemichordates *B. carnosus* and *S. kowalevskii* appear to be more closely related to vertebrates than to invertebrates.

Keywords: amino acid composition, complete mitochondrial genome, vertebrate, invertebrate, hemichordate, *Xenoturbella*, phylogenetic tree, evolution

1. Introduction

Evolution can be regarded as a branching process, whereby populations are altered over time and may speciate into separate branches. Approximately 150 years ago, Charles Darwin and Alfred Wallace established the concept of biological evolution by natural selection. Based on this theory, Charles Darwin and Ernst Haeckel developed the idea of phylogenetic trees as a visualization tool to aid readers' understanding of biological evolution.

Because evolution generally occurs over long periods of time and hence cannot be observed directly, scientists must construct phylogenies to infer evolutionary relationships among present-day organisms. Two different approaches — cladistics and phonetics — have been used to construct phylogenetic trees; the differences between these methods are beyond the scope of our study and are not described here.

To construct phylogenetic trees representing organismal relationships, similarities or differences in organismal traits are analyzed using implicit or explicit mathematical models. These traits can be morphological or molecular. Morphological characters include anatomical characteristics of extant organisms and changes in the fossil record; the latter data are limited and tend to be ambiguous (Cobbett et al., 2007). Molecular data, including protein and DNA sequences, have been widely used for constructing phylogenetic trees (Zuckerkandl

& Pauling, 1962; Dayhoff et al., 1977; Sogin et al., 1986; Doolittle & Brown, 1994; Maizels & Weiner, 1994; DePouplana et al., 1998; Woese & Fox, 1977; Weisburg et al., 1991). Complete genome sequence data are not readily applied to phylogenetic tree construction, however, because of the enormous number of nucleotides constituting many different genes among organisms. We have recently shown that ratios of amino acid composition and nucleotide content are useful for genome research because they can characterize entire genomes (Sorimachi et al., 2001). Based on such indices, we were able to generate phylogenetic trees in which vertebrates were classified into two groups, terrestrial and aquatic, even though the analyzed organisms were chosen randomly without pre-defined criteria (Sorimachi et al., 2013a). Similar classification results were also obtained in the previous study when deliberately selected samples were added to the pre-existing baseline data set (Sorimachi & Okayasu, 2013). Our previously developed method is thus applicable to phylogenetic tree construction. In this study, we therefore used amino acid composition and nucleotide content of complete mitochondrial genomes to ascertain the phylogenetic position of *Xenoturbella bocki* and hemichordates, which occupy an ambiguous evolutionary position between vertebrates and invertebrates.

2. Materials and Methods

Mitochondrial genome data were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/sites). In an earlier study, organisms were chosen according to the alphabetical order of their scientific names without considering their characteristics (Sorimachi et al., 2013a), because cluster analyses are significantly influenced by different sampling. Vertebrate species included 39 species from a previous study (Sorimachi et al., 2013a) to which 24 other vertebrates had been subsequently intentionally added to evaluate phylogenetic tree reconstructions (Sorimachi & Okayasu, 2013). For the current study, we added 58 invertebrate species, which were randomly chosen based solely on the alphabetical order of their scientific names. Cluster analysis has five methods based on differences in calculation procedures to estimate the distance between two samples; Ward's, nearest neighbor, furtherest neighbor, group average and centroid methods. Ward's method has been widely used, and we used this method to classify various organisms (112 bacteria, 15 archaea and 18 eukaryotes) (Okayasu & Sorimachi, 2009). In our recent study (Sorimachi & Okayasu, 2013), cetaceans (*Balaenoptera musculus*, *Sousa chinensis*, and *Phocoena phocoena*) form a small cluter that is closely related to hippopotamus. This placement is consistent with results of other studies (Catesy, Hayashi, Cronin, & Arctander, 1996; Ursing & Arnason, 1998), and supports the evolution of cetaceans from terrestrial vertebrates within the mammalian evolution. Based on these evidences, Ward's method has been mainly used in the present study. Cluster analyses were carried out on the data using Ward's (Ward, 1963) and neighbor-joining (Saitou & Nei, 1987) methods. Nucleotide contents of coding and non-coding regions of mitochondrial genomes were compared with the content of their complete corresponding single-strand DNA and normalized to 1 (G + C + T + A = 1). Predicted amino acid compositions of mitochondrial genome coding regions were estimated (Sorimachi et al., 2001; Sorimachi & Okayasu, 2004). Calculations were performed using Microsoft Excel (version 2003). Classifications based on Ward's clustering method (Ward, 1963) were conducted using commercially purchased-multivariate software developed by ESUMI (Tokyo, Japan).

3. Results

3.1 Relationships Based on Nucleotide Content

When nucleotide contents calculated from coding regions of complete mitochondrial genomes were plotted against those of entire genomes, invertebrates were classified into two groups: high C/G and low C/G (Figure 1). *X. bocki* and hemichordates (*Balanoglossus carnosus* and *Saccoglossus kowalevskii*) were located in the region where vertebrates and invertebrates overlapped. Vertebrates widely overlapped with high C/G invertebrates; on the plot, they were positioned in the upper right portion of the high-C/G invertebrate distribution (Figure 1).

When relationships based on nucleotide content were examined between homonucleotides using linear regression in a previous study (Sorimachi et al., 2013b), linear regression lines with high regression coefficients were obtained. Two distinct lines corresponding to invertebrates and vertebrates were observed. Because these two lines form a "wedge-shape" when superimposed, we concluded that vertebrates and invertebrates diverged from a common ancestor (Sorimachi et al., 2013b), and that extant organisms diverged from a single origin (Sorimachi, 2010). *X. bocki* and the hemichordates were located between these two lines (Figure 2).

3.2 Cluster Analysis of Invertebrates

Based on nucleotide content from complete mitochondrial genomes, the 58 randomly chosen invertebrates (Sorimachi & Okayasu, 2008) were analyzed using Ward's method (Ward, 1963). Three major clusters were obtained (Figure 3). Similar results were obtained using predicted amino acid composition, although the groups were not as well-differentiated (data not shown); this may be because the high- and low C/G invertebrate

classification is based on nucleotide relationships. In the tree generated using nucleotide content, one major cluster, consisting of Echinodermata, Mollusca, Arthropoda, and Placozoa — all high-C/G invertebrates except for *Trichoplax adhaerens* — is divided into two sub-clusters. The hemichordates *B. carnosus* and *S. kowalevskii* are found in one of these sub-clusters, with the deuterostome *X. bocki* falling into the other (Figure 3). The second major cluster, all low-C/G invertebrates, consists of Cnidaria, Arthropoda, Nematoda, and Platyhelminthes. Chordata (*Branchiostoma belcheri*) also falls into this major cluster, grouped with Platyhelminthes. The third major cluster consists of three sub-clusters. One of the three sub-clusters comprises Amoebozoa, Protozoa, Loukozoa, Mycetozoa, and Nematoda, all low-C/G invertebrates except for *Dictyostelium citrinum*. The second sub-cluster, containing high-C/G invertebrates, consists of Mollusca and Arthropoda. The final sub-cluster contains low-C/G organisms — Arthropoda, Protista (*Tetrahymena pyriformis*), and Nematoda (*Strelkovimermis spiculatus* and *Romanomermis spiculatus*), as well as high-C/G invertebrates.

Of seven species pairs belonging to the same genera, three (*Bactrocera*, *Reticulitermes*, and *Acanthaster*) remained joined in the phylogenetic tree; the other four species pairs (*Tigriopus*, *Dictyostelium*, *Drosophila*, and *Hemichordata*) were divided between separate clusters.

Similar classifications were obtained when nucleotide content calculated from coding and non-coding regions of complete mitochondrial genomes was analyzed.

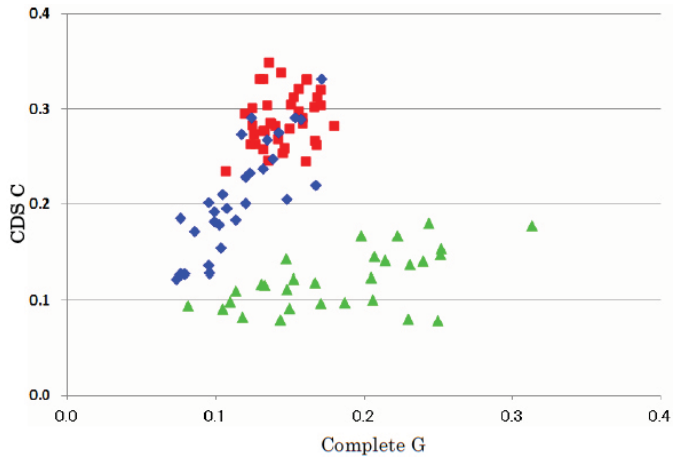

Figure 1. Relationships based on nucleotide content of complete mitochondrial genomes. Values are normalized to 1 (G + C + T + A = 1). Closed blue squares, closed green triangles, and closed red squares represent high-C/G invertebrates, low-C/G invertebrates, and vertebrates, respectively

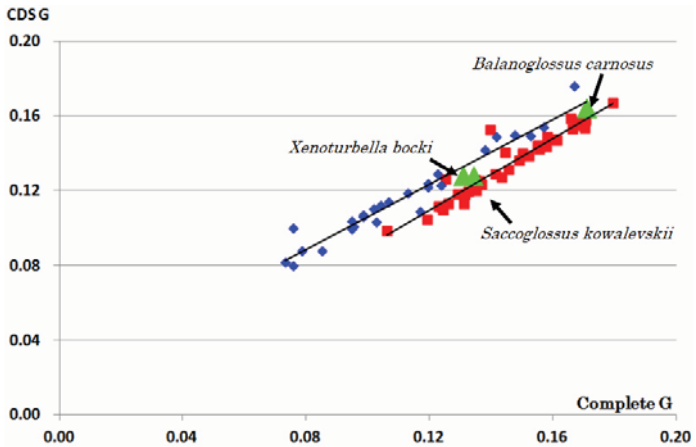

Figure 2. Nucleotide relationships based on normalized vertebrate (red squares) and high-C/G invertebrate (blue squares) valuesThe G nucleotide content in the coding region (vertical axis) is plotted against G content in a complete single DNA strand (horizontal axis). Closed green triangles represent *Xenoturbella* and Hemichordata

3.3 Phylogenetic Analysis of Vertebrates and High-C/G Invertebrates Using Ward's Method

In our previous study (Sorimachi et al., 2013a), vertebrates were classified into terrestrial and aquatic groups based on cluster analysis of amino acid composition using Ward's method. This classification was corroborated through analysis of 16S rRNA gene sequences using neighbor-joining. In this study, we consequently used Ward's method to analyze amino acid composition of a sample set consisting of 39 vertebrate species and 31 high-C/G invertebrates (Figure 4). In the resulting tree, vertebrates and invertebrates correspond to the two major groups, with the exception of the hemichordates and *X. bocki*, which are placed with the vertebrates. *X. bocki* is found in one of the two vertebrate sub-clusters, which comprises mammals and some reptiles, and is most closely related to reptiles. The hemichordates belong to the sub-cluster consisting of fish, amphibians, reptiles, and birds; within that sub-cluster, they are closely related to birds and reptiles. Hagfish (*Eptatretus burgeri*) is placed in the cluster consisting of mammals, consistent with our previous results (Sorimachi et al., 2013a). This placement may be due to its controversial characteristics (Janvier, 2010).

We also analyzed this sample set using nucleotide content calculated from complete mitochondrial genome data, but similarly clear classifications were not obtained (data not shown).

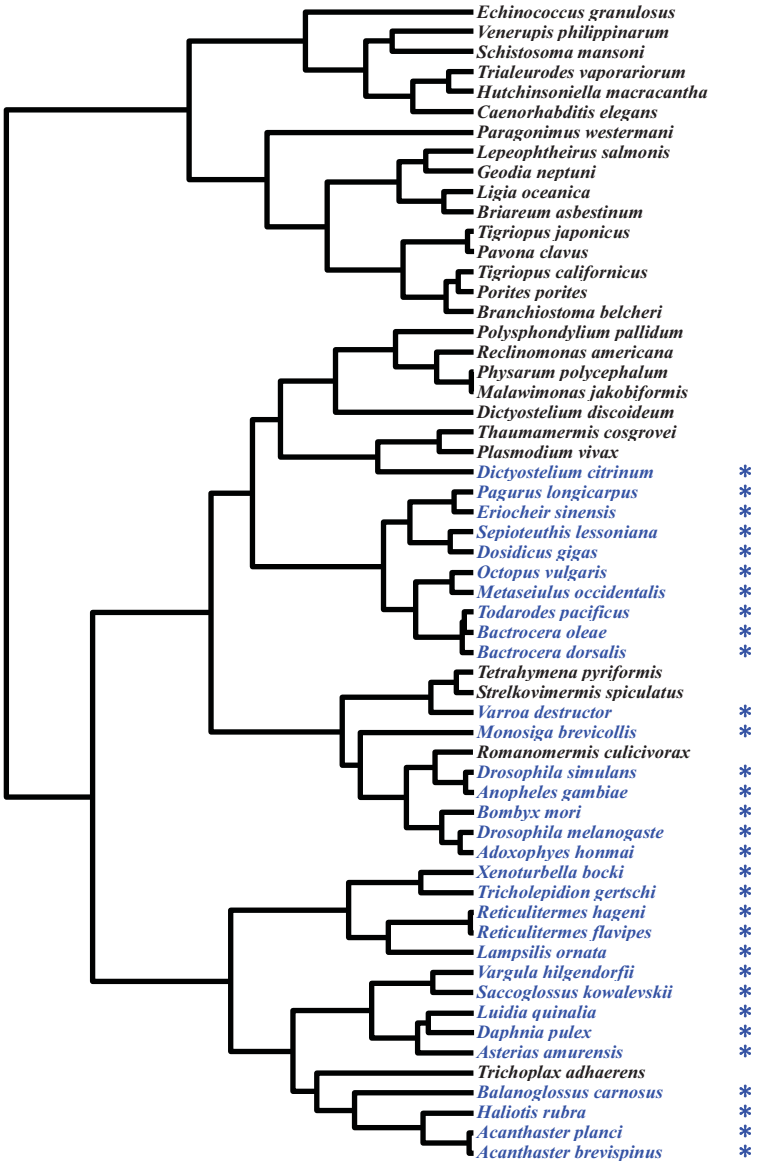

Figure 3. Phylogenetic tree generated using Ward's cluster analysis method (Ward, 1963) from nucleotide content of complete mitochondrial genomes. High-C/G and low-C/G invertebrates are indicated in blue (*) and black (null), respectively

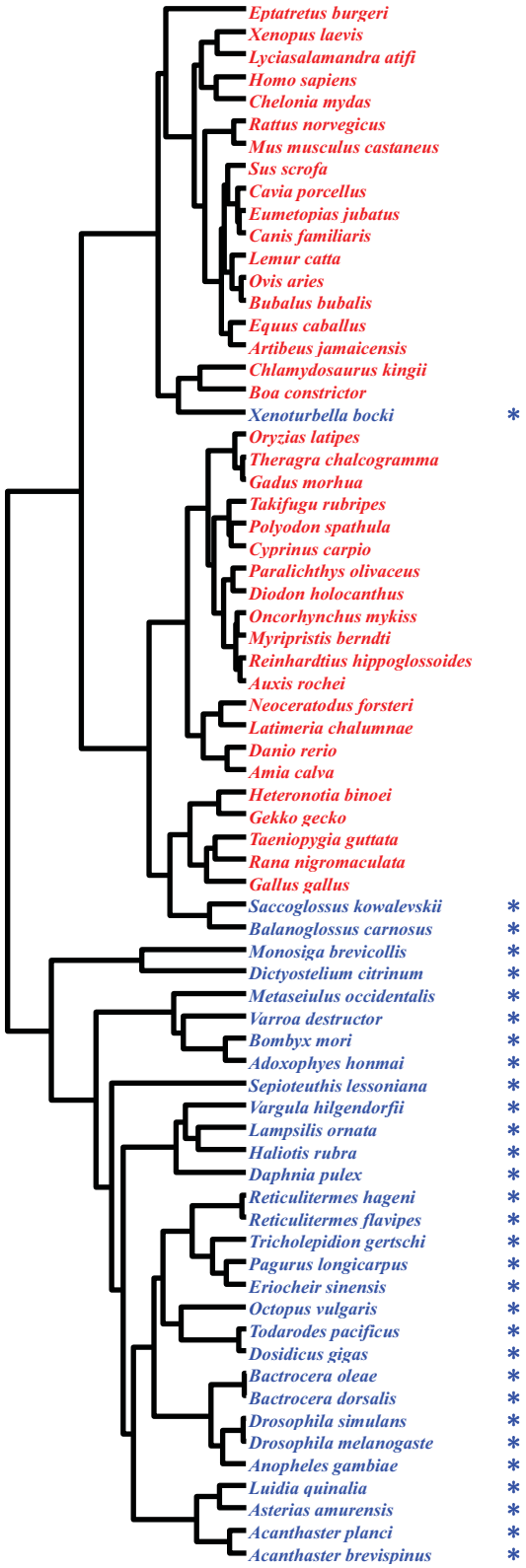

Figure 4. Phylogenetic tree generated using Ward's cluster analysis method (Ward, 1963) from predicted amino acid composition of complete mitochondrial genomes of 31 invertebrates and 39 vertebrates. High-C/G invertebrates and vertebrates in indicated in blue (*) and red (null), respectively

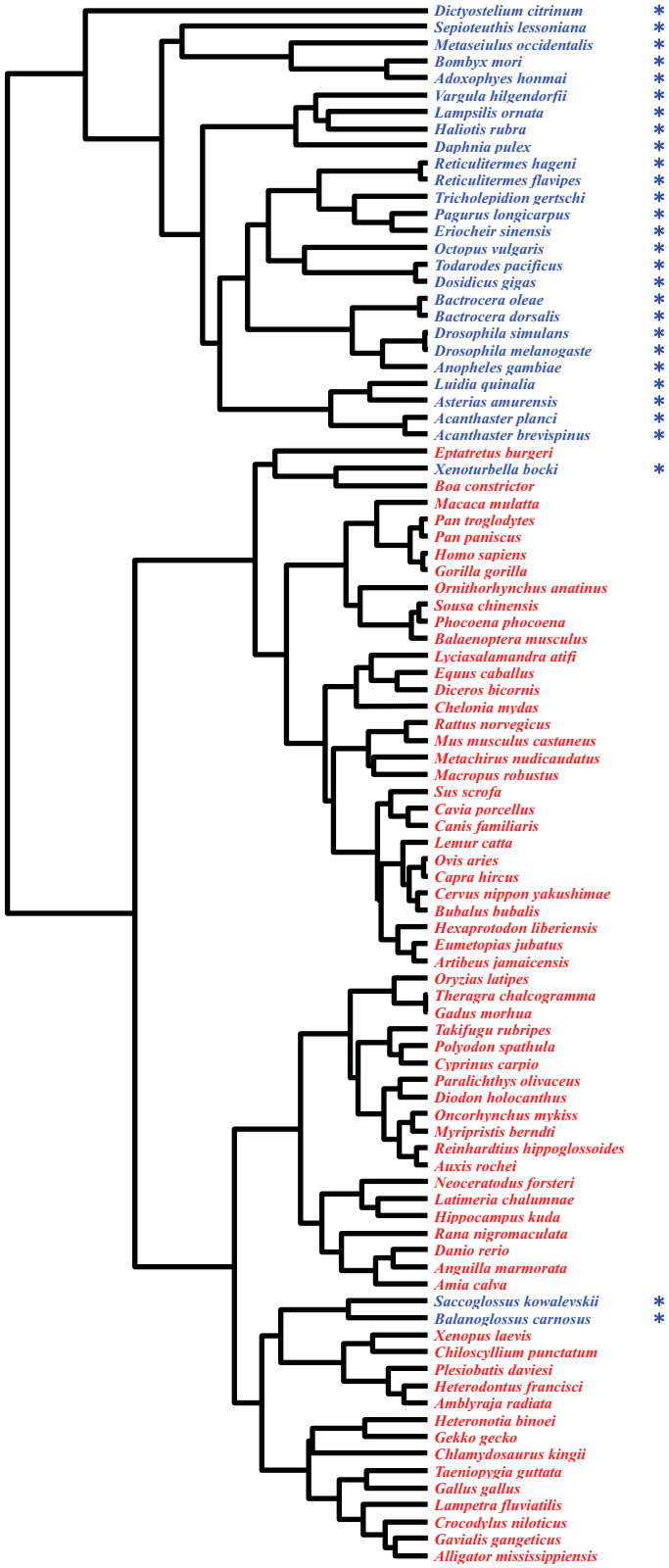

Figure 5. Phylogenetic tree generated using Ward's cluster analysis method (Ward, 1963) from predicted amino acid composition of complete mitochondrial genomes of 29 invertebrates and 63 vertebrates. High-C/G invertebrates and vertebrates are shown in blue (*) and red (null), respectively

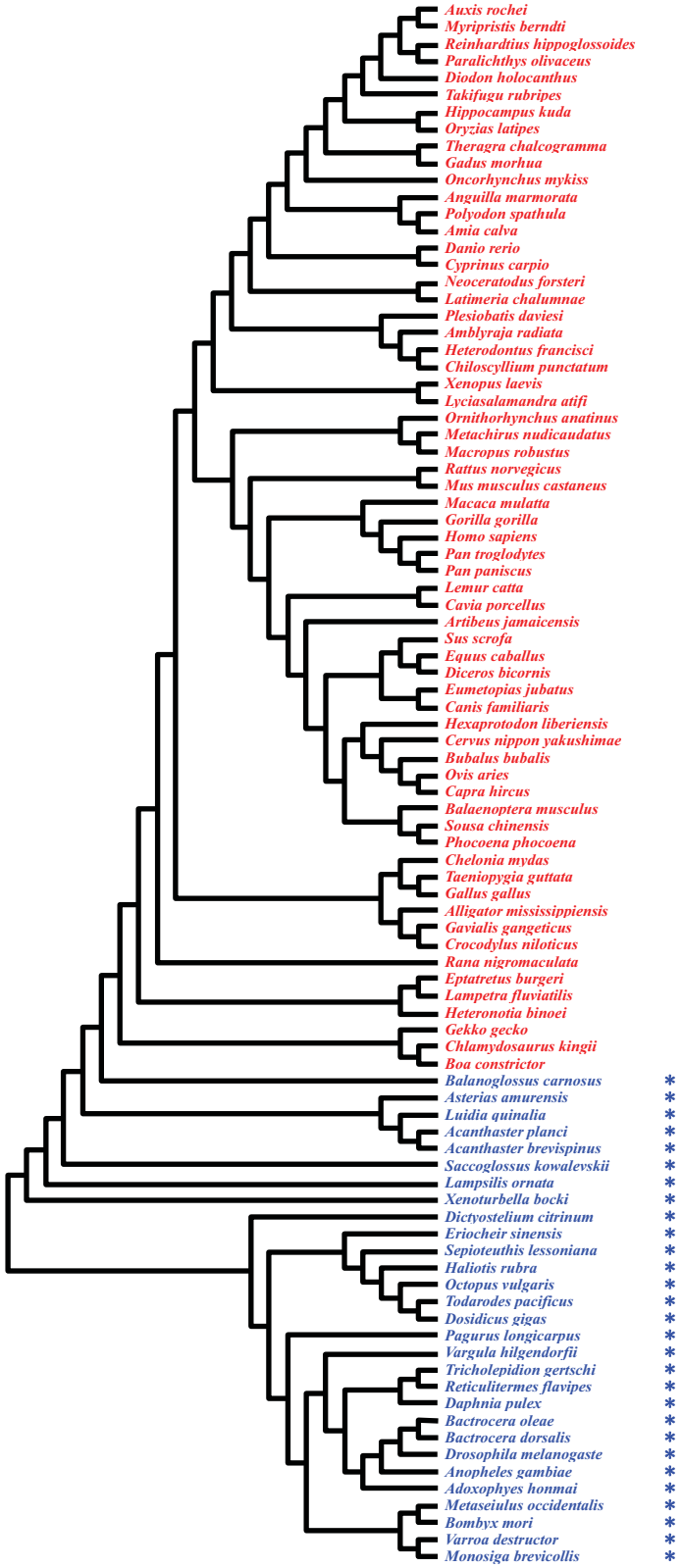

Figure 6. Phylogenetic tree generated using neighbor-joining (Saitou & Nei, 1987) from predicted amino acid composition of complete mitochondrial genomes. High-C/G invertebrates and vertebrates are designated by blue (*) and red (null), respectively

In our previous studies, additional vertebrates were deliberately selected to aid evaluation of reconstructed phylogenetic trees and were added to a set of randomly chosen vertebrates from an earlier study (Sorimachi & Okayasu, 2013). The number of vertebrate species in the expanded data set was increased to 63 in this fashion. Predicted amino acid compositions of these samples were analyzed using Ward's method, resulting in classification into three major clusters (Figure 5). High-C/G invertebrates form one major cluster, with vertebrates classified into two clusters. One vertebrate cluster consists of a large mammalian group and a small cluster that includes hagfish (*E. burgeri*), snake (*Boa constrictor*), and *X. bocki*; and the other comprises a large fish sub-cluster and various other sub-clusters including hemichordates, amphibians, reptiles, and birds. The Hemichordata are closely related to amphibians and reptiles. In this analysis, the hemichordates were separated from *X. bocki*, belonging to different clusters. All of these species fall into vertebrate groups, however, and are separate from invertebrates.

3.4 Neighbor-Joining Analysis Using Complete Mitochondrial Genome Nucleotide Sequences

Using nucleotide sequences of complete mitochondrial genomes, neighbor-joining analysis generated a different topology, as shown in Figure 6. Two groups, vertebrates and invertebrates, are evident, although clearly distinct major clusters were not obtained. *X. bocki* and hemichordates *B. carnosus* and *S. kowalevskii* are placed among the invertebrates, but do not form a separate cluster. They are apparently intermediate between vertebrates and invertebrates.

3.5 Neighbor-Joining Analysis Using 16S rRNA Gene Sequences

When 16S rRNA gene sequences were analyzed, the hemichordates and *X. bocki* were not found in the major cluster containing most of the invertebrates (Figure 7). According to the phylogenetic tree, these three species are closely related to one another, and also to *Lampsilis ornata* and *Metaseiulus occidentalis*. Hemichordata and *X. bocki* are more closely related to vertebrates than to invertebrates.

3.6 Phylogenetic Analysis of Vertebrates and Invertebrates

Using predicted amino acid composition from complete mitochondrial genomes, low-C/G invertebrates were also examined. We added 29 low-C/G invertebrate species to the data set consisting of 29 high-C/G invertebrates and 63 vertebrates. Using Ward's method, vertebrates and invertebrates were completely classified into two major clusters (Figure 8). In the resulting tree, *X. bocki* and Hemichordata (*S. kowalevskii* and *B. carnosus*) are placed into the vertebrate group. Hemichordata belongs to a fish sub-cluster, while *X. bocki* is grouped with *Chlamydosaurus kingii* and *B. constrictor* in the mammalian sub-cluster. Hagfish (*E. burgeri*) falls into the invertebrate group, in contrast to its placement among the vertebrates in our other analyses (Figures 4-7) and in our previous studies (Sorimachi et al., 2013a; Sorimachi & Okayasu, 2013).

Among the 7 species pairs in the set of 58 invertebrates analyzed, only three were paired in the phylogenetic tree generated from analysis of invertebrates alone (Figure 3). When 63 vertebrate species were analyzed along with 58 invertebrates, all 7 species pairs were found in the phylogenetic tree (Figure 8), even though increased sampling increases coincidental similarities and leads to worsening clustering results. These results support the appropriateness of Ward's method using amino acid composition. The results of cluster analysis of the data set consisting of 58 invertebrate and 63 vertebrate species are consistent with our previous results (Sorimachi et al., 2013a; Sorimachi & Okayasu, 2013).

4. Discussion

In our previous study (Sorimachi & Okayasu, 2003), we demonstrated that a universally representative phylogenetic tree is not currently available because of various factors affecting phylogenetic results. Increased sampling and reductions in the number of character states increase coincidental similarities, leading to worsening clustering results. Conversely, factors that reduce coincidental similarities yield better phylogenetic constructions. When amino acid composition was analyzed using two independent clustering methods, vertebrates and invertebrates were well-differentiated, even when the number of vertebrates was increased from 32 to 63 (Figures 3-8). In addition, hemichordates and *X. bocki* were placed with vertebrates, and were separated from invertebrates. Based on these results, *X. bocki* and hemichordates *B. carnosus* and *S. kowalevskii* are more closely related to vertebrates than to invertebrates.

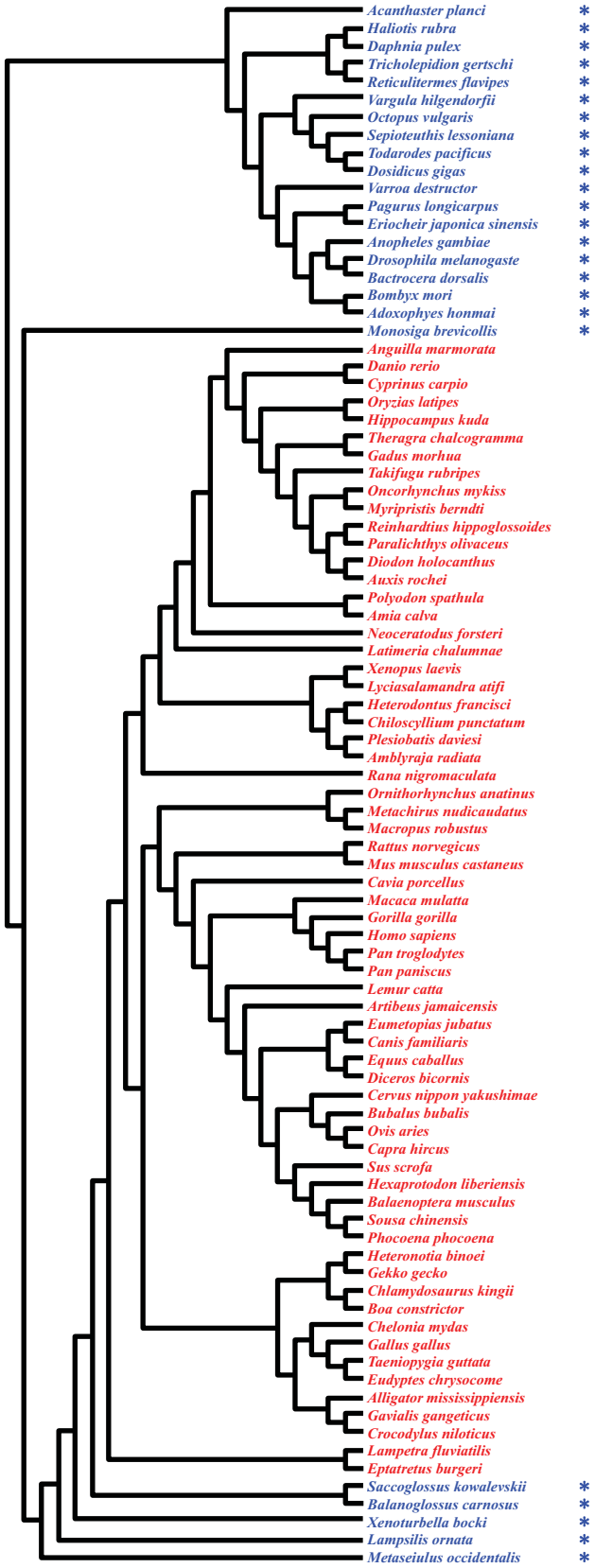

Figure 7. Phylogenetic tree generated using neighbor-joining (Saitou & Nei, 1987) from 16S rRNA gene sequences. High-C/G invertebrates and vertebrates are shown in blue (*) and red (null), respectively

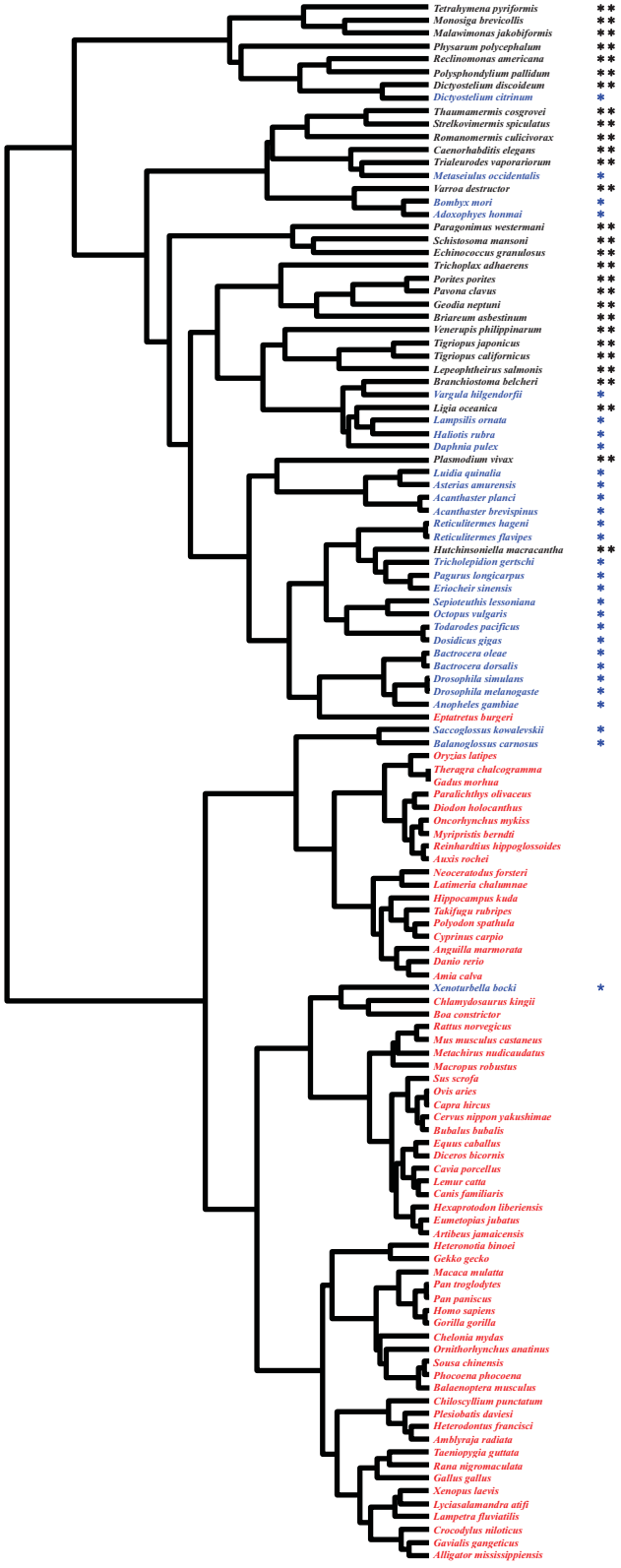

Figure 8. Phylogenetic tree generated using Ward's cluster analysis method (Ward, 1963) from predicted amino acid composition of complete mitochondrial genomes of 58 invertebrates and 63 vertebrates.High-C/G invertebrates, low-C/G invertebrates, and vertebrates are indicated in blue (*), black (**), and red (null), respectively

Neighbor-joining analysis of 16S rRNA gene sequences separated *X. bocki* and hemichordates from the invertebrate group and placed them within the vertebrates, whereas *L. ornata* and *M. occidentalis* were distinct from invertebrates (Figure 6). *X. bocki* and Hemichordata are obviously more closely related to vertebrates than invertebrates, although some exceptions were observed. In addition, neighbor-joining using predicted amino acid composition from mitochondrial genomes suggested that *X. bocki* and Hemichordata are closely related to vertebrates (Figure 7). Based on these results, *X. bocki* and hemichordates (*B. carnosus* and *S. kowalevskii*) are closely related to vertebrates.

The taxonomic position of the genus *Xenoturbella* has long been uncertain (Westblad, 1949). It was once thought to be closely related to mollusks (Noren & Jondelius, 1997), but this conclusion was due to DNA contamination from mollusks, a food source for *Xenoturbella* (Bourlat et al., 2006; Israelsson & Budd, 2006). The genus currently comprises its own phylum, and a close relationship with Acoelomorpha is strongly supported based on both morphological and molecular data (Lundkin, 1998; Raikova et al., 2000; Hejnol et al., 2009). A sister group relationship with echinoderms and hemichordates was also recently reported (Philippe et al., 2011). In our study, in contrast, *Xenoturbella* is closely allied with reptiles (*B. constrictor*) and hagfish (*E. burgeri*) (Figure 5), or to reptiles (*B. constrictor* and *C. kingii*) (Figure 4). These results are based on Ward's method using amino acid composition. According to cluster analyses using mixtures of vertebrates and invertebrates, *Xenoturbella* is more closely related to vertebrates than to invertebrates, falling into a large cluster consisting of all mammals. In addition, *Xenoturbella* is separated from another large cluster consisting of all fish, although hemichordates belong to this cluster. Ultimately, *Xenoturbella* is closely related to neither Echinodermata nor Hemichordata in our present study.

5. Conclusion

Among the invertebrates, the Hemichordata are the closest extant phylogenetic relatives of chordates, and have been described as a sister group to echinoderms such as the sea urchin (Philippe et al., 2011). In our study, consistent with this classification, hemichordates fall into a cluster consisting of echinoderms, mollusks, and arthropods (Figure 2). *Xenoturbella* is also contained in this cluster. Cluster analyses using samples of vertebrates and invertebrates, however, revealed that hemichordates are more closely related to vertebrates than to invertebrates, and that they are phylogenetically distant from *X. bocki* (Figures 4-8). Based on these results, both hemichordates and *Xenoturbella* are more closely related to vertebrates than to invertebrates, but do not fully belong in either group.

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Detection of *Beet Necrotic Yellow Vein Virus* by Double Stranded RNA Analysis

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Abstract

Beet necrotic yellow vein virus (BNYVV), the type member of the *Benyvirus* genus, has a multipartite, positive-sense single-stranded RNA genome, which consists generally of four, or in some isolates five, distinct RNA species. In this study, 108 BNYVV infected soil samples were collected from Isparta province, Turkey. Sugar beet plants cv Kasandra were grown in these soil samples using bait plant techniques and root samples were then analyzed by dsRNA analysis. The RNA was purified by CF-11 cellulose chromatography and gel electrophoresis. In 108 samples tested, dsRNA profiles were observed in 53 samples. No dsRNA bands were observed in negative control used in the analysis.

Keywords: sugar beet, BNYVV, rhizomania, dsRNA analysis

1. Introduction

Rhizomania is caused by *Beet necrotic yellow vein virus* (BNYVV). Rhizomania causes serious disease of sugar beet. It was first reported in Italy in the 1950s (Canova, 1959) but now it is present in sugar beet areas all over the world (Chiba et al., 2011). BNYVV is transmitted in soil by zoospores of plasmodiophorid, *Polymyxa betae* (Keskin, 1964). BNYVV is member of the genus *Benyvirus* (Tamada, 1989). BNYVV is characterized by rod-shaped particles, 20 nm in diameter and four different model lengths 85, 100, 265 and 390 nm (Putz, 1977) containing four seperate single stranded genomic RNAs of 1467, 1774, 4612 and 6746 base pairs, respectively.

In some Asian, French and English isolates, 5th RNA, which is 1349 nucleotide long has been described (Saito et al., 1996; Koenig et al., 1997; Harju et al., 2002; Ward et al., 2007). RNA 1 and RNA 2 have "housekeeping" genes involved in replication, assembly and cell to cell movement, whereas RNA 3, RNA 4 and RNA 5 are associated with vector-mediated infection and disease development in sugar beet roots (Tamada, 2007).

The first sign of rhizomania disease in a sugar beet crop appears as light green or yellow irregularly shaped patches in the field. Individual plants show the characteristic proliferation of fibrous roots around the tap root, "the root madness symptoms" of rhizomania. In severely infected plants, the tap root and lateral roots become necrotic and die then and the vascular tissue develops a pale brown coloration (Brunt & Richards, 1989).

BNYVV leads to serious decreases in root yield and quality of sugar. Virus reduces sugar content in the roots by 3-4% and yields of sugar beet more than 50-60% (Henry, 1996).

In Turkey, Rhizomania was first detected in Alpulu Sugar Refinery area. Later the presence of the disease was reported in different beet growing areas of Turkey (Vardar & Erkan, 1992; Kıymaz & Ertunç, 1996; Ertunç et al., 1998; Kutluk-Yılmaz & Yanar, 2001; Kaya, 2009).

More than 90% of plant viruses have single or double stranded RNA genomes. During the replication of single-stranded RNA viruses, a complementary strand of viral RNA is synthesized. An annealed dsRNA can be isolated by phenol extraction (Zaitlin & Hull, 1987). Morris & Dodds (1979) developed methods for the isolation and analysis of viral dsRNAs in diseased plant tissue.

Analysis of dsRNA has been used as a means of virus detection in various crops (Rezaian & Krake, 1987; Monette et al., 1989; Yardımcı & Açıkgöz, 1997; Bostan & Açıkgöz, 2000; Yardımcı &Korkmaz, 2004; Yardımcı & Eryiğit, 2006) including sugar beet (Hutchinson et al., 1992; Ilhan & Ertunç, 2001; Ertunç & Ilhan, 2002).

This study aimed to identify BNYVV on sugar beet plants by dsRNA analysis.

2. Materials and Methods

2.1 Sampling

Soil samples were collected in August and September 2011 from soils used in sugar beet culture of Isparta Isparta province. The samples were selected considering the visual indications for the presence of rhizomania in field-grown sugar beet plants, such as yellow coloration of leaves and beard-like appearance of the roots (Figure 1). Each sample consists on a mixture of 5 sub-samples collected from different parts of the same fields.

Figure 1. Typical root symptoms of Rhizomania in field-grown sugar beet plants in Gonen region, Isparta Province

2.2 Bait Plant Technique

The soil samples were air dried for 3-4 weeks placed in sterilized pots and 10 seeds of *Beta vulgaris* cv. Kasandra were sown in each pot. Sugar beet plants were grown for 9 weeks in the greenhouse at 23 °C then roots from each pot were harvested separately. Pots containing sterilized soil were used as negative control. Roots were placed into polyethylene bags labeled and stored at -20 °C until dsRNA analysis was performed.

2.3 dsRNA Analysis

The method for dsRNA extraction was performed according to Morris and Dodds (1979). 20 g of bait plant roots was homogenized with a mortar and pestle. It was added 1 ml of 10% SDS, 1 ml of 2% Bentonit, 10 ml of 1XSTE (0.1 M NaCl, 0.05 M Tris- HCl, 1mM EDTA, pH: 6.9), 10 ml of water-saturated phenol and 5 ml of Chloroform: pentanol (25:1). The homogenate was mixed for 60 min. The homogenate was centrifuged at 8000 g for 20 min. The upper aqueous phase was withdrawed and placed it in a 50 ml centrifuge tube and 2.1 ml of 96% Ethanol were added. The samples were stored overnight at 4 °C. 1 g portion of Whatman CF-11 cellulose per sample was added. Cellulose colons were prepared with 20 ml plastic syringe. The samples were added to one colon and let it drain completely. The colon was washed with 60 ml of 1XSTE containing ethanol (16%). Double-stranded RNA was then eluted with STE buffer and precipitated by adding 0.5 ml of 3 M Sodium acetate (pH 5.5) and 20 ml of 96% Ethanol to each sample.

The dsRNA was stored at -20 °C overnight. The precipitated dsRNAs were collected by centrifuged at 8000 g for 25 min. The pellet was dried and resuspended in 200 µl TBE (Tris, Boric Acid, EDTA). The samples were mixed with 30 µl 3M Sodium acetate and 0.9 ml of 96% Ethanol then incubated at -20 °C overnight. After centrifugation at 5000 g for 20 min. The pellet was resuspended in sterile distilled water and used in electrophoresis.

Double- stranded RNA was analysed by electrophoresis in agarose gels. The gels were electrophoresed at 100 V for 1 hour, stained with ethidium bromide and then visualized and photographed by Doc-It gel imaging and documentation system (UVP, England).

3. Results and Discussion

Typical rhizomania symptoms on leaves and roots were observed on plants growing under the greenhouse

conditions after 9 months (Figure 2). Typical symptoms of BNYVV which are beard-like apperance of the roots, light green coloration of leaves.

dsRNA profiles typical of BNYVV were observed in 53 of 108 samples tested. However, healthy sugar beet did not show any dsRNA bands onto agarose gel. In 28 samples were observed RNA 1+2+3+4, in 9 samples were observed RNA 3, in 7 samples were observed RNA 1+2+3, in 9 samples were observed RNA 1+3 (Figures 3-4). In this research, RNA 5 has not been identified in the analyzed samples. Profiles of dsRNA obtained in this study are shown in Table 1.

The dsRNA profile detected in this study was same as previously reported BNYVV dsRNA profiles (Hutchinson et al., 1992; Ilhan & Ertunç, 2001; Ertunç & Ilhan, 2002). Non-specific bands were observed in agarose gel. The main reason is thought to be the lack of enzymatic treatment.

Variable dsRNA profiles were observed in BNYVV isolates. These alterations in the deletion mutations of smaller RNAs were associated with changes in symptoms expression of BNYVV, molecular differences between the strain of BNYVV or low virus concentration in the isolates (Henry et al., 1986; Koenig et al., 1986). The yield low amount of dsRNA in infected plants is related specifically to the time of infection and incubation temperature (Valverde et al., 1990).

Figure 2. Beard like appaerance of bait plant roots

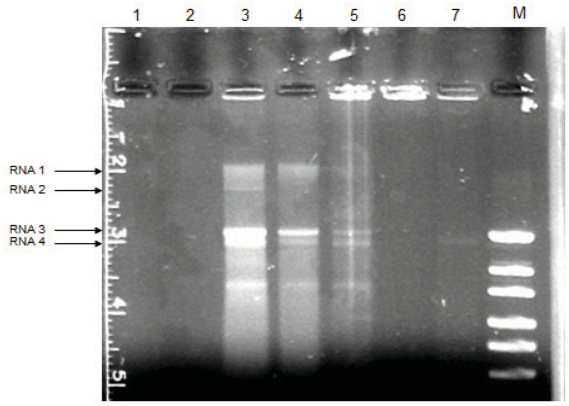

Figure 3. Agarose gel electrophoresis of dsRNA from BNYVV- Infected roots (M: Marker (100 bp-1.5 kb DNA Ladder, Biobasic); 1: Atabey-3 isolate; 2: Gonen-44 isolate; 3: Islamkoy-40 isolate; 4: Yalvac-11 isolate; 5: Keciborlu-17 isolate; 6: Kuleonu-60 isolate; 7: Negative control

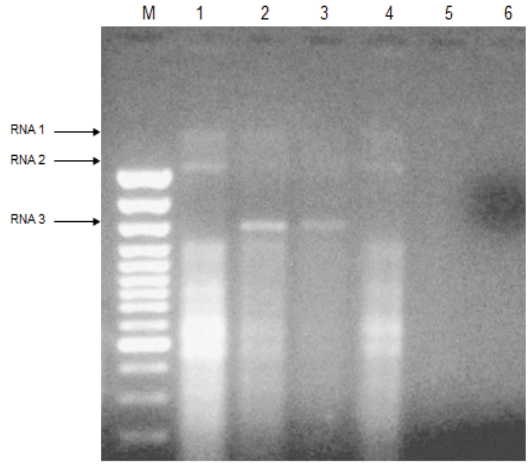

Figure 4. Agarose gel electrophoresis of dsRNA from BNYVV- Infected roots (M: Marker (100 bp DNA Ladder, Biobasic)); 1: Gonen-33 isolate; 2: Kuleonu-14 isolate; 3: Keciborlu-7 isolate; 4: Yalvac-22 isolate; 5: Senirkent 20 isolate; 6: Negative control

dsRNA analysis is simple, quick, efficient and relatively inexpensive. The purified dsRNA can be used as template for cDNA synthesis and subsequent PCR, molecular cloning, prob preparation and a reagent for mechanical inoculations (Valverde et al., 1990). dsRNA analysis is more sensitive when compared to serological tests (Ertunç & Ilhan, 2002). A disadvantage is that this procedure can generally not be used to process a large number of samples at a time. Also, since dsRNA is related to replication of the virus the titre of the dsRNA molecule may increase or decrease in the plant at certain times of the year, thereby influencing detection.

4. Conclusions

We can say that practicality of using analysis of dsRNA as an alternative or complementary method for diagnosis of BNYVV. At the very least it provides a jumping-off place in the diagnostic process before proceeding to more specific techniques.

Table 1. dsRNA Profile of BNYVV on samples in different districts of Isparta Province

Samples No	dsRNA Profile
Atabey-5	RNA 1+2+3+4
Atabey-24	RNA 1+2+3+4
Atabey-25	RNA 1+2+3+4
Atabey-78	RNA 1+2+3+4
Islamkoy-1	RNA 1+2+3+4
Islamkoy-21	RNA 1+2+3+4
Islamkoy-40	RNA 1+2+3+4
Islamkoy-48	RNA 1+2+3+4
Yalvac-2	RNA 1+2+3+4
Yalvac-11	RNA 1+2+3+4
Yalvac-41	RNA 1+2+3+4
Yalvac-23	RNA 1+2+3+4
Keciborlu-8	RNA 1+2+3+4
Keciborlu-22	RNA 1+2+3+4

Keciborlu-20	RNA 1+2+3+4
Keciborlu-17	RNA 1+2+3+4
Senirkent-5	RNA 1+2+3+4
Kuleonu-66	RNA 1+2+3+4
Kuleonu-23	RNA 1+2+3+4
Kuleonu-88	RNA 1+2+3+4
Kuleonu-29	RNA 1+2+3+4
Kuleonu-67	RNA 1+2+3+4
Sarkikaraagac-77	RNA 1+2+3+4
Sarkikaraagac -1	RNA 1+2+3+4
Sarkikaraagac -95	RNA 1+2+3+4
Sarkikaraağac-15	RNA 1+2+3+4
Sarkikaraağac-58	RNA 1+2+3+4
Gonen-3	RNA 1+2+3+4
Gonen-33	RNA 1+2+3
Gonen-70	RNA 1+2+3
Gonen-2	RNA 1+2+3
Gonen-56	RNA 1+2+3
Gonen-68	RNA 1+2+3
Gonen-23	RNA 1+2+3
Gonen-99	RNA 1+2+3
Islamkoy-43	RNA1+3
Islamkoy-9	RNA1+3
Kuleonu-77	RNA1+3
Kuleonu-78	RNA1+3
Kuleonu-86	RNA1+3
Kuleonu-95	RNA1+3
Atabey-33	RNA1+3
Atabey-11	RNA1+3
Atabey-10	RNA1+3
Keciborlu-7	RNA 3
Keciborlu-12	RNA 3
Islamkoy-12	RNA 3
Islamkoy-61	RNA 3
Islamkoy-10	RNA 3
Islamkoy-88	RNA 3
Kuleonu-14	RNA 3
Gonen-28	RNA 3
Gonen-81	RNA 3

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