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# CLASSIFICATION OF POPULATION STRUCTURE FOR ALLELOPATHIC PROPERTIES IN ITCHGRASS (*Rottboellia cochinchinensis*)

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## ABSTRACT

Biodiversity of itchgrass from different areas were studied by morphological traits and AFLP analysis for classify of an allelopathic ability. The correlation of the similarity/distance between AFLP markers (Jaccard coefficient) and morphological traits (Euclidean distance) was significant with r = − 0.84**. Itchgrass were divided into two groups from both UPGMA and STRUCTURE analyses: the group A consisted of itchgrass from Chaehom-Lampang, Si Thep-Phetchabun, Phrom Phiram-Phitsanulok, Amphur Muang-Nakhon Sawan, Kamalasai-Kalasin, Amphur Muang-Chachoengsao and Bang Yai-Nonthaburi, whereas itchgrass from Amphur Muang-Chiang Mai, Pak Chong-Nakhon Ratchasima and Kamphaeng Saen-Nakhon Pathom constituted the group B. Allelopathic properties of itchgrass as representative from different group were determined in bioassay test, the aqueous extract of itchgrass from Chaehom-Lampang area has a strong allelopathic ability on growth of *Echinochloa crus-galli*, *Bidens pilosa* and *Lactuca sativa* than the other group. Molecular analysis was strongly supported in morphological clustering with bioassay test, specific morphological traits were soft trichomes, dark purple stems and roots which can be used for the preliminary classification of allelopathic ability. This suggests that itchgrass classification by morphological traits is related to the analysis of the genetic relationship of itchgrass with AFLP analysis, that allowing the assessment of bio-diversity of itchgrass and their allelopathic potentials.

Keywords: AFLP analysis, allelopathy, genetic diversity, itchgrass, morphological traits

## INTRODUCTION

A synergistic relationship between the allelopathic potential and genetic, chemicals, and ecological factors, not only affects both the stable and unstable allelopathic potentials, but also relatively strong or weak allelopathic abilities (Zuo *et al.*, 2007). Allelopathic activity was identified in many plant species. Such as *Avena sativa* L. (Kato-Noguchi *et al.*, 1994), *Helianthus annuus* L. (Ohno *et al.*, 2001), and *Rottboellia exaltata L. f.* (Kobayashi *et al.*, 2008, Meksawat and Pornprom, 2010), have been found to have allelopathic properties. However, the age and genotype, location or environment, and cropping system, all of which influence the production of allelochemicals that impact on plant growth, in the field, laboratory, or greenhouse (Weston *et al.*, 2013). Therefore, a better understanding of the allelopathic potential of physiological, biochemical, and genetic levels will advance our knowledge on the role of allelopathic properties.

Itchgrass [*Rottboellia cochinchinensis* (Lour.) W.D. Clayton] is an annual upland weed, self-pollinated, and widely distributed in the tropical and subtropical regions (Alloub *et al.*, 2005, Alves *et al.*, 2003, Millhollon and Burner, 1993). It is a dominant species in some regions including Thailand where has a serious weed problem from itchgrass in several crops. Conversely, farmers in Chaehom-Lampang area in northern Thailand cultivated itchgrass as a mulching material for weed control in vegetable fields (Kobayashi *et al.*, 2008, Meksawat and Pornprom, 2010). Itchgrass is a wide diversity in the morphological, biological, and physiological features between itchgrass populations in Thailand. The climatic, edaphic, and biotic factors can affect the morphological and physiological

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characteristics of weeds and cause the evolution of new weed biotypes (Zimdahl, 1993). Thus, the appearance and dispersal of itchgrass in different areas needs to be considered to classify itchgrass accessions into certain phenoltypic groups relevant to itchgrass management. Millhollon and Burner (1993) analyzed itchgrass collected from 34 countries and identified five biotypes based on general morphologies and growth patterns. Alves *et al.* (2003) classified itchgrass collected from six Brazilian regions using both molecular marker and morphological traits. Furthermore, three major groups were reported in Peninsular Malaysia based on the variations in growth behavior of itchgrass (Alloub *et al.*, 2005).

Techniques based on DNA sequencing have great accuracy in taxonomic studies, and amplified fragment length polymorphism (AFLP) markers are used to determine genetic variation across different populations (Powell *et al.*, 1996). AFLP analysis was successfully utilized to reveal genetic diversity among *Echinochloa* species (Tabacchi *et al.*, 2006). To assess genetic diversity, AFLPs were applied on *Glycine max* L. Merr. (Tara Satyavathi *et al.*, 2006) and *Triticum aestivum* L. (Eivazi *et al.*, 2007). Molecular markers can establish associations between genotype and morphological traits. Zhao *et al.* (2007) reported the genetic variation of *Brassica rapa* with AFLP and the morphological data. Kantartzi and Stewart (2008) identified 58 cotton germplasms to evaluate fiber trait and their diversity by means of DNA markers. Skot *et al.* (2005) studied the heading date of natural populations of perennial ryegrass and identify genetic diversity with AFLP markers. Thus, a formal comprehension of the genetic diversity and morphological traits is called for the purpose of the classification of the allelopathic potential of itchgrass. The limited information is available on the morphological and genetic diversity of itchgrass in Thailand is not fully understood, in each morphotype may have different allelopathic abilities. Therefore, the objectives of this study were taken in order to determine genetic diversity, population structure, and a potent allelopathic of itchgrass using morphological and AFLP markers.

## MATERIALS AND METHODS

### Plant Materials

Itchgrass seed from ten areas within the main crop-growing regions were investigated for classification of the population structure. Each location were represented a distinct geographical region in each part of the different areas. For example, Chaehom-Lampang (CH-LP) and Amphur Muang-Chiang Mai (AM-CM) were located in northern region. The sampling areas in central region were Phrom Phiram-Phitsanulok (PR-PL), Si Thep-Phetchabun (ST-PB), Amphur Muang-Nakhon Sawan (AM-NS), Bang Yai-Nonthaburi (BY-NB), and Kamphaeng Saen-Nakhon Pathom (KPS-NP). Pak Chong-Nakhon Ratchasima (PC-NR) and Kamalasai-Kalasin (KL-KS) itchgrass were located in north-east region whereas Amphur Muang-Chachoengsao (AM-CS) was situated in east region (Figure 1). The seeds were stored at 7°C until the initiation of the experiment.

The itchgrass-powder was prepared from mature itchgrass plants, which were grown under uniform environmental and agronomical conditions. They were harvested at maturity and then separated into shoot and root. These plant portions were individually cut and air-dried at 40°C for one week, and ground into powder with an electrical grinder to pass a 0.5 mm screen mesh and stored in plastic bottles at −20°C.

Three test plant species such as *Bidens pilosa* L. var. *radiata* Sch. Biq., *Echinochloa crus-galli* L. P. Beauv., and *Lactuca sativa* L. var. OP were used in bioassay test. Test plant seed were germinated (radicle~0.02 m) before used in the experiment.

### Morphological Traits

A single itchgrass seedling in each location was transplanted to a pot and placed in a greenhouse under controlling condition. The data were collected at different growth stages of itchgrass that included the 2−3 leaf stage, tillering, flowering, and maturation with measurements of inflorescence length, leaf length, plant height, number of seeds: inflorescence, 1000 seeds weight, seed size, trichomes, leaf blade color, leaf sheath color, stem color and root color. The morphological characteristic data were standardized and the Euclidean distance coefficients were calculated for all pairs of the samples using the similarity of interval data (SimInt) module of the software package. The cluster analysis was performed according to the unweighted pair group method with arithmetic averages (UPGMA method) based on: trichomes, the color (ligule, leaf sheath, and stem), as well

as seed size (width, length, and 1,000 seed weight) with the SAHN algorithm. The goodness of fit of the clustering compared with the basic data matrix was tested by computation of the cophenetic correlation coefficient using the normalized Mantel statistics Z test through the Coph and MxComp algorithm. All commands were created with the application of the NTSYSpc software package version 2.01e (Mantel, 1967, Rohlf, 1998).


Figure 1. Geographical locations of the 10 fields where itchgrass samples were collected from four regions of Thailand, Chaehom-Lampang (CH-LP), Amphur Muang-Chiang Mai (AM-CM ), Si Thep-Phetchabun (ST-PB), Phrom Phiram-Phitsanulok (PR-PL), Amphur Muang-Nakhon Sawan (AM-NS), Pak Chong-Nakhon Ratchasima (PC-NR), Kamalasai-Kalasin (KL-KS), Amphur Muang-Chachoengsao (AM-CS), Bang Yai-Nonthaburi (BY-NB), and Kampha-eng Saen-Nakhon Pathom (KPS-NP)

**AFLP Analysis**

Genetic diversity of itchgrass from ten locations was determined. Fresh leaf tissue at the 2-3 leaf stage was harvested from each location as mentioned before and was frozen in liquid nitrogen. Subsequently, 100 mg of the frozen leaf was used for DNA extraction with the modified method as described by Doyle and Doyle (1987), and Cullings (1992). The DNA analysis using AFLP markers was conducted as described by Vos *et al.* (1995). The isolated genomic DNA was digested with *Eco*RI and *Mse*I restriction enzymes, a total of five AFLP selective primer combinations were used for selective amplification (E-ACA : M-CGA, E-ACA : M-CGC, E-ACA : M-CGG, E-ACA : M-CGT, and E-ACC : M-CGG) and amplication products were detected with denaturing Polyacrylamide Gel Electrophoresis and silver straining. AFLP bands throughout the gel profiles were scored visually as present (1) or absent (0) across ten locations. The total number of fragments scored, the number of polymorphic fragments, and the percentage of polymorphic fragments were determined for each primers used. Genetic similarity based on the Jaccard coefficient was calculated and the UPGMA method was used. Cluster analysis with the application of the NTSYSpc program for Windows Version 2.01e was utilized to generate a Phylogenetic tree (Jaccard, 1908, Rohlf, 1998).

**Population Structure Analysis**

The identification groups of population structure of sampled itchgrass in ten locations, they were estimated by STUCTURE program version 2.1 using Bayesian approach available at http://pritch.bsd.uchicago.edu (Pritchard *et al.*, 2000, Falush *et al.*, 2003). Principal component analysis of morphological data based on correlation matrix and the relationship of genetic similarity/distance between AFLP and morphological traits as simple correlation were determined with PAST.exe version 2.10 (http://folk.uio.no/ohammer/past).

**Bioassay Test**

Phytotoxicity of the aqueous extract from itchgrass on growth of test plants was determined for allelopathic properties through bioassay test. Aqueous extract were prepared by saturating 500 mg of itchgrass-powder in 100 mL distilled water for 24 h and filtrated before used. Distilled-water was used to establish various quantities of the extracts from the original extract equivalent to 0, 5, 10, 50, and 100 mg/mL with a pH level in the range of 6.4−6.6. A twenty five seedling of test plant species was placed in a petri dish lined with filter paper and saturated

with 4 mL of aqueous extract in various required concentrations. The petri dish was kept in an incubator at 25°C for 24 h. Both of shoot and root length of the test plant was measured at 5 days after transplant in accordance with the method outlined by Meksawat and Pornprom (2010).

Inhibition percentage was calculated using the following equation: (%) Inhibition = [1-(length with aqueous extract/length with control)] × 100. The data were analysed for analysis of variance (ANOVA) including the calculation of the means, standard errors of means (SEs) and degrees of freedom. The statistical analyses were conducted using the R-program.

## RESULTS AND DISCUSSION

### Morphological Traits

The morphological of itchgrass was investigated for clustering, itchgrass accessions were characterized by means of examination of the main morphological traits for the clustering of the specimens. Principal coordinate analysis of morphological traits of itchgrass from ten locations was reported. Based on the morphological traits and the data from ten locations, the cophenetic correlation between clusters, and the morphological traits similarity data resulted a good fit (r = 0.91936). To assess genetic diversity of itchgrass, ten accessions of itchgrass seed collected from the main crop-growing regions of Thailand were investigated. Cluster analysis with the application of the UPGMA method separated itchgrass populations into two main clusters: Group A included seven locations, with all further specimens categorized into Group B, which was composed of three locations (Figure 2A). Group A consisted of itchgrass from CH-LP, ST-PB, PR-PL, AM-NS, KL-KS, AM-CS, and BY-NB; whereas Group B consisted of samples from AM-CM, PC-NR, and KPS-NP.

The morphological traits related to allelopathic potential of itchgrass from CH-LP with soft trichomes and dark purple stem and root had the high potential for allelopathy compared with a diminutive with a little soft trichomes and green stem and root. It is similar to the result of principal component analysis (PCA) that explained the morphological data with 83.16 % total variability where all seven morphological traits provided suitable data for the morphological analysis. Principal component 1 (PC1) explained for 50.69% variability of seed width, seed length, ligule contrasted with leaf sheet color, and thousand seed weight whereas PC2 explained 32.47 % variability with stem color and trichomes. The itchgrass population from CH-LP was separated from others by morphological trait and agreed to the allelopathic potential of itchgrass where CH-LP was clarified for morphological and allelopathic potential trait with trichomes character and stem color. The other populations were grouped into two groups by PCA where AM-CM, KPS-NP, and PC-NR were grouped with green leaf sheet color and slightly green ligule. In comparison, the second group composed of itchgrass populations from AM-NS, PR-PL, ST-PB, KL-KS, AM-CS, and BY-NB with red pink leaf sheet color and purple ligule (Figure 2B).

The relationship of itchgrass population in each group was explained by the UPGMA dendrogram. Itchgrass in Group A possessed some distinct morphological traits such as, the color (leaf sheath, stem and ligule), trichomes, size of seed (length and width), and 1,000 seed weight in comparison to the itchgrass samples in Group B. Plant height, number of leaves, number of seeds/plant, secondary culm diameter, and the weight of 100 intact or dehulled seeds were the most significant characteristics which provides strong evidence for the existence of three itchgrass groups in Peninsular Malaysia (Alloub *et al.*, 2005). Even though the limitation of morphological traits for species classification is affected by both genetic and environment factors in identical conditions, the morphological traits do not differentiate from those of the itchgrass in the original region. This is an indication that the morphology of itchgrass is dependent on the genotype rather than the environment.

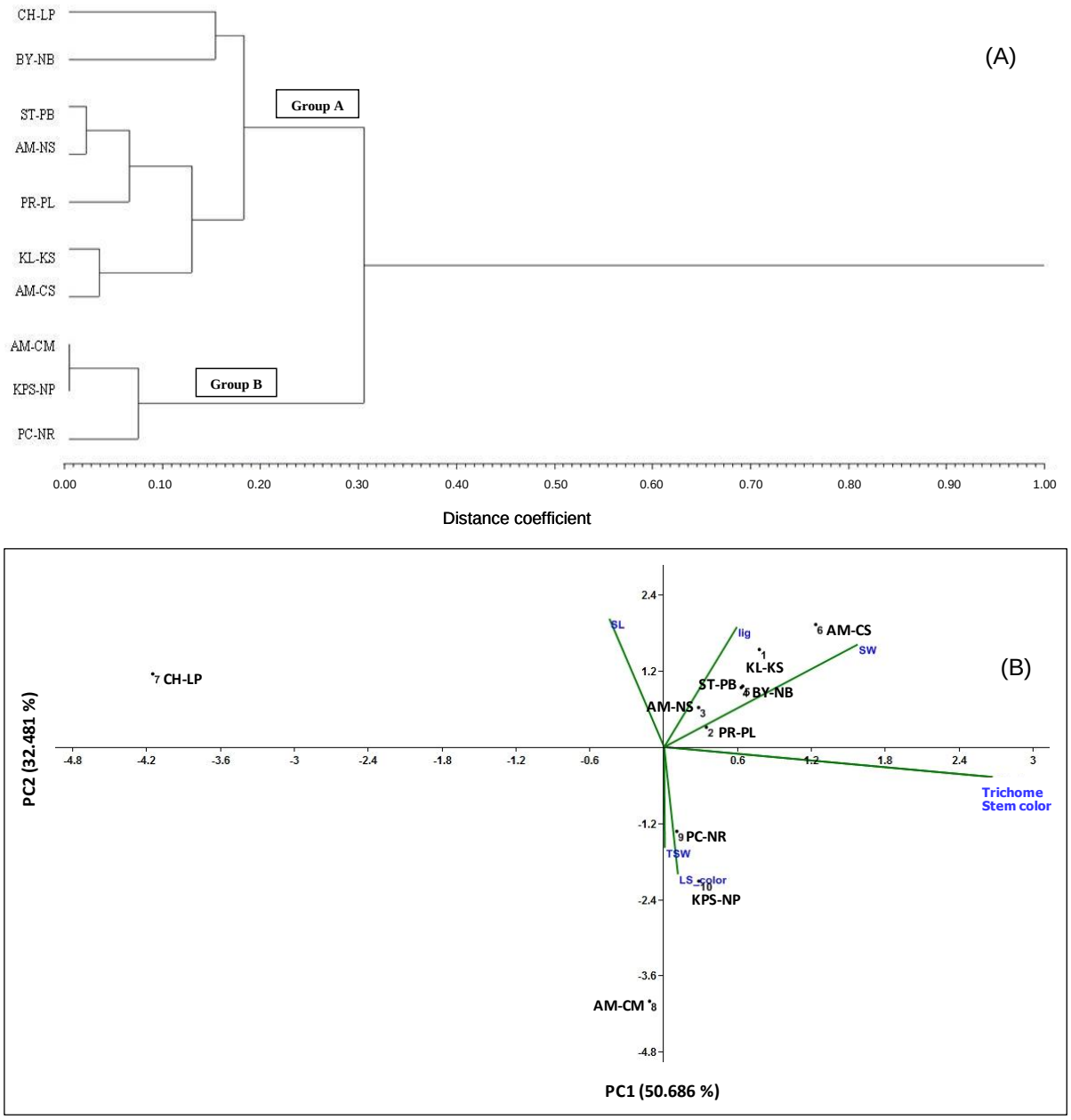



Figure 2. The cluster analysis based on morphological traits for itchgrass from ten locations; (A) the grouping of morphological data from UPGMA method using NTSYS, and (B) principal component analysis of itchgrass from ten location based on correlation coefficient of seven morphological traits

**AFLP Analysis**

A total of five AFLP primer combinations were used to classify ten diverse locations of itchgrass which yielded clearly distinct bands on clustering. There was a cophenetic correlation between clusters and the genetic similarity data (r = 0.9997). The cluster analysis was performed on all the data obtained from all five primer pairs with 206 polymorphic alleles. The dendrogram was obtained using the Jaccard's coefficients. The Jaccard's similarity coefficients ranged from 0.1991 to 0.9907. The highest similarity (0.9907)

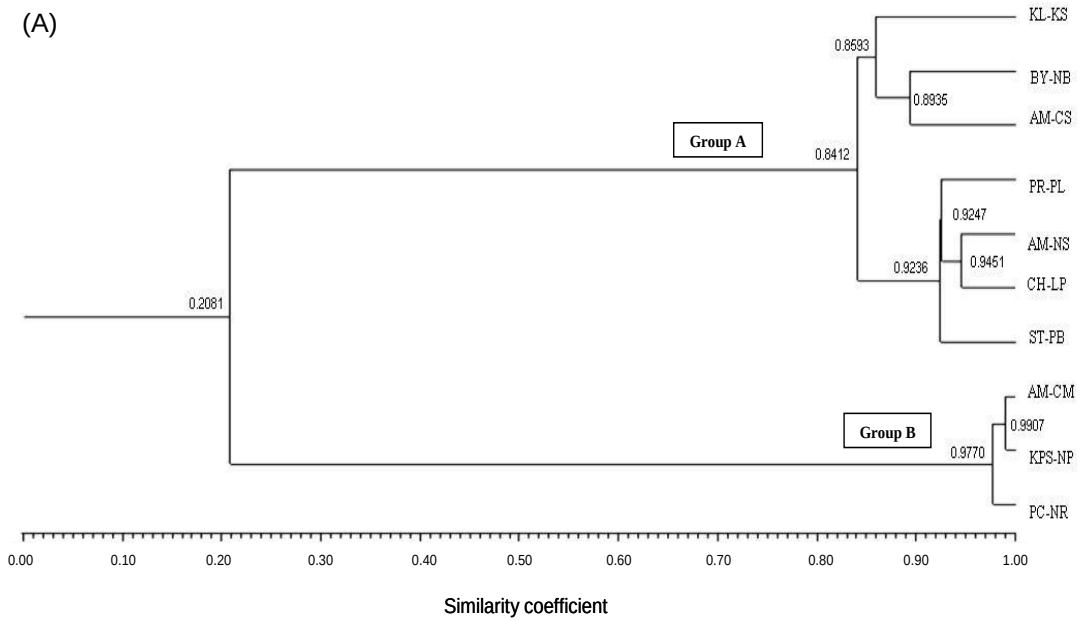
for pair wise comparisons among the ten accessions was observed between accessions AM-CM and KPS-NP, and the lowest similarity (0.1991) was obtained between KL-KS versus PC-NR and for CH-LP versus PC-NR. Two main groups (A and B) were revealed by dendrogram. Group A, which comprised the seven locations of the itchgrass samples, had an average similarity of 0.84. Group B had the highest average similarity (0.98) including itchgrass from three locations. The percentage similarity was clearly elevated among these samples. There was a segregation of itchgrass into two groups: the first group consisted of itchgrass from CH-LP, ST-PB, PR-PL, AM-NS, KL-KS, AM-CS, and BY-NB, whereas itchgrass from AM-CM, PC-NR, and KPS-NP constituted the second group (Figure 3A).

AFLP analysis suggests that samples from ST-PB, PR-PL, AM-NS, KL-KS, AM-CS, and BY-NB are closely related to CH-LP with respect to the allelopathic potential in itchgrass, while itchgrass from AM-CM, PC-NR, and KPS-NP is a weak-allelopathic properties group. Itch-grass can be described by two large groups which are suspected to release allelochemicals and/or is a major weed widely distributed in crop fields. Our experiments are the first study of population structure and genetic diversity for the potential allelopathy of weed in Thailand and AFLP analysis revealed the population structure of ten accessions separated into three sub-populations agreed with the sampling and distribution areas. In another similar study, 134 durum wheat accessions mainly from North America and Mediterranean region were analyzed with SSR markers and population structures by geographical origin of the accessions were classified (Maccaferri *et al.*, 2005). The findings of population structure analysis of 56 *Gossypium arboreum* germplasm accessions based on SSR markers were able to identify cotton corresponding to their geographical region (Kantartzi and Stewart, 2008).

**Population Structure Analysis**

The relationship of itchgrass population in each group seems to be well explained by the UPGMA dendrogram. However, another program was utilized to evaluate the relationship among populations and their classified population group to confirm the population structure of ten itchgrass samples. The population structure was analyzed by STUCTURE program. Three subpopulations (K=3) were identified with the highest likelihood value [Pn(D) = -492.3] by K1 to K5 running. The average distance between individual in the subpopulation group 1-3 (K1-3) was 0.4047, 0.0871, and 0.0093, respectively (Figure 3B). As there were also three clusters from the UPGMA method, the three UPGMA clusters were compared with STRUCTURE program analysis to determine how the programs discriminated among populations. The result from STRUCTURE was similar with cluster from UPGMA analysis. Subpopulation 1 included three itchgrass from AM-CS related with BY-NB and KL-KS for 79.2% and 67.3 %, respectively. These three populations were collected from central and north-east of Thai-land. The second subpopulation was the group of itchgrass collected from closed area consisting of itchgrass from AM-NS, ST-PB, CH-LP, and PR-PL, and were closely related of samples from central through north of Thailand. The highly closed relationship of three itchgrass populations were the third subpopulation includ-ing itchgrass collected from AM-CM, PC-NR, and KPS-NP, and were similar in morphological characters.

The correlation of the similarity/distance between AFLP markers (Jaccard similarity coefficient) and morphological traits (Euclidean distance) was significant with $r = -0.84^{**}$. Therefore, all similarity and distance between itchgrass accessions were related and itchgrass from CH-LP had an allelopathic effect on test plant species, hich clustered in different group by both morphological and AFLP clustering. Another group clustered itchgrass from PC-NR and KPS-NP in the same group. When morphological clustering was compared with AFLP clustering for Group A from morphological data, seven itchgrass accessions (KL-KS, BY-NB, AM-CS, PR-PL, AM-NS, CH-LP, and ST-PB) were grouped in the same as group A based on the AFLP data. Group B from morphological data clustered three itchgrass accessions (AM-CM, KPS-NP, and PC-NR) into the group that was the same AFLP clustering.



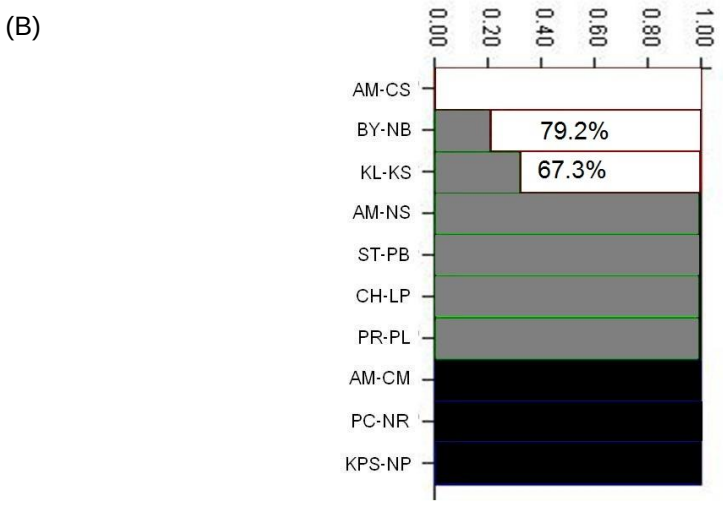




Figure 3. The dendrogram obtained with cluster analysis of AFLP data in each accession (A); Population structure of itchgrass in Thailand which K=3 and each color is one cluster analyzed by structure and given 3 clusters (B)

The genetic variation of itchgrass was investigated to classify the allelopathic potential of itchgrass morphotypes from different areas of Thailand by examining both the main morphological traits and the genetic analysis using AFLP. The molecular analysis was strongly supported by the dendrogram in the preliminary study in morphological analysis clustering. The significance of similarity/distance coefficient from morphological traits and AFLP markers correlation points out that some morphological characteristics are related to the genetic diversity of itchgrass. These results are in line with those of Na *et al.* (2010), who reported a positive correlation between the genetic distance and the geographic distance among *Typha* species in East Asia. The morphological traits and AFLP analysis generally confirmed the

distinction of two separate groups obtained from the cluster analysis. This suggests that the classification of itchgrass by morphological traits is related to the analysis of the genetic relationship of itchgrass with AFLP. This can be used to assess the genetic diversity of itchgrass with a potential for allelopathy. However, the allelopathic potential of itchgrass from different areas should be determined to confirmed the preliminary classify a potent allelopathic of itchgrass species using main morphological traits and AFLP analysis.

**Allelopathic Potential of Aqueous Extract from Itchgrass**

The aqueous extract was investigated to confirm for allelopathic potential of ichgrass in each areas. From the clustering using morphological traits and AFLP analysis results, the aqueous extract of itchgrass from three sites such as itchgrass from CH-LP area was represented as Group A, and itchgrass from KPS-NP and PC-NR areas were represented as group B were determined the phytotoxic on growth of test plant species through the bioassay test.

In general, the aqueous extract from CH-LP had inhibitory effect on seedling growth of *Bidens pilosa* L., *Echinochloa crus-galli* L., and *Lactuca sativa* L., except for the shoot growth of *Echinochloa crus-galli* L. was not significant. In addition, increased concentrations of the aqueous extract accentuated the inhibition of shoot and root growth. In conversely, both of the aqueous extract of itchgrass from KPS-NP and PC-NR area were not significantly inhibited to shoot length and root length of test plant seedling growth (Figure 4). Allelochemicals present in weed extracts reduced shoot and root growth by an average of 50% and 70%, respectively, at the highest concentration of the extract. These results indicated that itchgrass from farmer's field in CH-LP area had a strongly allelopathic effect on seedling growth of test plant species than the other areas.

The investigation of allelopathy potential of itchgrass from three sites suggested that itchgrass fom CH-LP area had an allelopathic property. Similarly, Kobayashi *et al.* (2008) also observed the allelopathic activity of itchgrass from upland fields in Lampang province in northern Thailand under field situations. The phytotoxic potential of itchgrass plants is due to the direct release of a possible toxic substance from the plant parts and/or the indirect release of a toxic substance after degradation of the plant (Meksawat and Pornprom, 2010).

From the phytotoxicity of aqueous extract from itchgrass on test plant growth result, the aqueous extracts of itchgrass from a farmer's field in CH-LP in northern Thailand had a greater inhibitory effect than itchgrass from KPS-NP and PC-NR areas. This is an indication of the inconstancy in the allelopathic potential of itchgrass from diverse origins. Such a divergence in allelopathic potential is potentially related to genetic and environmental factors. These results are similar to those of Zuo *et al.* (2007), who reported a synergistic relationship between the allelopathic potential, genetic, chemicals, and ecological factors in *T. aestivum* L.

Biodiversity of itchgrass was investigated in order to allow the classification of the potential of itchgrass with allelopathic characteristics of diverse origin through analysis of the main morphological traits and AFLP analysis. The main morphological traits and AFLP analysis suggested that itchgrass sample from CH-LP area (strong-allelopathy potential) belonged to Group A, and the itchgrass samples from KPS-NP and PC-NR with weak-allelopathy potential were assigned to Group B. This study has shown that itchgrass populations from CH-LP located in northern of Thailand had a strongly allelopathic potential. Genetic diversity based on both morphological and AFLP markers are related, and high potential for allelopathic itchgrass was clustered separately from a high potential for allelopathic itchgrass supported by both data sets. Therefore, the specific traits as soft trichomes, the dark purple color of the stems, and roots can be used for the preliminary classify of an allelopathic ability in itchgrass.

The utilization of allelopathy research as an agricultural tool against weeds may be due to its historical baggage. The development of weed management with less dependence on synthetic herbicides is one of the major challenges in agrochemical research. In recent years, the development of herbicides from naturally-occurring plant chemicals has become an important issue. The information obtained can be potentially utilized in the development of bio-herbicide for future weed management. Therefore, more research is needed to identify the allelochemicals from itchgrass.

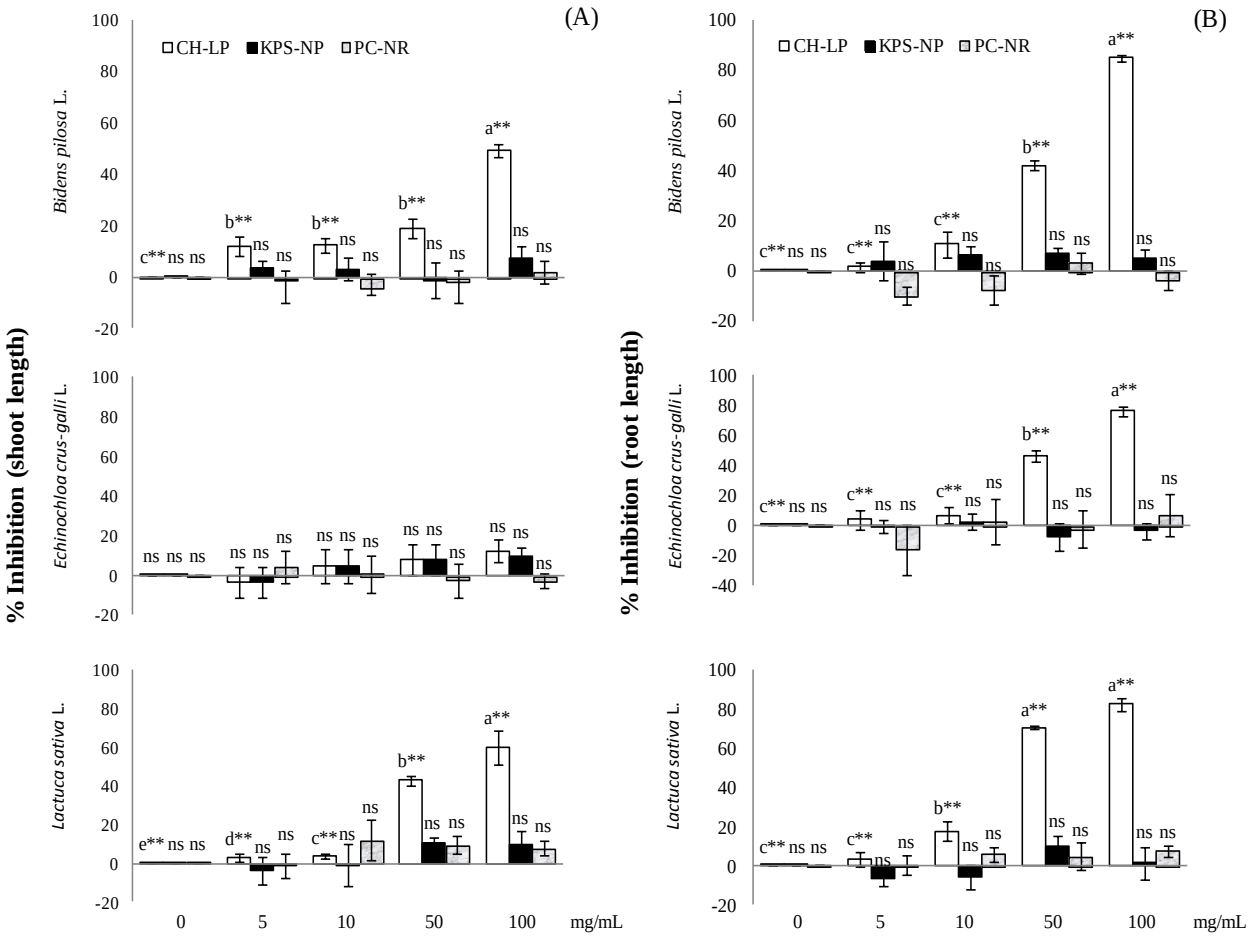



Figure 4. Inhibition of aqueous extract from itchgrass; on shoot length (A) and root length (B) of test plant seedling. The data are presented as the mean and standard errors, Least Significant Difference (LSD) indicate significant differences at $^{**}P < 0.01$ (ns = not significant)

Research on allelopathy genetics is significant and should thus be continued in order to attain further comprehension of the function of genes responsible for the presence of allelochemicals in plant.

## CONCLUSIONS AND SUGGESTIONS

Itchgrass is a weed widely distributed in maize and sugarcane fields and is suspected to release allelochemicals. The specific traits as soft trichomes, the dark purple color of the stems, and roots can be used for the preliminary classify of an allelopathic ability in itchgrass. The molecular analysis was strongly supported in morphological analysis clustering with bioassay test for allelopathic ability. The results demonstrate that the classification of itchgrass by morphological traits is related to the analysis of the genetic relationship of itchgrass with AFLP. This can be used to assess the genetic diversity of itchgrass with a potential for allelopathy in the future.

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