Variabilities in karyotype and molecular ISSR in *Gonospermum fruticosum* (C. Smith ex Link) Less., *Argyranthemum coronopifolium* (Willd.) Humphries and two strains of *A. foeniculaceum* (Willd.) Webb ex Schultz-Bip. (Asteraceae the tribe Anthemideae) collected in the Canary Islands

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**ABSTRACT.** *Gonospermum fruticosum* (C. Smith ex Link) Less, *Argyranthemum coronopifolium* (Willd.) Humphries and two strains of *A. foeniculaceum* (Willd.) Webb ex Schultz-Bip all placed in the family Asteraceae, the tribe Anthemideae, were collected in Tenerife, the Canary Islands, Spanish Territory, Africa. They were analyzed in order to clarify their relationships and variabilities by karyotypes and molecular ISSR (inter simple sequence repeat). *Gonospermum fruticosum* and *Argyranthemum coronopifolium* studied showed the common karyotype different from that of two strains of *A. foeniculaceum*. Among the 54 ISSR bands obtained in the four species studied 46 were polymorphic, and 22 were species-specific. *Gonospermum* and *Argyranthemum* showed quite different band patterns from each other. Two strains of *A. foeniculaceum* showed different, large polymorphic patterns. Thus, the ISSR must be useful tool to isolate and evaluate intergeneric, interspecific, down to intraspecific genic differences, furthermore,introgressive hybridization within the members of the tribe Anthemideae, the Asteraceae.

**KEYWORDS:** *Argyranthemum coronopifolium*, *Argyranthemum foeniculaceum*, Asteraceae, Canary Islands, *Gonospermum fruticosum*, Inter Simple Sequence Repeat (ISSR), Tribe Anthemideae,

Twenty-three species of *Argyranthemum* revised by Humphries (1976) plus some more newly recorded species (Santos personal communication) grow in Macaronesia in the Canary Islands, Madeira and the Salavage Island, Spanish Territory in Africa (Bremer and Humphries 1993). In contrast, *Gonospermum* consisted of four species in the Canary Islands and has close relatives such as *Inulanthera* and *Lugea* (Bremer and Humphries 1976). They are taxonomically placed in the Asteraceae, the tribe Anthemideae Cass., the subtribe Chrysantheminae Less. (Bremer and Humphries 1993) or Gonosperminae (Francisco-Ortega et al. 2001). The majority of the species of *Argyranthemum* such as *A. foeniculaceum* (Willd.) Webb ex Schultz-Bip. and *A. frutescens* (L.) Schultz-Bip. have crowded to non-crowded succulent leaves with two to 14 deeply filiform to linear-lanceolate lobes but some species have leaves with primary leaf-lobes ovate to obovate in outline, cypselas creamish or have leaves oblong to obovate. Leaves of *Arbyranthemum adauctum* (Link in Buch) Humphries and those of *Gonospermum fruticosum* are very similar in morphological character to each other.

*Argyranthemum* was seemed to be morphologically and karyotypically similar to *Chrysanthemum* (formerly *Dendranthera*) and *Glebionis* (Trehane 1998) together with other taxonomic groups such as *Ismelia* and *Heteranthemis* by Humphries (1976) and Bremer and Humphries (1993). *Argyranthemum frutescens* (L.) Schultz-Bip can be artificially hybridized with *Glebionis carinatum* (Schousb.) Tzvelev and with *G. coronaria* (L.) Cassini ex Spach. with aid of the ovule culture (Ohtsuka and Inaba 2008).

Inter-Simple Sequence Repeats (ISSR) are partially inserted into the microsatellite region in the genome. The ISSR is amplified by using the sequence of an adjacent microsatellite region for a primer (Zietkiewicz et al. 1994; Kondo et al. 2003), and is applicable to polymorphism analysis. Since the ISSR is a random primer, it does not need the sequence information for a primer design, and its operation is simple and easy like the RAPD (random amplified polymorphic DNA) method (Hiraoka et al. 2009), and it is used for phylogeography analyses or identification of individual species. It can be applied in many studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species (Kondo et al. 2003; Racharak et al. 2007).
**Materials and Methods**

*Plant materials.* Seeds of *Gonospermum fruticosum* (C. Smith *ex* Link) Less., *Argyranthemum coronopifolium* (Willd.) Humphries and two strains of *A. foeniculaceum* Webb *ex* Sch. were collected in the Canary Islands, Spain and were sown, germinated and grew up in the greenhouse (Table 1; Fig. 1), to analyze and compare their relationships of karyotypes and molecular ISSR.

*Karyotype analysis.* After young plants of the species (Table 1) grew well (Fig. 1), their growing root tips ca 5 mm long were collected and pretreated in 2 mM 8-hydroxyquinoline at 18°C for 2 h. Then, they were fixed in 45% acetic acid at 4°C for a few min., before, they were macerated in the 2:1 mixture of 1N HCl and 45% acetic acid at 60°C. Root tips less than 1 mm long were cut and stained by 1% aceto-orcein on glass slides for 30 min. in a chamber moistened by 45% acetic acid. Then, they were covered by cover slips and were tapped by stick to spread chromosomes in cells.

Karyotype formulas of *Gonospermum fruticosum*, *Argyranthemum coronopifolium*, and two strains of *A. foeniculaceum* (Table 1) were prepared on the basis of mean based on the size data of chromosome characters of ten somatic metaphase cells. Position of the primary constriction in chromosome followed Levan *et al.* (1964): Arm ratio was calculated by long arm/short arm: 1.0-1.7 was grouped as the median centromeric chromosome, 3.1-7.0 the subterminal centromere, and 7.1 or more as the terminal centromere.

*DNA extractions.* Total genomic DNA of each species of *Argyranthemum* studied was extracted from fresh leaves by using the CTAB method described by Doyle and Doyle (1987).

<table>
<thead>
<tr>
<th>Accession number*</th>
<th>Species name</th>
<th>Locality in the Canary Islands</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Gonospermum fruticosum</em> (C. Smith <em>ex</em> Link) Less.</td>
<td>Anaga Area, Tenerife</td>
</tr>
<tr>
<td>3</td>
<td><em>Argyranthemum coronopifolium</em> (Willd.) Humphries</td>
<td>Anaga Area, Tenerife</td>
</tr>
<tr>
<td>12</td>
<td><em>A. foeniculaceum</em> Webb <em>ex</em> Sch.</td>
<td>Mt. Teide, Tenerife</td>
</tr>
<tr>
<td>15</td>
<td><em>A. foeniculaceum</em> Webb <em>ex</em> Sch.</td>
<td>Mt. Teide, Tenerife</td>
</tr>
</tbody>
</table>

*I.V. Tatarenko’s accession number for seed collection*

Fig. 1. Inter- and intra-specific variation of leaves. A. *Gonospermum fruticosum* (C. Smith *ex* Link) Less. (Accession No. 1). B. *Argyranthemum coronopifolium* (Willd.) Humphries. (Accession No. 3). C. and D. *A. foeniculaceum* Webb *ex* Sch. (Accession Nos. 12 and 15).
DNA amplification. Five out of 21 primers were selected by the primary screening and were selected for analysis. The 10 µl reaction mixture included 5 µl of 2 X KOD FX neo buffer, 1.25 µl of 2 mM dNTP, 10 pmol of ISSR primer, 0.1 µl of KOD FX Neo (TOYOBO), 10 ng of template DNA. PCR was performed using Gene Amp PCR System 9700 (PE Applied Biosystems) and TaKaRa PCR Thermal Cycler Dice (TAKARA BIO Inc.) and DNA amplification were carried out with one cycle of 94°C for 5 min, followed by 40 cycles of denature temperature 94°C for 30 sec, annealing temperature 52°C, 54°C, 58°C and 60°C (depending on primers used, Table 1) for 30 sec, and 72°C for 1.5 min, and concluded by one cycle of 72°C for 10 min. PCR products was electrophoresed on 1.5% agarose gel with DNA size marker (200bp DNA Ladder, TaKaRa) at 100 V for 30 min in Mupid-2 plus (ADVANCE) filled by 1 X TAE Buffer. After staining of the electrophoresed gel with Ethidium Bromide solution for 20 min observation and photography of the band were performed using Printgraph AE-6933 FXCF-U (ATTO) under the ultraviolet exposure.

RESULTS AND DISCUSSION

The chromosome numbers in Gonospermum fruticosum, Argyranthemum coronopifolium, and A. foeniculaceum Accession numbers 12 and 15 studied were commonly 2n=18 (diploid; the basic chromosome number of X=9 is common to the members of the tribe Anthemideae) (Ardevol-Gonzales et al. 1993; Kondo et al. 2003) and relatively big chromosome sizes and the largest chromosomes were bigger than 10 µm and the smallest chromosomes were less than 3.8µm (see Fig. 2). These chromosome characters in Gonospermum and Argyranthemum studied were different from those of Asiatic members in the tribe Anthemideae, especially in Chrysanthemum, although they have common basic chromosome numbers of X=9 (Bramwell et al. 1976; Ortega and Navarro 1977; Brockmann 1987; Febles et al. 1989; Kondo et al. 2003). Thus, the karyotypes of Gonospermum fruticosum, Argyranthemum coronopifolium and A. foeniculaceum Accession numbers 12 and 15 studied showed very less differences in karyotypes from those of the majority of the diploid species of Asiatic polyploid Chrysanthemum such as K(2n=18)=16m+2m sat in Gonospermum fruticosum (Accession No. 1). B. K(2n=18)=16m+2sm sat in A. coronopifolium (Accession No. 3). C. K(2n=18)=14m+4sm sat in A. foeniculaceum (Accession No. 12). D. K(2n=18)=14m+4sm sat in A. foeniculaceum (Accession No.15). Bar = 10µm.

Argyrantehum coronopifolium and A. foeniculaceum Accession numbers 12 and 15 studied showed very less differences in karyotypes from those of the majority of the diploid species of Asiatic polyploid Chrysanthemum such as K(2n=18)=14m or sm+4st of which 2-4 chromosomes have satellites (e.g., Borgen et al. 2003; Suda et al. 2003; Tatarenko et al. 2011; Kondo et al. 2012). Moreover, hybridization and introgression might be common to the species of Argyranthemum in Tenerife after certain isolation mechanisms would be broken and they might have homoploid hybrid speciation and repeatedly species complexity (Fjellheim et al. 2009) in compared to Asiatic Chrysanthemum such as polyploidization from diploid up to much higher ploids such as hexaploid (2n=54; Tanaka and Watanabe 1972; octoploid (2n=72; Tanaka and Watanabe 1972; Nakata and Kumagai 1999) and aneuploidization in high-ploids.

Table 2. Karyotypes obtained in Gonospermum fruticosum, Argyranthemum coronopifolium and two strains of A. foeniculaceum

<table>
<thead>
<tr>
<th>Accession number*</th>
<th>Species name</th>
<th>Chromosome Number</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gonospermum fruticosum (C. Smith ex Link) Less</td>
<td>18</td>
<td>16m+2sm sat</td>
</tr>
<tr>
<td>3</td>
<td>A. coronopifolium (Wild.) Humphries</td>
<td>18</td>
<td>16m+2sm sat</td>
</tr>
<tr>
<td>12</td>
<td>A. foeniculaceum Webb ex Sch.</td>
<td>18</td>
<td>14m+4sm sat</td>
</tr>
<tr>
<td>15</td>
<td>A. foeniculaceum Webb ex Sch.</td>
<td>18</td>
<td>14m+4sm sat</td>
</tr>
</tbody>
</table>

*Tatarenko’s number of seed collection
in Asiatic *Chrysanthemum* especially hybridization of cultivars by artificial cross-hybridization by artificial hybridization in high-ploids in Asiatic *Chrysanthemum*, especially hybridization of cultivars by artificial hybridization (Endo 1969; Endo 1994; Aoyama et al. 1997). Concerning Canary’s species of the tribe Anthemideae, the Asterceae, currently much more human activities including tourisms and floricultural, artificial hybridizations would make more diversified flowering *Argyranthemum*. For instance, triploid cultivars were found in cultivars of *Argyranthemum frutescens*.

**ISSR profiles.** In order to evaluate the genetic relativity among the four species, five ISSR markers (Table 3) were used. Out of 54 bands generated, 46 were found to be polymorphic (86.7% polymorphism). The band pattern of four *Argyranthemum* species with ISSR primer 2, 5, and 6 was shown in Fig. 5 and the primer 11 and 15 was shown in Fig. 4. *Gonospermum fruticosum* had 12 specific bands (22.2%) out of 54 bands, *A. coronopifolium* had five specific bands (9.3%), *A. foeniculaceum* Accession number 12 had one specific band (1.9%) and *A. foeniculaceum* Accession number 15 had four specific bands (7.4%). Three species such as *A. coronopifolium* and *A. foeniculaceum* Accession numbers 12 and 15 had eight common bands, and *Gonospermum fruticosum* had only two common bands of other three species. Two strains of *A. foeniculaceum* showed nine common bands. On the other hand, two strains of *A. foeniculaceum* had respectively different band patterns and two distinctively common bands which had the great common feature of 76.0%.

The result of PCR amplification of the ISSR region in *Argyranthemum* confirmed the polymorphic bands of *Argyranthemum* including specific bands of each species obtained were reproducible and sharp. *Gonospermum fruticosum* showed specific banding pattern different from those of the *Argyranthemum* species studied here. However, they had two bands common to those two genera studied. They must be somewhat closely related to each other. Additionally, respective taxa studied had own specific bands. Thus, the ISSR must be important tool to identify estimation of genetic diversity.

**Table 3.** Selected primers and genetic variations among *Gonospermum fruticosum*, *Argyranthemum coronopifolium* and two stains of *A. foeniculaceum* Accession numbers 12 and 15 in the Canary Islands in ISSR analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’~3’)</th>
<th>Tm/Ta (°C)</th>
<th>Total number of bands</th>
<th>Number of polymorphic bands</th>
<th>Number of specific bands for species</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISSR2</td>
<td>(GA)8C</td>
<td>52/54</td>
<td>10</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>ISSR5</td>
<td>(AG)8C</td>
<td>52/54</td>
<td>12</td>
<td>12</td>
<td>100.0</td>
</tr>
<tr>
<td>ISSR6</td>
<td>(AG)8A</td>
<td>50/52</td>
<td>9</td>
<td>9</td>
<td>100.0</td>
</tr>
<tr>
<td>ISSR11</td>
<td>(AC)8CTG</td>
<td>58/60</td>
<td>15</td>
<td>11</td>
<td>73.3</td>
</tr>
<tr>
<td>ISSR15</td>
<td>(AC)8GG</td>
<td>56/58</td>
<td>8</td>
<td>8</td>
<td>100.0</td>
</tr>
<tr>
<td>Total number of bands</td>
<td>54</td>
<td>46</td>
<td>-</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>10.8</td>
<td>9.2</td>
<td>86.7</td>
<td>4.4</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. ISSR banding pattern produced by each ISSR primer 2, 5 and 6. Lane M is 200bp DNA Ladder. Lane 1, *Gonospermum fruticosum*; lane 3, *A. coronopifolium*; lane 12, *A. foeniculaceum* Accession number 12; lane 15, *A. foeniculaceum* Accession number 15. A thick, white arrow point the bands pointing respective common bands seen in the four species.
Argyranthemum frutescens (L.) Schultz-Bip is one of the commonest, horticulturally meritorious species which has been performing great many cultivars so-called ‘Marguerite’. They have been made out of hybridization with different species, mutation, and so on. Their breeding lines and origin of cultivars would be able to be analyzed by PCR amplification of ISSR. Since those species of Argyranthemum studied were very closely related without no barrier, the ISSR analysis concerning presence or absence of some certain markers might be suitable for speculating and explaining relationships among very closely related species.

The specific bands of each species has had the sufficient reproducibility and specificity for the generating a marker identifying respective species of Gonospermum and two species Argyranthemum and two strains of A. foeniculaceum. Therefore, the ISSR method was effective for estimating of genetic relationship and identification of species of Argyranthemum.

Further karyotypic and molecular comparisons among the species of Argyranthemum should be made and deposited to clarify and justify the classification and genetic systems devised for the genus sensu stricto in the past, which inadequately reflect the natural groupings within Argyranthemum sense stricto. A thorough system of Argyranthemum sense stricto taxonomy is essential.

**LITERATURE CITED**


