**MICROSATELLITES FROM *Fosterella christophii* (Bromeliaceae) BY DE NOVO TRANSCRIPTOME SEQUENCING ON THE PACIFIC BIOSCIENCES RS PLATFORM**

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**Key words:** Bromeliaceae; Fosterella christophii; microsatellites; Pacific Biosciences; single-molecule real-time (SMRT) sequencing; transcriptome.

**METHODS AND RESULTS**

Total RNA was isolated from fresh leaves of one *F. christophii* plant (NW09.030-11) using the RNeasy Plus Micro Kit (QIAGEN, Venlo, The Netherlands). RNA quality and quantity were assessed by capillary electrophoresis on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Polyadenylated RNA was isolated with the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, Massachusetts, USA), followed by an integrity check via capillary electrophoresis. An aliquot of 1 ng of poly(A) RNA was selected as an input for cDNA synthesis with a SMARTer PCR cDNA Synthesis Kit (Clontech Laboratories, Mountain View, California, USA). PCR cDNA Synthesis Kit (Clontech Laboratories, Mountain View, California, USA). A SMARTbell library was prepared as recommended by Pacific Biosciences (PacBio, Menlo Park, California, USA). The amplified cDNA was size-fractionated on agarose gels, and fragments with insert sizes >1.5 kb were recovered. SMARTbell templates were bound to polymerases using the PacBio DNA/polymerase binding kit P4 and v2 primers. Polymerase-template complexes were bound to magnetic beads using a MagBead Kit (PacBio, part #100-133-600). Sequencing was carried out on the PacBio RS II sequencer using C2 sequencing reagents with a movie length of 180 min. Full-length cDNAs were identified with the PacBio SMRT analysis software (version 2.2.0). High-quality sequences were achieved by running the protocol with a filter for a minimum of three full passes of a cDNA and discarding all non–full length cDNAs and chimeric products. The read output was further trimmed and assembled into unigenes using CAP3 (Huang and Madan, 1999).

A total of 1590 high-quality consensus isoforms with an average size of 1322 bp were assembled into 971 unigenes. BatchPrimer3 (You et al., 2008) was applied to detect perfect microsatellites, accepting minimum thresholds of seven repeat units for di-, six for tri-, five for tetra-, and four for penta- and hexanucleotide repeats, respectively. A total of 421 microsatellites were present in 275 unigenes. Motif types are compiled in Appendix S1. Flanking sequences of appropriate quality and length were present at 335 microsatellite loci.

Microsatellite-flanking primers were designed using the BatchPrimer3 interface (You et al., 2008), applying the following criteria: length ranging from 18 to 23 nucleotides, product size ranging from 100 to 300 bp, annealing

**Fosterella christophii** Ibisch, R. Vásquez & J. Peters, *F. micrantha* (Lindl.) L. B. Sm., and *F. villosula* (Harms) L. B. Sm. form a well-circumscribed species group within the genus *Fosterella* L. B. Sm., known as the *F. micrantha* group (Pitcairnioideae; Bromeliaceae) (Wagner et al., 2013). The three species are morphologically very similar terrestrial rosette plants with small, whitish, insect-pollinated flowers (Peters, 2009). Such high levels of similarity are surprising, given that *F. micrantha* is endemic to Central America, whereas the other two species reside in the Bolivian Andes. Controlled pollination experiments indicated that all three species are self-compatible but also form viable hybrids (Wagner et al., 2013). The species *F. christophii* (Peters, 2009) to develop a set of genic microsatellite markers in *F. micrantha*. 

**Premise of the study:** Microsatellite markers were developed in *Fosterella christophii* (Bromeliaceae) to investigate the genetic diversity and population structure within the *F. micrantha* group, comprising *F. christophii*, *F. micrantha*, and *F. villosula*.

**Methods and Results:** Full-length cDNAs were isolated from *F. christophii* and sequenced on a Pacific Biosciences RS platform. A total of 1590 high-quality consensus isoforms were assembled into 971 unigenes containing 421 perfect microsatellites. Thirty primer sets were designed, of which 13 revealed a high level of polymorphism in three populations of *F. christophii*, with four to nine alleles per locus. Each of these 13 loci cross-amplified in the closely related species *F. micrantha* and *F. villosula*, with one to six and one to 11 alleles per locus, respectively.

**Conclusions:** The new markers are promising tools to study the population genetics of *F. christophii* and to discover species boundaries within the *F. micrantha* group.

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| Locus   | Primer sequences (5′–3′) | Repeat motif | Allele size (bp) | T<sub>a</sub> (°C) | GenBank accession no. | BLASTX description                                      | %c | SSR location |
|---------|-------------------------|--------------|------------------|------------------|----------------------|----------------------------------------------------------|----|--------------|
| Foc_01  | F: CTCACACTTGGCTACTACAT | (AG)<sub>3</sub> | 146              | 55.2             | KT036677             | PREDICTED: cinnamoyl-CoA reductase 1-like isoform X1 [Phoenix dactylifera] | 80 | 5′UTR        |
|         | R: GTCTACACTACATTGGC    |              |                  |                  |                      |                                           |    |              |
| Foc_02  | F: GAGGAGTTGTTTTCCTC   | (TC)<sub>17</sub> | 147              | 55.3             | KT036678             | No match found                                        |    |              |
|         | R: AGATCTGGCCTACTACCTC |              |                  |                  |                      |                                           |    |              |
| Foc_03  | F: CCTATTCGCAAAATCATAAA | (AG)<sub>17</sub> | 148              | 55.8             | KT036679             | PREDICTED: tetraspanin-3-like [Musa acuminata subsp. malaccensis] | 80 | 5′UTR        |
|         | R: ACAGCCAGGCAATATTACA  |              |                  |                  |                      |                                           |    |              |
| Foc_04  | F: GCCATGGCTCCTCAACAAGT | (AG)<sub>20</sub> | 145              | 55.5             | KT036680             | PREDICTED: tricetin 3′,4′,5′-O-trimethyltransferase [Phoenix dactylifera] | 77 | 3′UTR        |
|         | R: ATCCACCTGCTCTCTCTTCTC|              |                  |                  |                      |                                           |    |              |
| Foc_05  | F: CTCATTCCCTCTCCATCT  | (TCG)<sub>15</sub> | 136              | 55.4             | KT036681             | PREDICTED: ribonuclease 2 [Musa acuminata subsp. malaccensis] | 78 | 5′UTR        |
|         | R: ATATGCTGAGAGTTAGCTGC|              |                  |                  |                      |                                           |    |              |
| Foc_06  | F: CTCATCTCAAACCCTTC   | (CCT)<sub>21</sub> | 138              | 55.3             | KT036682             | PREDICTED: secretary carrier-associated membrane protein 2-like isoform X1 [Elaeis guineensis] | 88 | 5′UTR        |
|         | R: ACCTGACCTACGAGAA     |              |                  |                  |                      |                                           |    |              |
| Foc_07  | F: TCTACGCGCTCTCCTAT    | (CTC)<sub>15</sub> | 130              | 57.9             | KT036683             | PREDICTED: uncharactertized protein LOC103707719 isoform X2 [Phoenix dactylifera] | 86 | 5′UTR        |
|         | R: TCTCCGCTCTACCTCAGAGT |              |                  |                  |                      |                                           |    |              |
| Foc_09  | F: AGAGGAGGAGAGGAGAGA   | (AGA)<sub>3</sub> | 168              | 55.3             | KT8581625            | PREDICTED: phosphoribulokinase, chloroplastic [Pyrus pyrifolia] | 93 | 5′UTR        |
|         | R: ATTCAGTTGCTGCTTCTG   |              |                  |                  |                      |                                           |    |              |
| Foc_10  | F: CCGGTCTGTCTTGCTTAT   | (GAA)<sub>9</sub> | 140              | 54.6             | KT8581626            | Ubiquitin-conjugating enzyme 32 [Theobroma cacao] | 87 | 5′UTR        |
|         | R: ACCTGGCTGCTTGCTTGCT  |              |                  |                  |                      |                                           |    |              |
| Foc_11  | F: GAGGGTTAAAATTTCTCTTCT | (GGA)<sub>8</sub> | 204              | 55.2             | KT8581627            | Best match <75% sequence similarity                   |    |              |
|         | R: ATGAGTTGAGAGTTAGCTGC |              |                  |                  |                      |                                           |    |              |
| Foc_12  | F: CACAAATGGCTCTCTCTGG  | (TCT)<sub>10</sub> | 153              | 55.0             | KT8581664            | Best match <75% sequence similarity                   |    |              |
|         | R: GGTGAGTTGCTCTATCTG   |              |                  |                  |                      |                                           |    |              |
| Foc_15  | F: GAGGATCTGCTGTTAATTGT | (ATTTT)<sub>4</sub> | 185              | 55.2             | KT8581628            | Best match <75% sequence similarity                   |    |              |
|         | R: CAAACGGAGACTTCAATAA  |              |                  |                  |                      |                                           |    |              |
| Foc_16  | F: CTCAGCTGAGACTCTCTAG  | (GAAAGA)<sub>5</sub> | 194              | 54.3             | KT8581629            | PREDICTED: sec-independent protein translocase protein TATC, chloroplastic-like [Oryza brachyantha] | 90 | 5′UTR        |
|         | R: ACTGCTGCTGCTCTCTCTCTT|              |                  |                  |                      |                                           |    |              |
| Foc_17  | F: GCCATGCTCGACAGAATCC  | (TCCTC)<sub>4</sub> | 153              | 55.1             | KT8581630            | PREDICTED: carboxyvinyl-carboxyphosphonate phospholipomutase, chloroplastic [Amborella trichopoda] | 82 | CDS          |
|         | R: TAAAATAGGGAGTAGACAG   |              |                  |                  |                      |                                           |    |              |
| Foc_18  | F: CTTGCTCTCTCCTACCTCG  | (CCGCTC)<sub>4</sub> | 193              | 54.8             | KT8581685            | Best match <75% sequence similarity                   |    |              |
|         | R: GCCCTCTGCTGTTACCTCG  |              |                  |                  |                      |                                           |    |              |
| Foc_19  | F: GAAAGAGGAGAAACCCTGAG | (TCTCCT)<sub>5</sub> | 156              | 55.6             | KT8581631            | PREDICTED: PTI1-like tyrosine protein kinase 1 isoform X1 [Elaeis guineensis] | 91 | 5′UTR        |
|         | R: ATCAAAAGATGGAGGAGAG   |              |                  |                  |                      |                                           |    |              |
| Foc_20  | F: GAGGAGAGGAGGAGAAGAGA | (TCTTCC)<sub>4</sub> | 134              | 54.4             | KT8581632            | RING/FPFV/PETD zinc finger superfamily protein [Theobroma cacao] | 80 | 5′UTR        |
|         | R: AGGATGAGGAGGCTCTCG    |              |                  |                  |                      |                                           |    |              |
| Foc_21  | F: CTCACACACGACCCACAC  | (GA)<sub>12</sub> | 134              | 55.9             | KT8581633            | PREDICTED: cation transport regulator-like protein 2 [Elaeis guineensis] | 76 | 5′UTR        |
|         | R: AGAACGGAGCTGGTTGTTT   |              |                  |                  |                      |                                           |    |              |
| Foc_25  | F: GCCATGCTGAGAGAGGA    | (AG)<sub>12</sub> | 159              | 55.8             | KT856686             | PREDICTED: UFP0235 protein At5g63440 isoform X1 [Elaeis guineensis] | 95 | 5′UTR        |
|         | R: GCTTTAGCTAGAAGCTGAAGA|              |                  |                  |                      |                                           |    |              |
| Foc_27  | F: TACCTCCATGACCTCCTC   | (AG)<sub>6</sub> | 122              | 56.0             | KT856687             | Membrane steroid-binding protein 1, partial [Oryza sativa Indica group] | 84 | 5′UTR        |
|         | R: CTCCACGCTCTCCACAT    |              |                  |                  |                      |                                           |    |              |
| Foc_28  | F: AAGGAGAGAGGAAAGAGAGG | (GCA)<sub>20</sub> | 147              | 54.9             | KT856688             | Best match <75% sequence similarity                   |    |              |
|         | R: GAGGAGAGAGGCTCCTG    |              |                  |                  |                      |                                           |    |              |
| Foc_30  | F: CAATCTTATAATTCAGAGAG | (CT)<sub>14</sub> | 150              | 55.2             | KT856689             | PREDICTED: uncharactertized protein LOC102721803 [Oryza brachyantha] | 79 | 5′UTR        |
|         | R: TCCATCTTATCTCCTCTTCTC|              |                  |                  |                      |                                           |    |              |

Note: 5′UTR = 5′ untranslated region; 3′UTR = 3′ untranslated region; CDS = coding region; T<sub>a</sub> = annealing temperature.

*a* All loci were amplified using a standard touchdown PCR.

*b* Loci that were monomorphic (*) among the seven initially tested individuals of *F. christophii*, *F. villosula*, and *F. micrantha*; loci that were monomorphic (**) within each of the three tested species but showed some potential to differentiate between species.

*c* Sequence similarities of unigenes with more than 75% identity (%) to known genes obtained using BLASTX (Altschul et al., 1990).
temperature from 50°C to 70°C, and GC content from 30% to 70%. Based on optimal primer characteristics, 30 loci representing all repeat types (12 di-, 10 tri-, two tetra-, three penta-, and three hexanucleotide repeats) were selected for further analysis. Primer functionality was validated by genotyping 29 *F. christophii* plants from three natural populations, with nine to 11 individuals each (Appendix 1). DNA was extracted from dried leaves according to Tel-zur et al. (1999). PCR amplifications were conducted in 12.5-μL final volumes in a T-Gradient thermocycler (Biometra, Göttingen, Germany), following the touchdown protocol previously described (Wöhrmann et al., 2012).

For the initial screens, PCR products from three *F. christophii* individuals (including NW09.003-11 as a positive control) and two plants each of *F. micrantha* and *F. villosula* were electrophoresed on an automated sequencer (LI-COR 4300 IR²; LI-COR Biosciences, Lincoln, Nebraska, USA). Fragment sizes were scored manually as described by Wöhrmann et al. (2012). Twenty-two of the 30 primer pairs yielded one or two distinct bands of the expected size range in each tested individual, depending on the homo- or heterozygous state of the respective amplification characteristics, primer sequences, GenBank accession numbers, and the results of a BLASTX similarity search in GenBank of these 22 loci are summarized in Table 1. Eight primer pairs failed to amplify in any of the specimens and were not considered further.

### Table 2. Results of primer screening for 13 polymorphic loci developed for *Fosterella christophii*.

| Locus   | NW09.005 (N = 11) | NW09.030 (N = 9) | NW09.034 (N = 9) | F. christophii (N = 29) | F. micrantha (N = 31) | F. villosula (N = 21) | Total (N = 81) |
|---------|-------------------|------------------|------------------|------------------------|-----------------------|----------------------|---------------|
|         | H<sub>a</sub> (A) | H<sub>e</sub> (H) | H<sub>e</sub> (H) | H<sub>a</sub> (A)     | H<sub>e</sub> (H)     | H<sub>e</sub> (H)     | H<sub>a</sub> (A)     |
| Foc_01  | 4                  | 0.09*            | 0.59             | 5                      | 0.44                  | 0.72                 | 4              | 0.78          | 0.78          | 7              | 9              | 4              | 13             |
| Foc_02  | 2                  | 0.00             | 0.42             | 5                      | 0.78                  | 0.75                 | 5              | 1.00          | 0.83          | 8              | 4              | 6              | 13             |
| Foc_03  | 3                  | 0.09             | 0.50             | 5                      | 0.56                  | 0.60                 | 4              | 0.22          | 0.47          | 9              | 8              | 2              | 16             |
| Foc_04  | 4                  | 0.22             | 0.52             | 6                      | 0.89                  | 0.80                 | 6              | 0.11          | 0.57          | 7              | 2              | 1              | 9              |
| Foc_05  | 2                  | 0.00*            | 0.52             | 5                      | 0.78                  | 0.71                 | 4              | 0.11          | 0.57          | 7              | 2              | 1              | 9              |
| Foc_06  | 1                  | —                | —                | 4                      | 0.89                  | 0.66                 | 3              | 0.56          | 0.57          | 6              | 4              | 2              | 8              |
| Foc_07  | 2                  | 0.00             | 0.42             | 2                      | 0.22                  | 0.21                 | 4              | 0.67          | 0.75          | 4              | 2              | 2              | 4              |
| Foc_12  | 2                  | 0.00*            | 0.52             | 4                      | 0.56                  | 0.61                 | 4              | 0.67          | 0.70          | 7              | 1              | 3              | 15             |
| Foc_18  | 2                  | 0.00             | 0.17             | 5                      | 0.44                  | 0.66                 | 4              | 0.00*         | 0.65          | 7              | 4              | 2              | 11             |
| Foc_25  | 2                  | 0.09             | 0.25             | 3                      | 0.89                  | 0.69                 | 4              | 3            | 2              | 6              |
| Foc_27  | 3                  | 0.09             | 0.18             | 4                      | 0.78                  | 0.71                 | 3              | 0.23          | 0.29          | 7              | 5              | 4              | 8              |
| Foc_28  | 1                  | —                | —                | 3                      | 0.56                  | 0.60                 | 2              | 0.33          | 0.29          | 4              | 4              | 2              | 6              |
| Foc_30  | 2                  | 0.00             | 0.51             | 2                      | 0.67                  | 0.52                 | 4              | 0.22          | 0.78          | 6              | 4              | 5              | 9              |
| Mean    | 2.08               | 0.04             | 0.41             | 3.54                   | 0.57                  | 0.61                 | 3.85           | 0.54          | 0.66          | 6.54           | 4.69           | 2.85           | 9.77           |

*Note:* A = number of alleles; A<sub>total</sub> = number of alleles across all tested accessions; H<sub>e</sub> = expected heterozygosity; H<sub>a</sub> = observed heterozygosity; N = number of individuals sampled.

*See Appendix 1 for locality and voucher information.

*Highly significant deviation from Hardy–Weinberg equilibrium (P < 0.001).*

CONCLUSIONS

So far, PacBio’s SMRT technology has only rarely been applied to microsatellite marker development (e.g., Grohme et al., 2013; Wei et al., 2014). To our knowledge, the present report is the first using cDNAs as source material for this purpose. The increasing popularity of the PacBio RS II system compared with earlier sequencing technologies is primarily attributed to its high sequencing accuracy obtained by circular consensus sequencing and the extraordinarily long reads of up to 20 kb. The analysis of full-length cDNAs is appealing not only for detecting genomic microsatellite markers but also for many other applications such as gene mapping or gene expression profiling. The cDNA-based microsatellite markers developed for *F. christophii* represent promising tools for population genetic analyses and species delimitation within the *F. micrantha* group and presumably other species complexes of the Pitcairnioideae.

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APPENDIX 1. Plant material analyzed in this study. Representative samples of F. christophii, F. villosula, and F. micrantha populations were collected with the largest possible distances from each other (between 20 cm and 3–4 m, depending on the total size of the patch).

| N  | Species                     | Location            | Plant ID/Voucher (Herbarium)* | GPS coordinates |
|----|----------------------------|---------------------|-------------------------------|----------------|
| 11 | Fosterella christophii Ibisch, R. Vásquez & J. Peters | Florida, Santa Cruz (BOL) | NW09.005 (LPB) | -18.09952 -63.60210 |
| 9  | Fosterella christophii      | Larecja, La Paz (BOL) | NW09.030 (LPB) | -15.66240 -67.71320 |
| 5  | Fosterella villosula        | Larecja, La Paz (BOL) | NW09.034 (LPB) | -15.46787 -67.97005 |
| 3  | Fosterella villosula        | Caranavi, La Paz (BOL) | NW09.012 (LPB) | -16.03293 -67.63168 |
| 7  | Fosterella villosula        | Sud Yungas, La Paz (BOL) | NW09.019 (LPB) | -15.45160 -67.17057 |
| 6  | Fosterella villosula        | José Ballivián, Bení (BOL) | NW09.024 (LPB) | -14.54075 -67.49693 |
| 5  | Fosterella micrantha (Lindl.) L. B. Sm. | Oaxaca (MEX) | Schütz 11.04 (KAS) | 18.41262 -95.09465 |
| 6  | Fosterella micrantha       | Oaxaca (MEX) | Schütz 11.05 (KAS) | 17.85203 -96.21658 |
| 4  | Fosterella micrantha       | Oaxaca (MEX) | Schütz 11.17 (KAS) | 17.73767 -96.32755 |
| 1  | Fosterella micrantha       | Oaxaca (MEX) | Schütz 11.19 (KAS) | 15.86467 -96.47219 |
| 1  | Fosterella rusbyi (Mez) L. B. Sm. | South Yungas, La Paz (BOL) | JP 06.0078 (LPB) | -16.36167 -67.46333 |
| 1  | Dyckia marnier-lapostollei L. B. Sm. var. estevesii Rauh | Goias (BRA) | BGHD 130151 | -16.66667 -49.26666 |
| 1  | Deuterocohnia longipetala (Baker) Mez | Tarija (BOL) | BGHD 09068 (KAS) | -22.50463 -64.41242 |
| 1  | Encholirium spp. var. ex Schult. f. | NA (BRA) | BGHD 125585 | NA NA |
| 1  | Ananas comosus (L.) Merr. | NA (NA) | HERRH 0000-G-33 | NA NA |
| 1  | Catopsis morreniana        | Veracruz (MEX) | Schütz 11.05 (KAS) | 17.85203 -96.21658 |
| 1  | Puya mirabilis (Mez) L. B. Sm. | Salta (ARG) | BGDH 131731 | NA NA |

Note: ARG = Argentina; BGHD = Botanical Garden Heidelberg; BOL = Bolivia; HERRH = Botanical Garden Hannover; MEX = Mexico; N = number of individuals per sampling location; NA = not available; PE = Peru.

*Herbarium abbreviations are according to Index Herbariorum (http://sweetgum.nybg.org/science/ih/).

APPENDIX 2. Cross-species amplification of 13 microsatellite markers developed for Fosterella christophii in seven heterologous bromeliad species.

| Species                     | Foc_01 | Foc_02 | Foc_03 | Foc_04 | Foc_05 | Foc_06 | Foc_07 | Foc_12 | Foc_18 | Foc_25 | Foc_27 | Foc_28 | Foc_30 |
|-----------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Fosterella rusbyi           | +      | -      | +      | +      | +      | +      | -      | +      | +      | -      | +      | +      | +      |
| Dyckia marnier-lapostollei var. estevesii | +      | -      | +      | +      | +      | +      | -      | +      | +      | -      | +      | +      | +      |
| Deuterocohnia longipetala   | -      | -      | -      | +      | -      | -      | -      | -      | -      | +      | -      | +      | +      |
| Encholirium spp.            | -      | -      | -      | +      | -      | -      | -      | -      | -      | +      | -      | +      | +      |
| Ananas comosus              | -      | -      | +      | +      | +      | +      | -      | +      | +      | -      | +      | +      | +      |
| Catopsis morreniana         | -      | -      | +      | +      | +      | +      | -      | +      | +      | -      | +      | +      | +      |
| Puya mirabilis              | +      | -      | -      | +      | +      | +      | -      | +      | +      | -      | +      | +      | +      |

Note: + = successful amplification as evidenced by the occurrence of distinct single or double bands on sequencing gels; — = no amplification.