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# Differentiation and Dynamics of some *Epipactis* Species

Daniel Prat<sup>1</sup> and Jérôme Briolay<sup>2</sup>

<sup>1</sup> UMR 5023 LEHNA, bâtiment A. Forel, Université Claude Bernard – Lyon 1

<sup>2</sup> DTAMB, Bâtiment G. Mendel, Université Claude Bernard – Lyon 1  
69622 Villeurbanne Cedex, France

## ABSTRACT

In Orchidaceae, many species are subjected to decline due to direct or indirect effects of human activities. Accurate information is needed to prioritize protection actions. Within terrestrial species, genus *Epipactis* provides a suitable model to analyze its susceptibility to environmental changes. It consists of several groups of close species, varying in reproductive patterns and growing in various ecological conditions. Published phylogeny studies allowed identification of various clades but relationships and gene flows among taxa remain unclear. Hybrids can be observed even between species from separated clades, revealing a low differentiation. Controlled interspecific hybridization leads to normal seeds. Seed germination and protocorm development remains erratic in natural conditions, as in *in vitro* conditions, but much faster in *in vitro* culture. Comparison of different seed types remained difficult due to the low success with mature seeds. Nevertheless, some protocorms obtained from controlled crosses between plants growing in different stands were much larger than those from other types of crosses. Such possible inbreeding address question on population genetic structure and differentiation. Few data on their genome organization are available. Genome size is not related to the allogamy to autogamy switch. Stomatal guard cell size is weakly correlated to genome size. The clade of *E. helleborine* with derived species shows a lower genome size than other species. The development of new molecular markers for diversity assessment has been tested using RNA-Seq to reduce the sequencing effort due to the large genome size of the genus (2C: 25-30 pg DNA). Reads from flower tissues collected in *E. helleborine* and *E. placentina* (14 10<sup>6</sup> in total) expanded on 120 10<sup>6</sup> bp of assembled sequences (approximately 1% of nuclear genome size). More than 7000 sequences exhibited polymorphic sites (116,000 polymorphisms; 5% being indels) in expressed sequences by sequencing different plants. Transitions are more frequent than transversions. The amount of reads varied greatly according to genes. Differentiation among plants was larger between species than within species.

## INTRODUCTION

Orchids consist one of the most threatened flowering plant family due to human activities and environmental changes. Land use changes with habitat destruction and unsustainable collection for medicinal uses for instance consist of major threats. Their conservation in natural areas requires pedagogical actions and scientific knowledge to promote an efficient management of populations for the long term. Studies in ecology, biotic interactions, population dynamics, gene flows... are thus of great help. Various approaches from field studies to analyses in laboratory can be developed to provide suitable information.

Several studies have reported inbreeding depression in orchids<sup>16</sup>, including *Epipactis*<sup>2,7</sup>. Controlled crosses, including selfing, allogamy (within and among stands) and interspecific hybridization can thus be tested in order to precise their impacts on population dynamics. Seed germination can be tested by burying them in soil and also in asymbiotic *in vitro* culture. Not only the amount of viable seeds produced but also their genetic quality are important for population dynamics and conservation.

Orchidaceae exhibit a very large range of variation in genome size<sup>6,8</sup>. DNA Content can be related to cell size and mitotic rhythm<sup>3</sup>. The genome size provides information suitable for a wide variety of biological fields from cell and molecular biology, to ecology and systematics, including the development of genome sequencing projects. Its adaptive meaning is largely debated.

Genetic studies to assess gene diversity, gene flows, population differentiation are suitable for species conservation and to improve stand management. The development of molecular markers is rather difficult in orchids due to the large genome size of many species. Recent evolution of molecular biology techniques with new generation sequencing provides tools for analyzing polymorphism. RAD-Seq have been used to produce numerous SNP markers<sup>13</sup>. Phylogeny studies and morphological description of taxa which can split closely related taxa in separated species lead to a different number of operational taxonomic units. Most molecular markers used are located in non-expressed genomic sequences while expressed sequences could be more related to phenotypic traits used in traditional taxonomy and to plant adaptation. RNA-Seq can be a suitable alternative to obtain numerous polymorphic

markers related to expressed genes. Gene sequences can be reconstructed by assembling short reads. In order to increase the level of detected polymorphisms, several plants from different stands can be investigated. An other technical advantage of RNA-Seq is to reduce the sequencing effort which is limited to messenger RNAs. More or less partial sequences for many genes can be obtained. Molecular markers will be then developed and applied for genetic studies.

We focus analyses on the genus *Epipactis* (subfamily Epipendoideae, tribe Neottiae) which consists of several species with various mating systems, from allogamy to full autogamy<sup>5</sup>. Different species can grow in the same area leading sometimes to interspecific hybrids. Capacity of seeds to develop into plants can be tested in natural stands<sup>15</sup>. Genera of tribe Neottiae, including *Cephalanthera* and *Neottia*, have different genome size and exhibit variation among species<sup>1,9</sup>. Relationships between genome size and cell size have been confirmed in Neottiae<sup>10</sup>. Genome size should be related with species differentiation and speciation but its relationships with phylogenetic signal have only been poorly assessed, especially in *Epipactis*. An published extensive study on species relationships and phylogeny in *Epipactis*<sup>13</sup> makes this possible.

The objectives of the study dealt with species conservation and included two main aspects: (1), to asses influence of mating systems on population dynamics; (2), to analyze relationships among species. For this purpose, RNA-Seq analyses have been carried out to assess the amount of variation observed among few plants and to provide molecular markers suitable for further genetic studies.

## MATERIALS AND METHODS

Mating system and interspecific sexual compatibility have been analyzed in *E. atrorubens*, *E. helleborine* and *E. palustris* by controlled crossing in natural stands. Autogamy, allogamy (within and among stands), and interspecific hybridization have been tested (Fig. 1). Seeds produced spontaneously have been also included in the study. Seeds were then introduced between two tissue layers (90 µm sieves) and buried into soil, close to native *Epipactis* plants, according to Tesitelová *et al.* (2012). Native symbiotic fungi can meet seeds and favor protocorm development, Seed development was also tested in asymbiotic germination in *in vitro* culture (media for terrestrial orchids, Plantigen Inc.).

Fresh leaves of various *Epipactis* species growing in different stands in France have been analyzed by flow cytometry after staining with propidium iodide to determine genome size<sup>9</sup>. Obtained values were plotted on the most complete phylogenetic tree of *Epipactis* published by Sramkó *et al.* (2019).

RNA-Seq have been carried out on two plants of *E. helleborine* (EH1: 210 m asl, poplar plantation; EH2: 1300 m asl, meadow) and one plant of *E. placentina* (EP). Flowers (flower buds to wilted flowers) were collected and bulked separately for each plant (immediate storage in liquid nitrogen). Both species exhibit very close genome size<sup>9</sup>. Messenger RNAs were extracted and fragmented (average length of 400 bases) prior to reverse transcription, amplification and sequencing by Illumina technics (~150 bases from each fragment end). Sequences were assembled with Trinity<sup>4</sup>, available on Galaxy platform). Reads of each plant were aligned with BWA on complete assembled sequences. Polymorphisms (SNPs and indels) have been searched using Varscan (Galaxy platform tools) within each sample (to detect heterozygosity) and between samples (Fig. 2). Polymorphisms were screened with a minimum read depth of 25 and visualized using Integrative Genomics Viewer<sup>12</sup>.



Fig. 1. Controlled crosses on *E. palustris*. Hand-pollinated flowers and inflorescences are protected by a net to avoid uncontrolled pollination by insects or ants; self-pollination and crosses between plants were identified by the thread color.

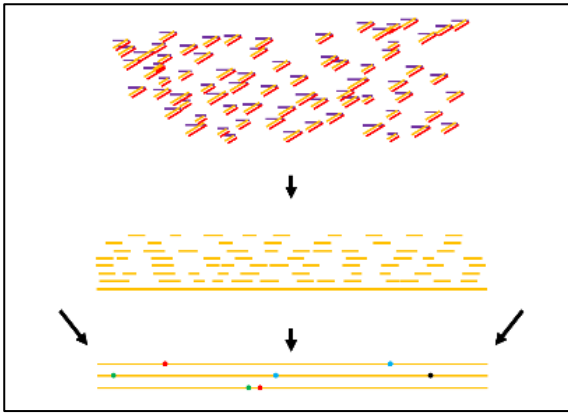


Fig. 2. Principle of marker development by RNA-Seq. Fragmented mRNA are reverse transcribed into DNA and then amplified prior to sequencing. Reads are then assembled to provide a more or less full gene sequence to which reads of the three investigated plants are aligned in order to detect polymorphic sites.

## RESULTS

No development of seeds buried into soil have been recorded within the first year. Two years after sowing, some protocorms were developed in some seed lots of *E. atrorubens* and *E. helleborine*. Their low frequency (1-5% within seed lots) did not allow suitable comparisons. Large protocorms (Fig. 3) were recorded only from crosses between plants growing in different stands. Seeds of *E. palustris* developed into protocorms in *in vitro* culture on medium BM-1 and BM-2. Some protocorms with rhizoids have been observed within 10 weeks at 22 °C (Fig. 4). Their frequency was remained at that stage below 1%. No difference was reported according to culture medium.

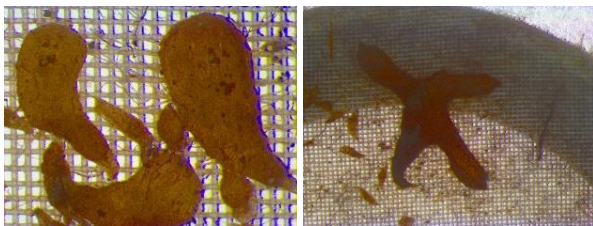


Fig. 3. *E. atrorubens* protocorm development observed after two years in natural stands.

Protocorms from native seeds on the left and from controlled crosses among plants from different stands on the right (scale provided by tissue, 90 µm sieve).

Genome size varied according to the species within genus *Epipactis* from 25.20 pg in *E. rhodanensis* to 30.54 pg in *E. kleinii* for 2C. Within clade A of *E. helleborine* including very closely related species and its derived clades G, H, I, J and K, genome size ranges from 25.20 pg in *E. rhodanensis* to 26.37 pg in *E. neerlandica* (Fig. 5). In other clades, genome size varied from 26.50 pg in *E. palustris* to 28.48 pg in *E. phyllanthes*. Both groups have a significantly different average value. No difference of genome size is related to mating systems (allogamous vs. autogamous).



Fig. 4. *In vitro* seed development of *E. palustris*. Protocorm observed 10 weeks after sowing on medium BM-1, besides undeveloped seeds.

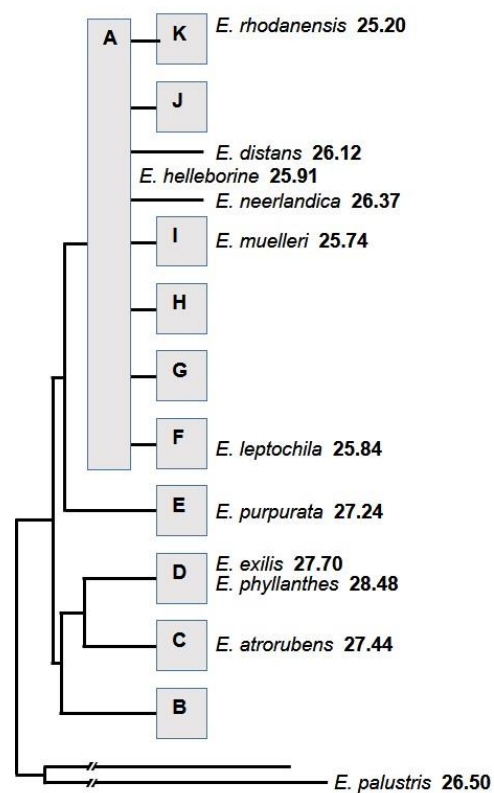


Fig. 5. Simplified phylogeny of genus *Epipactis* according to Sramkó *et al.* (2019) including genome size values for species in common.

Genome size values are indicated in pg.2C<sup>-1</sup> of DNA besides position of species within clades A to K defined by authors of phylogeny.

By RNA-Seq, more than 4 000 000 fragments were sequenced for each plant; their cumulated length represented more than 1 450 10<sup>6</sup> pb. The complete assembly consists of about 200,000 sequences, of an average size of 605 bp and median size of 345 pb stretching on 120 10<sup>6</sup> aligned bases. Sequencing depth reached thus about 12x. It can be more than 1000x for genes very expressed like phosphoenolpyruvate carboxylase 2 and even more than 40,000x for 5.8S and 18S ribosomal

genes which are tandemly duplicated. Several large assembled sequences (> 5000 pb) belonged to chloroplast genome (no polymorphisms within these sequences). Assembled sequences corresponded to genes of more than 11,000 proteins, some hits being related to retrotransposons. According to the parameters applied, 7527 assembled sequences showed polymorphic sites (24,978 polymorphic sites on 10.07 10<sup>6</sup> bp, approximately a SNP each 500 bp). Observed single nucleotide polymorphisms are more related to transition (7790 cases for each 4 combinations) than to transversion (2925 cases for each 8 combinations). Polymorphisms were detected at the level of individual plant (related to heterozygosity): *E. placentina* exhibited more SNPs than *E. helleborine* samples (Table 1). Plant EH2 of *E. helleborine* growing at higher elevation exhibited lowest polymorphisms. Differences between EP and EH1 or EH2 samples were more numerous than between EH1 and EH2 (Table 2). Indel distribution was similar among samples. Position of SNPs can be located along complete assembly (Fig. 6).

Table 1. Polymorphisms observed within plant. These polymorphisms are mostly related to heterozygosity.

Plant	Number of SNPs	Number of indels
EH1	12735	4472
EH2	7431	3121
EP	14125	5256

Table 2. Differentiation among plants. Table provide the number of polymorphisms observed between plants for SNPs and indels.

Polymorphism type	Plant	EH1	EH2
SNPs	EH1	19929	12735
	EP		19510
Indels	EH1	6550	4472
	EP		6618

## DISCUSSION

Allogamous species were able to produce seeds by selfing as already shown by Talalaj and Brzosko (2008). A low rate of seed development has already been reported for *Epipactis* species with buried seeds<sup>14</sup> in soil as well as with mature seeds introduced in *in vitro* cultures<sup>7</sup>. The cold requirement indicated by Rasmussen (1992) for *in vitro* germination of *E. palustris* seeds seems in fact not necessary. For the autogamous species *E. muelleri*, no protocorm was observed (data not shown). The largest protocorms observed in *E.*

*atrorubens* and *E. helleborine* originated from crosses between plants from distant stands; this suggests a clear inbreeding depression at germination stage, but it can not be confirmed statistically due to the very low success of germination. Brys and Jacquemyn (2016) provided evidence for an important inbreeding depression in allogamous *Epipactis* species. Population management should consider this point to preserve them for the long term.

Genome size varied in a limited range in *Epipactis* genus, only two triploid plants (data not shown) were noticed. Values were consistent with evolution patterns and phylogeny described by Sramkó *et al* (2019). *E. helleborine* and the derived species have a smaller genome than other *Epipactis* species.

RNA-Seq applied on flowers of three different plants revealed a large amount of polymorphisms, suitable for genetic studies. It provides also information on expressed genes. Polymorphisms observed on coding sequences could be more reliable to morphological variation among plants and species than non-coding ones. Data are now available for several orchid species. Various studies showed utilization of RNA-Seq for molecular marker development. The three plants investigated could then be compared: the level of heterozygosity was higher in *E. placentina* and lower in *E. helleborine* growing at higher elevation. The sample from *E. placentina* appeared the most differentiated, with significantly more polymorphic SNPs between the two species than within *E. helleborine*. Nevertheless, only three samples were analyzed and their comparison can not be generalized. Numerous markers can be tested for genetic investigation on *Epipactis*. This will be the next step of the study.

## CONCLUSION

*Epipactis* conservation in natural stands should take into account gene flows in order to avoid inbreeding depression. RNA-Seq is a suitable method to reveal polymorphisms mostly based on SNPs in order to produce molecular markers. These markers can be then used for genetic studies in *Epipactis* species. Since they are designed from expressed sequences, which are more conserved than non-coding sequences, these markers can be probably applied across a larger range of species. Primer pairs for DNA amplification will be designed and tested for genetic studies in *Epipactis*. The taxonomic status *E. placentina* as a separated species can not be confirmed with these partial results.

## ACKNOWLEDGEMENTS

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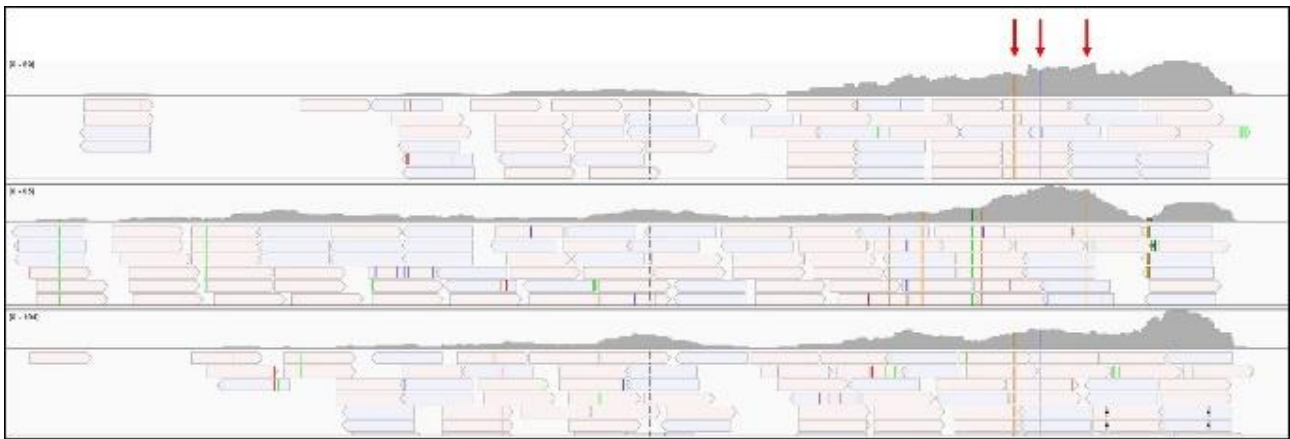


Fig. 6. SNP positions along partial sequences of  $\beta$ -D xylosidase 4 (2855 pb) obtained from RNA-Seq. The figure was obtained with Integrative Genomics Viewer; EP sequence is located above, EH2 in the middle and EH1 below; arrows indicate polymorphisms among both species.

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