Whole plastid genome-based phylogenomics supports an inner placement of the O. insectifera group rather than a basal position in the rapidly diversifying Ophrys genus (Orchidaceae)

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ABSTRACT

Some lineages of the orchid genus Ophrys exhibit among the highest diversification rates reported so far. As a consequence of such intense and rapid evolution, the systematics and taxonomy of this genus remain unclear. A hybrid assembly approach based on long-and short-read genomic data allowed us to outperform classical methods to successfully assemble whole plastid genomes for two new species of Ophrys: O. aymoninii and O. lutea. Along with three other previously Ophrys plastid genome sequences, we then reconstructed the first whole plastome-based molecular phylogeny including representatives of the three mains recognized Ophrys lineages. Our results support the placement of the O. insectifera clade as sister group of “non-basal Ophrys” rather than a basal position. Our findings corroborate recent results obtained from genomic data (RAD-seq and transcriptomes) but contrast with previous ones. These results therefore confirm that molecular phylogenetic hypotheses based on a limited number of loci (e.g. ratiS, matK, rbCL) may have provided a biased picture of phylogenetic relationships within Ophrys and possibly other plant taxa.

Introduction

Within orchids (Orchidaceae), the most speciose family of flowering plants, some lineages of the genus Ophrys L. display one of the highest diversification rates ever reported for plants (Givnish et al. 2015; Breitkopf et al. 2015). The adaptive radiation that Ophrys has experienced is likely to be due to their unusual pollination strategy (by sexual swindle) that leads to high levels of specialisation of these plants towards their insect pollinators, and favours evolutionary divergence (see Baguette et al. 2020 for a recent review). The phylogenetic relationships of such fast and highly diversifying groups are difficult to infer for two reasons. Firstly, because recent divergent times often render molecular signal of lineage delineation undetectable or at least ambiguous (incomplete lineage sorting), and secondly, because emerging species are still particularly prone to introgressive hybridization and reticulate evolution.

The systematics and taxonomy are particularly problematic in Ophrys in which the number of recognized species ranges from 9 to 354 according to authors (see Bateman et al. 2018; Bateman 2018; Bertrand et al. 2021). In particular, contrasting results still make the phylogenetic position of the (three) main lineages (sometimes considered as subgenera) debated.

Several molecular phylogenetic studies have suggested that the genus Ophrys is subdivided in three main sublineages: a first clade formed by the Ophrys insectifera group (also named group A since the study of Devey et al. 2008), a second clade consisting of the groups B to E (O. tentredinifera (B), O. speculum (C), O. bombyliflora (D), called “archaic Euophrys” by Tyteca and Baguette (2017), plus the so-called Pseudophrys group (E) and a third clade to which belong the groups F to J (O. apifera (F), O. sphegodes (G), O. fuciflora (H), O. scolopax (I) and O. umbilicata (J)), also called “recent Euophrys”). The terms Euophrys and Pseudophrys refer to the part of the pollinating insect body on which the orchid pollinia are glued during pseudocopulation. Pseudophrys corresponds to Ophrys species in which the pollinia are deposited on the abdominal region of the insect, whereas Euophrys corresponds to Ophrys species in which the pollinia are deposited on the cephalic region (see Bertrand et al. 2021). As Euophrys is a taxonomically incorrect term to refer to Ophrys groups, “section Ophrys” should be used instead of section Pseudophrys. However, because the section Ophrys is paraphyletic, we propose to use “basal Ophrys” and “non-basal Ophrys” instead of “archaic Euophrys” and “recent Euophrys” for clarity purpose.

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Out of the studies that could not unambiguously resolve tree topology for the three main *Ophrys* lineages (e.g. Soliva et al. 2001; Tytca and Baguette 2017) two contrasting hypthesis emerge concerning the phylogenetic position of the *O. insectifera* (A) group. Most of the molecular phylogenetic hypotheses have (historically) supported a basal position (*T*<sub>basal</sub>) for the *O. insectifera* (A) group (Devey et al. 2008; Breitkopf et al. 2015, Zitoun et al. in prep). However, recent findings based on genomic data such as SNPs derived from RAD-seq approaches (Bateman et al. 2018) or transcriptomes (Piñeiro Fernández et al. 2019) support a direct relationship between the group *insectifera* (A) and "non-basal *Ophrys*" (groups F to H) both of which being sister to the clade comprising "basal *Ophrys*" + *Pseudophrys* (groups B to E) (*T*<sub>inner</sub>).

In this study, we aim to reconstruct a phylogenomic hypothesis to test whether whole plastid genomic data support the inner placement of the *O. insectifera* (A) group or, alternatively, its basal position within the genus *Ophrys*. So far, three *Ophrys* plastid genomes have been published: *O. iricolor* Desf. (or *O. fusca* subsp. *iricolor* (Desf.) K.Richt) and *O. sphegodes* Mill. (Roma et al. 2018) and *O. aveyronensis* (J.J.Wood) P. Deljorge (or *O. sphegodes* subsp. *aveyronensis* J.J. Wood) (Bertrand et al. 2019). However, none of them is a member of the *O. insectifera* (A) group. To fill this knowledge gap, we generated genomic data for *Ophrys* *aymoninii* (Breistr.) Buttl. (or *O. insectifera* subsp. *aymoninii*, Breistr.) a representative of the *O. insectifera* clade that is endemic to a spatially restricted geographic area in the South of the Massif Central (France). We also provide similar data for *O. lutea* Cav., a widespread Western Mediterranean *Pseudophrys* species.

We relied on a hybrid approach to assemble the whole plastid genomes of the two *Ophrys* taxa mentioned above. In brief, this consists of a combination of long reads (here, Oxford Nanopore Technologies reads that can span repeated DNA regions known to be difficult to assemble) with the low error rate of short (paired-end) reads (here, Illumina reads). Although relatively recent, such hybrid strategy was found to outperform classical approaches (as recently supported by Wang et al. 2018; Scheunert et al. 2020). For this purpose, we used the Unicycler pipeline (Wick et al. 2017) that can analyse reads from both platforms simultaneously. Gene annotation and basic downstream analyses were then carried out as described in our former study (Bertrand et al. 2019, see also Appendix 1).

**Materials and methods**

**Field sampling and sample processing**

We collected fresh leaves from an individual of *O. aymoninii* and an individual of *O. lutea* near Causse-Bégon, France (N 44.05252°; E 3.35898°) and Versols-Et-Lapeyre, France (N 43.898677°; E 2.933099°), respectively, on 12–05-2018. As *O. aymoninii* is a nationally protected species in France, sampling was carried out under permit ‘Arrêté préfectoral n°2018-s-20’ issued by the “Direction Régionale de l’Environnement de l’Aménagement et du Logement (DREAL)” from the “Région Occitanie” on 11–06-2018. Back in the lab, samples were frozen and stored at −20°C until DNA extraction. We used a CTAB2X protocol to extract genomic DNA from the two specimens sampled (see Appendix 1 for details).

**DNA sequencing, plastid genome reconstruction and gene annotation**

**Long-read (Nanopore) sequencing**

Four Nanopore sequencing libraries (two for each individual) were prepared with the SQK-LSK108 kit following the ONT “1D genomic DNA by ligation protocol” or the “1D gDNA long reads without BluePippin protocol” from 2730/3632 ng and 2964/2500 ng of unfragmented DNA for *O. aymoninii* and *O. lutea*, respectively (see Appendix 1 for detail). Long read sequencing was carried out from four FLO-MIN106D R9 flowcells (two for each individual) on a MiniION (Oxford Nanopore Technologies, Oxford, UK) in the lab using MinKNOW v.1.15.1. FAST5 files were base-called with Albacore v.2.3.1 (see Appendix 1 for detail).

Adapters were removed with Porechop v.0.2.4 (https://github.com/rrwick/Porechop) with the – discard_middle option turned on. We then used Nanofilt v.2.7.1 (https://pypi.python.org/pypi/Nanofilt) to filter out reads shorter than 5 kb and bases with quality < 9 on both sides of reads. To facilitate assembly, we then extracted plastid reads by mapping short-reads onto a multi-fasta file comprising the three whole plastid genome sequences published for *Ophrys* species. As in Wang et al. (2018), we duplicated and concatenated each of the three sequences and included them in the reference set to avoid losing reads corresponding to the region where genomes were circularized. We then extracted reads that mapped onto this dataset with Minimap2 v.2.17 (Li 2018).

**Short-read (Illumina) sequencing**

Whole genomic libraries were prepared and sequenced in paired-end mode (2x150 bp, insert size: 350 bp) by Novogene Co., Ltd (HK) from
1.92 and 1.97 pg of DNA for *O. aymoninii* and *O. lutea*, respectively. Genomic DNA was extracted with the same protocol as the one used for long reads. Raw reads were trimmed with Trimmomatic v.0.39 (Bolger et al. 2014) and the resulting read quality was checked with FastQC v0.11.8 (Andrews et al. 2010). Plastid read extraction was carried out by mapping short-reads onto the *Ophrys* plastome dataset, as mentioned above, this time with bowtie2 v.2.3.4 (Langmead and Salzberg 2012). Because too high coverage is prone to disturb the assembly process, we subsampled the resulting read set to an expected coverage of 100X (i.e. by keeping 500,000 of both R1 and R2 reads assuming a plastid genome size of around 150 kb) before the assembly step.

**Plastid genome reconstruction and gene annotation**

Hybrid *de novo* assembly was performed with both long- and short-reads simultaneously using default settings in Unicycler v0.4.9b (Wick et al. 2017). Gene annotation and alignments were performed as in our previous study (Bertrand et al. 2019). Some genes (*ndhA* to *ndhK*) exhibited significant differences in length and similarity, even between the closely related *Ophrys* species considered here, probably as a result of pseudogenisation and were removed from the alignments for further analyses. In particular, *O. sphegodes*, *O. aveyronensis* and *O. aymoninii* presented truncation of most *ndh* genes and shared the loss of the partially duplicated gene of *ycf1* and a truncation of *ndhF* gene as already reported by Roma et al. (2018), see also Appendix 4.

**Phylogenetic reconstruction, concordance factors and tree topology tests**

We used the Maximum Likelihood approach implemented in IQ-TREE v2.0.6 (Minh et al. 2020a) to reconstruct gene and species trees with 1,000 replicates (-B 1000) of Ultrafast Bootstrap Approximation (UFBoot) to assess nodes support. The species tree was constructed based on three data set: i) whole plastome, ii) genes and iii) CDS alignments. The genealogical concordance in the dataset was also quantified with gene concordance factor (gCF) and site concordance factor (sCF) (Minh et al. 2020b). In addition, tree topology tests implemented in IQ-TREE were performed to test whether the inferred species tree was consistent with the inner (*T*inner) or basal (*T*basal) position of *O. aymoninii*. To further investigate which loci specifically support one or the other topology, and to what extent (by evaluating its phylogenetic signal), we then computed the difference in gene-wise Log-likelihood scores (ΔGLS, see Shen et al. 2017). For all phylogenetic analyses, the plastid genome of *Platanthera japonica* (Thunb.) Lindl. (GenBank Accession no.: MG925368) was used as outgroup. This species belongs to the Orchidoideae subfamily, like *Ophrys*.

**Characterization of the plastid genomes of *O. aymoninii* and *O. lutea* and comparison with previously published *Ophrys* plastid genomes**

Following the approach described in our previous study (i.e. using short-reads (Illumina) and NOVOPlasy (Dierckxksen et al. 2017; Bertrand et al. 2019)) we failed to infer a complete plastid genome sequence for *O. aymoninii*. For the two remaining species, namely *O. aveyronensis* and *O. lutea*, we were able to obtain a single contig, only when providing a closely related reference sequence of *O. sphegodes* Mill. and *Ophrys fusca* subsp. *tricolor* (Desf.) K.Richt., respectively (Table 1). The hybrid assembly implemented in Unicycler thus seems to outperform the short-read-based approach since we obtained a single contig of expected length for all three species (without reference sequence). The two new *O. aymoninii* and *O. lutea* genomes found to be very similar in size and structure to the three previously published *Ophrys* plastome sequences. The plastid genomes of *O. aymoninii* and *O. lutea* are described in Appendix 2 and have been deposited on Gen Bank with accession numbers MW309825 and MW309826, respectively. Raw reads are also available from the European Nucleotide Archive (Study Primary Accession PRJEB42431/Sencondary Accession ERP12689, see Appendix 1 for details).

| Genus and Species | Reference |
|-------------------|-----------|
| *Ophrys aveyronensis* | Bertrand et al. (2019) |
| *Ophrys aymoninii* | This study |
| *Ophrys lutea* | This study |

For *O. aveyronensis* we used the plastome sequence of *O. sphegodes* (Accession AP018717; Bertrand et al. 2019) and for *O. lutea* the sequence of *O. fusca* subsp. *tricolor* (Accession AP018716; Roma et al. 2018)
Phylogenetic relationships within the genus *Ophrys*

We found a full support for the inner placement ($T_{inner}$) of the *O. insectifera* (A) group within the genus *Ophrys*. Whatever the alignment considered (whole-plastome, concatenation of gene/CDS loci), bootstrap values fully support (UFBoot = 100) a topology according to which *O. aymoninii*, representative of the *O. insectifera* group, is sister to the “non-basal *Ophrys*” representatives, namely *O. sphegodes/O. aveyronensis*; the *Pseudophrys*: *O. lutea* and *O. iricolor* occupy a basal position (Figure 1). The gene and site concordant factor metrics (gCF and sCF) did not contradict bootstrap values even though they were found to be lower (especially gCF). This may be explained by very short branch lengths and the very limited amount of information contained in each gene/CDS sequence. All the tree topology tests also rejected the topology in which *O. aymoninii* is sister to all other species, when compared to the topology in which it has inner placement (Table 2). The distribution of ΔGLS values (Figure 2 and Appendix 3) confirms that most of the genes also support this topology and when they do not, they only weakly support the alternative one.

Altogether, our results are therefore congruent with the genomic-based findings recently reported by Bateman et al. (2018) and Piñeiro Fernández et al. (2019), and contrast with several previous studies. Although being located on a single molecule, plastid regions do not necessarily behave as a single locus, and experience certain forms of intra- and inter-molecular recombination (see Gonçalves et al. 2019; Walker et al. 2019). Since most of the plastid genes are also known to encode important biological functions, they may display sequence evolution patterns that deviate from the species tree topology, and even from non-coding plastid sequences, because of positive selection. Phenomena such as Incomplete Lineage Sorting (ILS), hybridization and introgression, gene duplication of loss as well as horizontal transfers may also affect gene tree topology. Finally, plastids are generally assumed to be maternally inherited in angiosperms but evidences of biparental inheritance have been

![Figure 1. Maximum likelihood phylogenetic tree (as inferred with iQtree) from whole plastid genome sequence alignment. UFBoot (Ultrafast Bootstrap Approximation) values were 100 at each node whatever the alignment considered (whole plastome, genes, CDS). Values next to the bootstrap indicate gene Concordance Factor (gCF) and site Concordance factor (sCF) inferred from gene-up and CDS-based (down) “gene” trees. *Platanthera japonica* (GenBank Accession no.: MG925368) is used as outgroup.](image)

![Table 2. Summary of the tree topology test statistics performed to compare the inner placement hypothesis of the *O. insectifera* group ($T_{inner}$) to the one supporting its basal position ($T_{basal}$).](image)

| Topology | logL | ΔL | bp-RELL | p-KH | p-SH | p-WKH | p-WSH | c-ELW | p-AU |
|----------|------|----|---------|------|------|-------|-------|-------|------|
| $T_{inner}$ | −258040.4949 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| $T_{basal}$ | −258129.4276 | 88.933 | 0 | 0 | 0 | 0 | 0 | 6.5×10⁻⁷⁵ | 1.21×10⁻⁷¹ |

ΔL: logLikelihood (logL) difference from the maximal logL in the set.
bp-RELL: bootstrap proportion using RELL method.
p-KH: p-value of one sided.
p-SH: p-value of test.
p-WKH: p-value of weighted KH test.
p-WSH: p-value of weighted SH test.
c-ELW: Expected Likelihood Weight.
p-AU: p-value of approximately unbiased (AU) test.
All tests performed 10001 resamplings using the RELL method. Tests for which the topology was significantly rejected are indicated in bold.
documented in this group. In spite of all these possible biases potentially affecting plastid gene trees in angiosperms, we did not find any gene strongly supporting the basal position of the *O. insectifera* group in *Ophrys*. We found that particular structural variation also supports the relative phylogenetic proximity of the *O. insectifera* lineage (here *O. aymoninii*) with the *O. sphegodes* relatives (*O. sphegodes* and *O. aveyronensis*). However, *Ophrys aymoninii* shows singular characteristics further confirming that the *O. insectifera* lineage is clearly distinct within the genus *Ophrys*. As already suggested for other angiosperms lineages, GLS shows that the phylogenetic signal of genes such as *matK* slightly outperform *rbcL* but that other loci such as *ycf1* (but also *ycf2*), *rpoC2* (see Walker et al. 2019), *rpoB* and *rpoC1* may be considered as good candidates for plastid-based phylogenetic analyses in *Ophrys*. Although our plastid-based findings are congruent with published genome-scale nuclear ones (RADseq- and transcriptome-based) and confirm the necessity to rely on a large number of loci to reliably infer the evolutionary history of such rapidly evolving groups.

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**Disclosure statement**

The authors declare no conflict of interest.

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**Author contributions**

**Joris Bertrand** conducted fieldwork, analyzed the data and wrote the manuscript.

**Anaïs Gibert** contributed to fieldwork and manuscript writing.

**Christel Llauro** generated the long-read data set.

**Olivier Panaud** contributed to research funding and manuscript writing.

**References**

Andrews, S, P Lindenbaum, B Howard, P Ewels. 2010. FastQC: a quality control tool for high throughput sequence data. Available online at [http://www.bioinformatics.babraham.ac.uk/projects/fastqc](http://www.bioinformatics.babraham.ac.uk/projects/fastqc).

Baguette, M, JAM Bertrand, V Stevens, B Schatz, C Noûs. 2020. Why are there so many Bee-orchids? Adaptive radiation by intraspecific competition for mnemonic pollinators. Biol Rev. 95:1630–1663. doi:10.1111/brv.12633.

Bateman, RM. 2018. Two bees or not two bees? An overview of *Ophrys* systematics. Ber Aus Arbeitkreisen Heimische Orchideen. 35:5–46.

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**Figure 2.** Genewise phylogenetic signal (ΔGLS) for $T_{inner}$ versus $T_{basal}$ the two alternative tree topologies for each gene (a) and CDS (b) along the plastid genome. Positive ΔGLS values support an inner placement of *O. aymoninii* whereas negative values rather support its basal placement.
Bateman, RM, G Sramkó, O Paun. 2018. Integrating restriction site-associated DNA sequencing (RAD-seq) with morphological cladistics analysis clarifies evolutionary relationships among major species groups of bee orchids. Ann Bot. 121:85–105. doi:10.1093/aob/mcx129.

Bertrand, JAM, M Baguette, N Joffard, B Schatz. 2021. Challenges inherent in the systematics and taxonomy of genera that have recently experienced explosive radiation: the case of orchids of the genus Ophrys. In M C Maugin, P Grandcolas, editors. Systematics and exploration of life. Paris:ISTE.

Bertrand, JAM, A Gibert, C Llauro, O Panaud. 2019. Characterization of the complete plastome of Ophrys aveyronensis, Euro-Mediterranean orchid with an intriguing disjoint geographic distribution. Mitochondrial DNA Part B. 4:3256–3257. doi:10.1080/23802359.2019.1670748.

Bolger, AM, M Lohse, B Usadel. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 30:2114–2120. doi:10.1093/bioinformatics/btu170.

Breitkopf, H, RE Onstein, D Cafasso, PM Schlüeter, S Cozzolino. 2013. Multiple shifts between different pollinators fueled rapid diversification in sexually deceptive Ophrys orchids. New Phytol. 207:377–386. doi:10.1111/nph.13219.

Devey, DS, RM Bateman, MF Fay, JA Hawkins. 2008. Friends or real tives? Phylogenet and species delimitation in the controversial European orchid genus Ophrys. Ann Bot. 101:385–402. doi:10.1093/aob/mcm299.

Dierckxsens, N, P Mardulyn, G Smits. 2017. NOVOPlasty: de novo assembly of organelle genomes from whole genome data. Nucleic Acids Res. 45:e18. doi:10.1093/nar/gkw955.

Givnish, TJ, D Spalink, M Ames, SP Lyon, SJ Hunter, A Zuluaga, WJD Iles, MA Clements, MTK Arroyo, J Leebens-Mack, et al. 2015. Orchid phylogenomics and multiple drivers of their extraordinary diversification. Proc R Soc B. 282:20151553. doi:10.1098/rspb.2015.1553.

Gonçalves, DIP, BB Simpson, EM Ortiz, GH Shimizu, RK Jansen. 2019. Incongruence between gene trees and species trees and phylogenetic signal variation in plastid genes. Mol Phylogenet Evol. 138:219–232. doi:10.1016/j.ympev.2019.05.022.

Langmead, B, SL Salzberg. 2012. Fast gapped-read alignment with bowtie 2. Nat Methods. 9:357–359. doi:10.1038/nmeth.1923

Li, H. 2018. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics. 34:3094–3100. doi:10.1093/bioinformatics/bty191.

Minh, BQ, HA Schmidt, O Chernomor, MD Schrempf, A Woodhams, A Von Haeseler, R Lanfear. 2020b. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol. 37:1510–1534.

Minh, BQ, MW Hahn, R Lanfear. 2020a. New methods to calculate concordance factors for phylogenomic datasets. Mol Biol Evol. 37:2727–2733. doi:10.1093/molbev/msaa106.

Piñeiro Fernández, L, KJRP Byers, J Cai, KME Seedeek, RT Kellenberger, A Russo, W Qi, C Aquino Fournier, PM Schlüeter. 2019. A phylogenomic analysis of the floral transcriptomes of sexually deceptive and rewarding European orchids, Ophrys and Gymnadenia. Front Plant Sci. 10:1553. doi:10.3389/fpls.2019.01553.

Roma, L, S Cozzolino, PM Schlüter, G Scopece, D Cafasso. 2018. The complete plastid genomes of Ophrys iricolor and O. sphegodes (Orchidaceae) and comparative analyses with other orchids. PLoS One. 13:e0204174. doi:10.1371/journal.pone.0204174.

Scheuert, A, M Dorfner, T Lingl, C Oberprieler. 2020. Can we use it? On the utility of de novo and reference-based assembly of Nanopore data for plant plastome sequencing. PLoS One. 15:e0226234. doi:10.1371/journal.pone.0226234.

Shen, X-X, CT Hittinger, A Rokas. 2017. Contentions relationships in phylogenomic studies can be driven by a handful of genes. Nat Ecol Evol. 1:0126. doi:10.1038/s41559-017-0126.

Soliva, M, A Kocyan, A Widmer. 2001. Molecular phylogenetics of the sexually deceptive orchid genus Ophrys (Orchidaceae) based on nuclear and chloroplast DNA sequences. Mol Phylogenet Evol. 20:78–88. doi:10.1006/mpve.2001.0953.

Tytca, D, M Baguette. 2017. Ophrys (Orchidaceae) systematics – when molecular phylogenetics, morphology and biology reconcile. Ber Aus Arbeitkreisen Heimische Orchideen. 34:37–103.

Walker, JF, N Walker-Hale, OM Vargas, DA Larson, GW Stull. 2019. Characterizing gene tree conflict in plastome-inferred phylogenies. PeerJ. 7:e7747. doi:10.7717/peerj.7747.

Wang, W, M Schalamun, A Morales-Suarez, D Kainer, B Schwessinger, R Lanfear. 2018. Assembly of chloroplast genomes with long- and short read data: a comparison of approaches using Eucalyptus pauciflora as a test. BMC Genomics. 19:977. doi:10.1186/s12864-018-5348-8.

Wick, RR, LM Judd, CL Gorrie, KE Holt. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol. 13:e1005595. doi:10.1371/journal.pcbi.1005595.