Molecular and metabolic traits of some Egyptian species of Cassia L. and Senna Mill (Fabaceae-Caesalpinioideae)

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Abstract
The genus Cassia and Senna have been classified under subfamily Caesalpinioideae of family Fabaceae (Leguminosae) of order Fabales. There is a scarce taxonomical studies of the genus Cassia and Senna inhabiting Egyptian environments, thus, the main objective of the current was to revise and authenticate the phylogenetic relationship between studied taxa of the species of the genera Cassia and Senna in Egypt using the recent tools of ITS barcoding, RAPD analysis and metabolic profiling, in comparing to the traditional taxonomical features. From the cluster analysis of the traditional 27 morphological characters, the studied taxa were categorized into two major clades with an average taxonomic distance of 4.3. The clade I include Cassia fistula, C. renigera, C. javanica L subsp. nodosa and C. roughia that belongs to series Obolospermae, and C. grandis that belongs to series Grandes. The clade (II) includes Senna surattensis and S. alata at taxonomic level 3.6. The taxonomical description of the studied taxa was confirmed from the molecular analysis of ITS sequences and RAPD analysis. The ITS sequences of the tested plants species C. fistula L, C. grandis MD4, C. javanica subsp. nodosa MD7, C. roxburghii MDS, C. renigera MDS were deposited at genbank with accession numbers MW367973, MZ960447, MW386305, MW326753 and MW32685, respectively. While, the ITS sequences of the S. surattensis and S. alata were deposited into genbank accession # MD14 MW367670 and MD20 MW412635, respectively. From the molecular analysis, two clades were clearly separated into Clade I of Cassia and Clade II of Senna. The cluster I represented by C. fistula, C. renigera, C. roxburghii, and C. javanica sub nodosa, and the cluster II represented by S. alata and S. surattensis. From the PCA of RAPD, a clearly discrimination between the two taxa was observed revealing the characteristic grouping of Cassia and Senna. The species Senna alata and Senna surattensis were grouped together, but the species of C. renigera, C. javanica, C. roxburghii and C. grandis was grouped on a distinct group. The separation of Cassia and Senna species into two clusters verify the segregation of the genus Cassia L. senso lato into two distinct genera namely Senna P. and Cassia L. The morphological, molecular traits of the studied plants were authenticated from the metabolic profiling by GC-MS analysis. Among the 23 identified metabolites, four compounds namely hexadecanoic acid, methyl ester, 9-Octadecenoic acid (Z)-ethyl ester and Vitamin E were detected with fluctuated concentrations, among C. fistula, C. grandis, C. javanica subsp. nodosa and C. roxburghii. Conclusively, the traditional morphological features, molecular barcoding using ITS sequences, RAPD analysis and metabolic traits by GC-MS analysis, authenticates the taxonomical diversity of the genus Cassia and Senna.

Keywords: Cassia, Senna, Taxonomical features, ITS sequence, RAPD analysis, GC-MS profile

Introduction
The genus Cassia L. and Senna Mill. have been classified under subfamily Caesalpinioideae of family Fabaceae (Leguminosae) of order Fabales [1, 2]. Cassia and Senna...
were segregated into the three genera: *Chamaecrista* Moench., *Senna* Mill. and *Cassia* L. [3–11]. This segregation was subsequently reinforced based on ontogenetic floral development studies Tucker [12] as well as using the molecular biology tools [13]. The genus *Cassia* include only 30 species [14]. Comparing to about 400 species in *Cassia* sens. Lat. as reported by Brenan [15]. Five species of *Cassia* sens. Lat. were introduced as horticulture plants in Egypt [16]. The genus *Senna* Mill, Gard. Dict. includes around 350 species and spread over the world [3, 4, 17] among them 17 species were introduced as horticulture plants in Egypt. Species of *Cassia* and *Senna* are widely grown as ornamentals [18] extensively used in various parts of the world as remedies for various human ailments [19, 20]. Species of *Cassia* sens. Lat. and *Senna* are well known for their laxative and purgative uses [21, 22] antioxidant activity [23] anticancer [24] and antimicrobial activities [25, 26]. In addition, these plants were used to treat gastrointestinal disorders, some skin diseases and wound healing [27, 28]. The extensive variability in its growth habit ranging from tall trees to delicate annual herbs, numbers and size of the leaflets, form and foliar characteristics has added difficulties to taxonomists in identification of species or the intraspecific taxa for influence of habitat conditions [7]. Taxonomically, *Senna* and *Cassia* are very complex genus owing to the strong polymorphism of a number of species and the absence of intrageneric incompatibility.

Recently, various taxonomical tools have been implemented such as anatomical, cytological, serological, genetic characteristics and metabolic traits [29–31], rather than floral and vegetative character, that have been reported as important features in determining the relationships and affinities of the plants. With the development of various taxonomical tools that based on the molecular and metabolic traits, new taxonomical systems have been evolved, exploring new criteria for assessing the evolutionary status of the individual taxa in the subtribe Cassiinae. Recently, several molecular markers for demonstrating the intra, and interspecific genetic variations have been implemented for the direct comparison of the plant variation at biochemical and molecular levels [32, 33]. Random amplified polymorphic DNA (RAPD) analysis and sequences of the internal transcribed spacer (ITS) are one of the recent molecular tools for separating the particular species from their ancestors. Unlike traditional morphological features, the molecular tools especially RAPD and ITS sequences are independent on the environmental changes that gave these approaches more credibility. RAPD is a reliable predictive, rapid tool to clarify areas of maximum diversity and evaluate natural genetic diversity in plant populations [34, 35]. The sequence of ITS region is one of the most authenticated molecular markers in the evolutionary investigations at various taxonomic levels. DNA sequence is the straightforward since the nrDNA sub-units contain large numbers of copies, with numerous copies of rRNA genes within a genome, relatively homogenous, coupled with the different subunits and spacer sections [36]. The ITS area is particularly common because of the highly nucleotide replacement rate of the transcribed intervals which allows a taxonomical comparison of the highly similar divergent species [37]. The ITS region is impacted by the coordinated evolution that homogenizes the tandem copies across individuals, making ribosomal DNA accessible to phylogenetic analysis [38, 39], as well as for determining the origin of plants and their derivatives [40, 41]. Comparison of DNA sequences within the species is a powerful approach for determining the evolutionary forces acting in specific gene regions, and also for determining the relevant aspects of the evolutionary history of the species [42]. Due to the higher rate of nucleotide substitution, relative feasibility of amplification and the large available sequence data, the internal transcribed spacer (ITS) regions of the nuclear ribosomal cistron (18S-5.8S-26S) has been considered as a very successful tool for species-level discrimination across flowering plants [43–45].

Recently, the emergence of DNA sequence data allows the quantitative comparison of nucleotide polymorphism levels at species and populations and corresponding degrees of population sequence divergence [46]. In addition, metabolic profiling by GC-MS of plants is one of the major recent trends for authentication of the morphological taxonomical and molecular features of plants [47]. From literature, there is a scarce taxonomical studies of the genus *Cassia* and *Senna* inhabiting Egyptian environments, thus, the main objective of this study was to implement the various molecular and biochemical tools for confirming and revisiting the taxonomical identities of these plants. Thus, the objective of this study was to revise and authenticate the phylogenetic relationship between studied taxa of the species of the genera *Cassia* and *Senna* in Egypt using the recent tools of ITS barcoding, RAPD analysis and metabolic profiling, in comparing to the traditional morphological and taxonomical features.

**Materials and method**

**Collection and identification of plant samples**

Seven horticultural taxa representing the genus *Cassia* and *Senna* comprising six species and one subspecies were the subject of this study. Fresh plant material, for each one of them, was collected since April 2020 (Table 1). *Cassia fistula* L. was collected from Parks at Faculty of Science, Zagazig University, Zagazig, Egypt. *Cassia grandis* L.f. and...
Senna surattensis (Dc.) Irwin and Barneby were collected from Zohria Trial Gardens, Gezira, Giza, Egypt. Cassia javanica L. subsp. nodosa, Cassia renigera Benth. and Cassia roxburghii DC were collected from Orman Botanic Garden, Giza, Egypt (Table 1). The plants were obtained after permission from Zagazig University, Orman and Zohria Botanical gardens. The voucher herbarium specimens were prepared and matched for identification with the authentic ones at the Orman Botanical and Zohria Botanical garden, Giza, Egypt. The plants were identified by the official staff members of the Orman Botanical Garden (OBG), Zohria Botanical Garden (ZBG), Cairo university Botanical garden, with the identification numbers as included on Table 1.

### Morphological studies

Twenty-eight characters were investigated according to the reference keys for taxonomic classification of Cassia and Senna [48–50]. The morphological characters and character states were determined by examining of the living specimens, and were coded as multistate characters. Ten individuals from each plant were used for the morphological description. One individual has been used for the molecular and biochemical analyses. The data matrix was analyzed using multistate matrix. The data matrix was subjected to cluster analysis using UPGMA (Unweighted pair group method with arithmetic mean) and the phylogenetic relatedness was constructed to show the relationship among the taxa. All analyses were carried out using the program Past (Version 4.3c) [51]. The Morphological Characters descriptions were recorded in Table 2.

### Molecular study

**Molecular identification of the plant samples**

The plant genomic DNA was extracted by CTAB lysis buffer [52]. Fresh weight of the plant tissue (0.1 g) was pulverized into fine powder in liquid nitrogen, the CTAB lysis buffer (500 μl) was added, vortex for 1 min, and centrifuged at 10000 rpm for 10 min [53]. Equal volume of chloroform was added to the supernatant, vigorously shaking, centrifuged at 10,000 rpm for 10 min, the upper layer was taken and amended with double volume of ethanol, incubated at −20 °C for 30 min, centrifugation for 10 min to pellet the DNA. The DNA pellets were dissolved in 50 μl distilled water and stored at −20 °C, and checked by 1.5% agarose gel, normalizing to 1 kb ladder (Cat. # PG010-55DI). The ITS primer sets were listed in Table 3. The reaction mixture contains 10 μl of 2 × PCR master mixture (i-Taq™, Cat. # 25,027), 2 μl of gDNA, 1 μl of the primers (10 pmol/μl), and completed to 20 μl with sterile distilled water. PCR amplification was performed at Thermal Cycler 006, programmed to initial denaturation 94 °C for 2 min, denaturation 94 °C for 20s, annealing 51 °C for 30s, extension 72 °C for 1 min for 35 cycles, with final extension 5 min at 72°C. The PCR amplicons were analyzed by 1.5% agarose gel in 1 × TBE buffer, and sequenced by Applied Biosystem Sequencer.

### Table 1

| Taxa                      | ID number | Locality                  | longitude & latitude | Series          |
|---------------------------|-----------|----------------------------|----------------------|-----------------|
| Cassia fistula L.         | ZUBG-09   | Parks at Faculty of Science, Zagazig University, Zagazig, Egypt. | 30.5765°N 31.5041°E | Cassia          |
| Cassia grandis L. f.      | ZBG-008   | Zohria Trial Gardens, Gezira, Giza, Egypt. | 30.0131°N 31.0289°E | Grandes         |
| Cassia javanica L. subsp. nodosa (Roxb.) K. Larsen & S. S. Larsen | OBG-109 | Orman Botanic Garden, Giza, Egypt. | 30.0444°N 31.235°E | Obolospermae    |
| Cassia renigera Benth. (Synonym: Cassia javanica L. subsp. renigera Benth.) | OBG-104 | Orman Botanic Garden, Giza, Egypt. | 30.0444°N 31.235°E | Obolospermae    |
| Cassia roxburghii DC. (Synonym: Cassia marginata Roxb.) | OBG-101 | Orman Botanic Garden, Giza, Egypt. | 30.0444°N 31.235°E | Obolospermae    |
| Senna surattensis (Dc.) Irwin&Banneby | ZBG-006 | Zohria Trial Gardens, Gezira, Giza, Egypt | 30.0444°N 31.235°E | Subverrucosae   |
| Senna alata (L.)Roxb      | CUBG-002  | Cairo University Botanical Garden | 30.0444°N 31.235°E | Pictae          |

ZUBG Zagazig University Botanical Garden, ZBG Zohria Botanical Garden, OBG Orman Botanical Garden, CUBG Cairo University Botanical garden.

### Table 2

| Name | Base Pair Primers (bp) | Prime Primer Sequence (5-3) | Source |
|------|------------------------|----------------------------|--------|
| RAPID ABI-07 | 10 bp | GGTGACGCAG | Chen et al., [52] |
| ABI-08 | 10 bp | GTCCACACGG | Chen et al., [52] |
| ABI-09 | 10 bp | TGGGGGACTC | Chen et al., [52] |
| ABI-10 | 10 bp | CTGCTGGGAC | Chen et al., [52] |
| ABI-11 | 10 bp | GTAGACCGTG | Chen et al., [52] |
| ABI-12 | 10 bp | CTTTGACGA | Chen et al., [52] |
| ITS  ITS2 2F | 20 bp | ATCGGATACCTGGTG TGAAT | Gao et al., [53] |
| ITS2 3R | 21 bp | GACCCTTCTCCAGAC TACAAT | Gao et al., [53] |
(HiSQV Bases, Version 6.0). The obtained sequences were non-redundantly BLAST on the NCBI, and the FASTA sequences were aligned with Clustal W muscle algorithm [54].

**Sequence analysis**

Alignment analysis of the ITS sequences were adjusted using BioEdit version 7.2.5 [55], for each sequence, length and GC contents were estimated using the Endmemo software (http://www.endmemo.com/bio/gc.php) (Table 4). The derived ITS nucleotide sequences were analyzed with MEGA version X software [56]. The sequences were manually checked and the pairwise sequence divergence between studied taxa in ITS1, 5.8S and ITS2 regions was calculated according to the Maximum Composite Likelihood (MCL) [56], verified by comparing with the sequences of other species by Basic Local Alignment Search Tool (BLAST). Positions containing gaps and missing data were eliminated from the dataset, support values of the internal branches of NJ tree were evaluated through bootstrap method (1000 replicates).

The transition/transversion ratio $r = \frac{A^*G^*k1 + T^*C^*k2}{[(A+G)^*(T+C)]}$ with A, G, C, T as the corresponding frequencies of four nucleotides [57]. The number of nucleotide substitutions per site between sequences was estimated. The aligned sequences in the Mega files were analyzed with DnaSP software version 4.0 [58] to

| Character                  | *Cassia fistula* L | *Cassia grandis* | *Cassia renigera* | *Cassia roxburghii* DC | *Cassia javanica* | *Senna alata* | *Senna surattensis* |
|---------------------------|--------------------|-----------------|-------------------|------------------------|------------------|---------------|---------------------|
| 1-Life Span               | perennial          | perennial       | perennial         | perennial              | perennial        | perennial      | perennial          |
| 2-Life form               | Tree               | Tree            | Tree              | Tree                   | Tree             | Shrub         | Shrub              |
| 3-Stem surfaces           | glabrous           | pubescent       | pubescent         | pubescent              | pubescent        | pubescent      | pubescent         |
| 4-Leaf duration           | deciduous          | deciduous       | deciduous         | deciduous              | deciduous        | evergreen      | evergreen          |
| 5-Leaflet pairs in numbers| 8-14 pairs         | 14-20pairs      | 16-20pairs        | 14-20 pairs            | 16-20 pairs      | 8-12 pairs    | 6-12 pairs         |
| 6-Leaflet shape           | ovate              | oblong          | oblong            | oblong                 | oblong           | obovate-oblong | obovate-oblong     |
| 7-Leaflet margin          | entire             | entire          | entire            | entire                 | entire           | entire         | entire             |
| 8-Leaflet apex            | acute              | obtuse          | obtuse            | obtuse                 | acute            | obtuse         | obtuse             |
| 9-Leaflet base            | Obtuse             | Obtuse          | Obtuse            | Oblique                | Obtuse           | Oblique        | Oblique            |
| 10-Leaflet adaxial surface| glabrous           | puberulent      | puberulent        | glabrous               | puberulent       | glabrous       | glabrous           |
| 11-Leaflet abaxial surface| puberulent         | tomentose       | tomentose         | puberulent             | puberulent       | puberulent     | puberulent         |
| 12-Leaflet length         | 7.5-15 cm          | 5-7 cm          | 5-10 cm           | 7-10 cm                | 5.5-10 cm        | 6-12 cm       | 2-5 cm             |
| 13-Leaflet width          | 2.5-7 cm           | 1-2 cm          | 0.4-2 cm          | 1-2 cm                 | 0.6-2 cm         | 3-6 cm        | 0.8-2 cm           |
| 14-Stipule                | caudacious         | caudacious      | caudacious        | caudacious             | caudacious       | Persistent     | caudacious         |
| 15-Stipule shape          | deltoid to ovate   | kidney          | kidney            | linear to lanceolate   | Triangular       | linear to lanceolate |
| 16-Bract shape            | ovate              | Linear to lanceolate     | Linear to lanceolate | Leave                | Linear to lanceolate |
| 17-Sepals shape           | ovate              | ovate           | ovate             | ovate                  | ovate            | obovate        | obovate            |
| 18-Sepals colour          | green              | redish          | redish            | redish                 | redish           | yellowish-green | yellowish-green    |
| 19-Petals shape           | obovate            | obovate         | obovate           | Ovate Obovate          | obovate          | obovate        | obovate            |
| 20-Petals colour          | yellow             | pink            | pink              | pink                   | pink             | yellow         | yellow             |
| 21-pod Curvature          | straight            | straight        | straight           | straight               | straight         | straight       | straight           |
| 22-Pod colour             | Black              | Dark brown      | Dark brown        | Dark brown             | Dark brown       | Brown          | Brown              |
| 23-Pod Texture            | glabrous           | glabrous        | glabrous          | glabrous               | glabrous         | glabrous       | hairy              |
| 24-POD APEX               | rounded            | rounded         | rounded           | rounded                | rounded          | ACUMINATE      | rounded            |
| 25-Dehiscence of pod      | indehiscent        | indehiscent     | indehiscent       | indehiscent            | indehiscent      | dehiscent      | dehiscent          |
| 26-Seed shape             | elliptic           | obovate-elliptic | obovate-elliptic | elliptic               | obovate-elliptic | deltoid        | obovate-oblong     |
| 27-Seed color             | Brown              | Brown           | Brown             | Brown                  | Brown            | Black          | Brown              |
estimate polymorphism indices. The average of nucleotide differences (k) and the minimum number of recombination events (Rm) are also estimated. Selection neutrality was tested by both Tajima’s D [59] and Fu and Li’s D* and F* methods [60].

**RAPD analysis**

The molecular diversity of the studied Taxa was assessed by RAPD analysis [61]. The primer set of 20 random decamer oligonucleotides were purchased (Metabion International AG, Planegg, Germany) as listed in Table 3. The reaction mixture contains 10 μl of 2 × PCR master mixture, 2 μl of gDNA, 1 μl of each primers (10 pmol/μl), and completed to 20 μl with sterile distilled water. PCR amplification was performed at Thermal Cycler 006, programmed to initial denaturation 94 °C for 2 min, denaturation 94 °C for 20 s, annealing 51 °C for 30 s, extension 72 °C for 1 min for 35 cycles, with final extension 5 min at 72 °C. The PCR amplicons were analyzed by agarose gel in 1× TBE buffer (Cat# AM9864). For each primer in RAPD PCR, the number of polymorphic and monomorphic bands was determined. Bands clearly visible in at least one genotype were scored (1) for present, and (0) for the absent and entered into a data matrix. Fragment size was estimated by interpolation from the migration distance of marker fragments. Percentage of Polymorphism Information Content (PIC) was calculated by applying the formula given by [62, 63], Where fi is the frequency of the ith allele, and the summation extends over alleles. Then PIC values were used to calculate marker index (MI). In addition, principal component analysis (PCA) scatter diagram was constructed based on Dice coefficient [65] using SIMQUAL module of the program. The hierarchical clustering analysis was generated using (UPGMA).

**Numerical analysis**

Data analysis was performed using the PAST, ver. 4.02 software [51]. Jaccard’s similarity coefficients were used to generate a dendrogram using Unweighted Pair Group Method with Arithmetic Average (UPGMA) [64] and relationships between the samples were represented. In addition, principal component analysis (PCA) scatter diagram was constructed based on Dice coefficient [65] using SIMQUAL module of the program. The hierarchical clustering analysis was generated using (UPGMA).

**GC-MS metabolic profiling**

**Preparation of plant leaves extracts**

Harvested healthy fresh leaves from the collected specimens, for each taxon, were shade dried in the laboratory for 2 weeks and crushed to a dry powder using a kitchen blender. The powdered leaves (10 g) were extracted by cold maceration [47, 66, 67] with 50 ml methanol (1:5 w/v) for 72 h at room temperature in tightly sealed conical flasks. Each extract was filtered using muslin cloth, the filtrates were collected and centrifuged. The supernatant was collected and the solvent was evaporated to 5 ml final volume, and then stored in tightly sealed dark vials at 4 °C till use.

**GC-MS analysis of the compounds from the leaves extracts**

The chemical constituents of each extract was determined with the Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) using a direct capillary column TG-5MS (30 m × 0.25 mm × 0.25 μm film thickness). The column oven temperature was initially hold at 50°C, then increased by 5°C/min to 230°C for 2 min, and increased to the final temperature 290°C by 30°C/min and hold for 2 min. The injector and MS transfer line temperatures were kept at 250°C, and 260°C respectively. Helium was used as a carrier gas at constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1 μl were injected automatically using Autosampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–1000 in full scan.

**Table 4** The studied taxa, location and their geographical distribution

| Scientific name | NCBI accession No. | Length bp | GC% |
|-----------------|-------------------|-----------|-----|
| 1-Cassia fistula L | MW367973 | 796 bp | 58.29 |
| 2- Cassia grandis. | MZ960447 | 439 bp |       |
| 3-Cassia renigera Benth (Synonym: Cassia javanica L. subsp. renigera Benth.) | MW326851 | 738 bp | 58.53 |
| 4-Cassia roxburghii DC. (Synonym: Cassia marginata Roxb.) | MW326753 | 732 bp | 63.25 |
| 5- Cassia javanica L subsp. nodosa (Roxb.) K. Larsen & S. S. Larsen | MW386305 | 737 bp | 59.52 |
| 6- Senna surattensis (Burm. f) Irwin & Barneby | MW367670 | 729 bp | 60.08 |
| 7- Senna alata (L.) Roxb. (Synonym: Cassia alata Linn.) | MW412635 | 403 bp | 59.55 |

\[
\text{PIC} = 1 - \sum_{i=1}^{n} f_i^2
\]

Where fi is the frequency of the ith allele and the summation extends over n alleles.
mode. The ion source temperature was set at 200 °C. The chemical identity of the components was identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral databases.

Results and discussions

Morphological analysis

Six species and one subsp. of *Cassia* and *Senna* were collected from different localities; Zagazig, Giza and Cairo Egypt, with different longitudes and latitudes as summarized in Table 1. Based on the traditional taxonomical criteria which approximately represented by 27 characters (Table S1), the different species of *Cassia* and *Senna* series were represented. *Cassia fistula* L. and *C. grandis* belonging to *Cassia* and Grandis series, respectively, were identified. While three species of namely; *Cassia javanica*, *C. renigera* and *C. roxburghii* were described to belongs to series Obolospermae. As well as based on the above 27 taxonomical feature, the species *Senna surattensis* and *S. alata* were described to be belonging to Subverrucosae and Pictae, respectively [3, 4]. The universal morphological features of *Cassia* and *Senna* plants were described in Table 2. The UPGMA dendrogram clusters generated from the 27 morphological characters (Fig. 1) classified all studied taxa into two major clades and have an average taxonomic distance of about 4.3. The first clade was divided into three groups; group one includes *Cassia fistula* which belongs to series *Cassia* at taxonomic level 4.4. *Cassia renigera*, *Cassia javanica* L subsp. nodosa and *Cassia roughii* which belongs to Series Obolospermae separated at in one group at a distance level at 2 taxonomic distance level third group includes *Cassia grandis* at taxonomic level 3.06. Clade (II) includes *Senna surattensis* and *Senna alata* at taxonomic level 3.6. The leaf morphological variations of all the plant species were shown (Table 3), that strongly agrees with the series level by [3, 4]. There is a considerable degree of genetic variety in several Cassiinae species derived via investigation by molecular markers, as coincide with other morphological markers [68–70].

**PCA analysis**

The PCA analysis reflects the distribution and incidence of the different morphological traits of the experimented plants, by plotting the PC1 and PC2. From the PCA scatter plot, a clearly discrimination between the two Taxa was observed revealing the characteristic assemblage of *Cassia* and *Senna*. The species of *S. alata* and *S. surattensis* were grouped together; the species of *C. renigera*, *C. javanica*, *C. roxburghii* and *C. grandis* was separated on a distinct group (Fig. 1). The interspecific genetic divergence refers to the genetic variation within the species, with the clear separation of the two genera *Cassia* and *Senna* as coincident with the criteria of morphological and molecular features. The
separation of Cassia and Senna species into two clusters proves the segregation of the genus Cassia L. senso lato into two distinct genera; Senna P. Mill., and Cassia L. senso stricto [3, 4].

Molecular analyses of the experimental plants

Internal transcribed spacers (ITS) analysis

The sequence of ITS region has been utilized as universal molecular phylogenetic marker for plant differentiation between various species [71]. The sequence of this has been frequently authenticated for differentiation of the interdependent and intra-specific interactions of plants [72, 73]. The genomic DNA of the plants were used as PCR primer for amplification of the ITS regions. From the PCR amplicons (Fig. 2), the size of DNA was around 600-700 bp, the products were sequenced and BLAST searched non-redundantly on NCBI database. According to the Neighbor-Joining (NJ) method, the studied taxa have been separated into two different clusters segregated the subtribe Cassiinae. The first cluster includes all Cassia species, while the second one includes all species of Senna. The first cluster (I) divided into two sub clusters, the first one include Cassia grandis MD4 MZ960447 that clearly separated, which belongs to Series Grandis while the other group includes and C. javanica subsp. nodosa MD7 MW386305. C. roxburghii MD5 MW326753, C. renigera MD5 MW32685, which belongs to Series oblospermaea the infra-generic arrangement of species in Cassia and Senna was in agreement with [3, 4], with an obvious deviations regarding to intrageneric relationships C. fistula MD1 MW3679973 in the same group. This might be due to the selection of a small number of species from such a large taxon for the present investigation and amplification of a small portion of the entire genome [74]. A significant difference in chromosome size, morphology and condensing behavior among members of the controversial subtribe Cassieae (Cassia, Chamaecrista and Senna) was revealed on the tribe to suggesting the heterogeneous group from the karyological view [3, 4, 75] (Irwin and Barneby 1982,1981, Souza

![Fig. 2](image-url) Molecular Phylogenetic analysis of the Cassia species based the ITS sequences for Cassia fistula (A), Cassia grandis (B), Cassia renigera (C) and Cassia javanica subsp nodosa (D)
and Benko-Iseppon, 2004). The second cluster includes \textit{S. surrattensis} MD14 MW367670 and \textit{S. alata} MD20 MW412635.

From the alignment profile, \textit{Cassia fistula} displayed 99% similarity with various species of \textit{Cassia fistula}, has been deposited on gene bank under accession number MW367793. \textit{Cassia fistula} displayed a 99% similarity with \textit{Cassia fistula} JX856431.1, JX856431.1, JX856431.1, MG283311.1, KJ638410.1, GU175310.1, MW326851.1 with E value zero and query coverage 96%. The ITS sequences of tested species of \textit{C. grandis} MD4, \textit{C. roxburghii} MD5, \textit{C. renigera} MD5, \textit{C. javanica} subsp. nodosa MD7 were deposited to the genbank with accession numbers MZ960447, MW326753, MW326851 and MW386305, respectively (Fig. 3). From the alignment profile, \textit{Cassia grandis} MD4 MZ960447 displayed 99% similarity with different species of \textit{C. fistula} MG283311.1, MW3674971.1, MW367522.1, MW326851.1, MW367973.1, and MW326753.1 with E value zero and query coverage 99%. From inspection of database deposited sequences there is no ITS sequences of \textit{C. grandis} on the genbank, so, this is the first report confirming the taxonomical identity of \textit{C. grandis}, inhabiting the Egyptian environment. \textit{Cassia roxburghii} MD5 MW326753.1 displayed a 98% similarity with \textit{C. fistula} MG283317, \textit{C. javanica} FJ980413.1, KX372778.1, MW386315.1, MW386314.1 and MW386305.1 with E value zero and query coverage 98%. Obviously, there is no ITS sequences of \textit{C. roxburghii} deposited on the database, so, the similarity has been conducted non-redundantly towards the database deposited sequences. \textit{Senna surattensis} MD14 MW367670 displayed 99% similarity with \textit{S. surattensis} KY611897.1, KY427088.1, KY611897.1, KY427088.1, KY427088.1, MW367547.1, MW367670.1 and MW325225.1 with E value zero and query coverage 90%. Based on the ITS sequence, the phylogenetic analysis of the experimented \textit{Cassia} and \textit{Senna} (Fig. 3), two phylogenetic clades, in which \textit{Cassia} belongs to Clade I, and \textit{Senna} belongs Clade II. From the molecular relatedness, the two species of experimented \textit{Senna} were apparently distinct from the tested \textit{Cassia} plants, ensuring the difference on the conserved sequences of ITS regions, or might be due to

![Fig. 3 Molecular Phylogenetic analysis of the Cassia and Senna species based the ITS sequences of \textit{S. surrattensis} (A), \textit{S. alata} (B), \textit{C. roxburghii} (C). The phylogenetic relatedness of the Cassia and Senna](image-url)
evolutionary. These molecular discriminations being consistent with the recent taxonomical traits based on the morphological features. Traditional taxonomical features such as macro-morphological and micromorphological characters are restricted by the deficiency of clear criteria for character selection, lacking the uniform standard and credible coding data, so causing somewhat misidentification. Therefore, confirmation of the morphological taxonomical features with the recent molecular tools such as DNA barcoding and molecular markers are one of the most recent trends for confirming the traditional morphological features, and exploring the phylogenetic relationships between closely related taxa and their effect on their morphological identification. From the traditional taxonomical traits, the subtribe Cassininae contains the genus Cassia and Senna [3, 4]. From the molecular analysis, two clades were clearly separated into Clade I of Cassia and Clade II of Senna, thus, conclusively the molecular analysis and morphological features being consistent. The taxonomical features of the subtribe Cassininae were described in details (Table S1), as result from the UPGMA dendrogram clustering algorithm using 27 morphological traits that indicated a strong relationship between seven taxa in two clusters (Fig. 1). The cluster I represented by C. fistula, C. renigera, C. Roxburghii, and C. javanica sub nodosa, and the cluster II represented by S. alata and S. surattensis.

The number of nucleotides substitution from sequences of the ITS sequences from the tested Cassia and Senna species were represented in Table 5. Three parameters and seven nucleotide sequences were used in the study, including 1st + 2nd + 3rd + noncoding codon positions [56]. For each pair of sequences all unclear locations were deleted (pairwise deletion option). The final dataset had a total of 837 locations. Analysis of distance matrix shows high level of genetic distance (1.470) was observed between MW367670, S. surattensis MD14, and MZ960447, C. grandis MD4. Low level of genetic diversity (0.0292) between MW367973 C. fistula MD1 and MW326851 C. renigera MD5 was observed.

**Length variation, GC content, nucleotide composition, and mutational events of ITS**

The obtained sequences demonstrating the differences in the GC content of the investigated species (Table 4). The sizes of ITS sequences were varied from 403 bp to 796 bp in Senna alata MD20 and Cassia fistula MD1, respectively. The GC contents were ranged between 58.29 and 63.25% in C. renigera, C. roxburghii. The transition/transversion rate ratios are $k_1 = 1.704$ (purines) and $k_2 = 3.238$ (pyrimidines). The overall transition/transversion bias is $R = 1.16$, where $R = (A^*G^* k_1 + T^*C^* k_2)/(A + G)(T + C)$. There were 7 nucleotide sequences in this study. Position 1st + 2nd + 3rd + noncoding was added for the codon. Every sequence pair of unclear places has been deleted (pairwise deletion option). The completed dataset has a total of 837 places, with the replacements (Table 5). The transitions on the intergenic spacer ITS are more common than transversion, there are 20.86% (A), 18.83% (T/U), 29.65% (C), and 30.67% of the nuclear frequencies (G) of species Cassia and Senna. According to these findings, the fluctuation in the composition of the ITS nucleotide alignment into the 837 character matrix indicated that there were 212 conserved sites, 582 variables comprising 159 informative sites, and 395 singleton loci (Table 6). The frequency of nucleotides composition was 20.86, 18.83, 29.65 and 30.67% accordingly for A, T, C and G, being consistent with that reported for Quercus spp. [76]. Similar studies were reported for Wheat (597 - 605 bp) and Barley (595 - 598 bp) [77]. The whole ITS variation spanned between 650 and 850 bp in the Asteraceae family, the average nucleotide frequency was A (25%), T (24%), C (26%), and G (25%) Average GC content was 51% and AT 49% [78]. The mean length of Ficus carica of ITS was 679.5 bp and its composition was 19.7% (18.6%) [37]. The Chili ITS1-5.8S-ITS2 analyses indicated nuclear frequencies of 18.85% (A), 17.56% (T), 33.95% (C), guanine (G) and 29.64% (A) and average length of 620 bp of thymine (T), respectively [79] The ITS region in Coniferales, Cycadales, Ginkgoales and Gnetales was ranged between 575 and 700 bp in angiosperms and between the species of 975 and 3125 bp in the range [80] Phoenix dactylifera with the mean ITS level of genetic diversity is 2% in the overall data set. These findings are comparable to those observed in Quercus suber and Q.

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**Table 5** Maximum composite likelihood estimate of the pattern of nucleotide substitution

|   | A    | T    | C    | G    |
|---|------|------|------|------|
| A | –    | 4.23 | 6.67 | 11.75|
| T | 4.69 | –    | 21.58| 6.9  |
| C | 4.69 | 13.7 | –    | 6.9  |
| G | 7.99 | 4.23 | 6.67 | –    |

Each entry shows the probability of substitution (i) from one base (column) 1 to another base (row) in the nucleotide matrix. The sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies are 20.86% (A), 18.83% (T/U), 29.65% (C), and 30.67% (G). The overall transition/transversion rate ratios are $k_1 = 1.704$ (purines) and $k_2 = 3.238$ (pyrimidines). The overall transition/transversion bias is $R = 1.16$, where $R = (A^*G^* k_1 + T^*C^* k_2)/(A + G)(T + C)$. This analysis involved 7 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 837 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].
Table 6: Nucleotide diversity, sequence polymorphism based on ribosomal DNA of Cassia and Senna species

| Parameter                  | Frequency |
|----------------------------|-----------|
| m                          | 7         |
| n                          | 837       |
| s                          | 582       |
| C                          | 212       |
| ps                         | 0.695341  |
| Θ                          | 0.283812  |
| π                          | 0.228936  |
| Eta                        | 310       |
| Tajima’s D                 | −1.24870  |
| Fu and Li’s D*             | −0.98694  |
| Fu and Li’s F*             | −1.16035  |
| Fu’s Fs statistic          | 1.474     |

This analysis involved 7 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Non-coding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 837 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].

Abbreviations: m Number of sequences, n Total number of sites, s Number of segregating sites, C Conserved sites, ps S/n, Θ ps/a1, π nucleotide diversity, and Eta Eta.

Random amplified polymorphic DNA (RAPD) analysis

The molecular similarity of the tested plants Cassia and Senna was verified from RAPD analyses. RAPD analysis has been recognized as one of the authentic molecular tools for confirmation of the traditional taxonomical features [13]. The genomic DNA of the plants was used as PCR template with a set of ten-mer oligonucleotide primers applied to the studied species of Cassia and Senna (Table 3). PCR was conducted with random six primer resulted reproducible profiles in the studied species of Cassia and Senna. The PCR amplicons for each primer for the tested plants were shown in Fig. 4. A total 130 bands were scored from PCR amplification of genomic DNA with all the species. In RAPD profiling, a total of 47 clear and reproducible bands was produced, of which 46 bands were polymorphic and only one band was monomorphic which generated by ABI-08 primer. The obtained bands were ranged in size from 100 to 1200bp. The largest amplicon 1200bp was amplified by the primers ABI-09, ABI-10 ABI-11, and the shortest amplicons 100bp by ABI-12. Maximum numbers of 9 amplification products were obtained with primer ABI-09 followed by 8 products with primer ABI-07, ABI08, ABI-10, and ABI-12. Minimum numbers of RAPD products were generated with primers ABI-11. The polymorphic information contents (PIC) ranged from 0.33 to 0.45 with an average of 0.37. The highest RAPD marker index (MI) (4.05) was found in primer ABI-09 and the lowest (2.31) in ABI-08 (Table 7). Jaccard’s similarity index was ranged from 0.575 to 0.068, as shown in Table 8. The highest similarity value (0.575) was recorded between C. grandis, C. javanica subsp. nodosa and the lowest similarity value (0.068) between C. grandis, Senna surattensis and C. javanica subsp. nodosa and S. surattensis. The phylogenetic relatedness of RAPD analysis was constructed using UPGMA and the hierarchical clustering using PAST 4.3e as shown in Fig. 4. The RAPD analysis of the current genera was consistent with the morphological, conventional taxonomical features of the subtribe of Cassininae as adopted by [3, 4]. From the results, the seven taxa of subtribe Cassinae were separated into two clusters for the genus Cassia and the genus Senna.
The UPGMA phenogram generated from the hierarchical clustering analysis of RAPD marker illustrated that *C. fistula* is delimited as a different identity at distance coefficient of 4.5 from the remainder taxa which are clustered together in one group (Fig. 4). Within this group *C. renigera* is delimited as a different identity at a distance coefficient of 4.0 as revealed from the UPGMA clustering, *Cassia javanica* subsp. *nodosa* was delimited at a distance coefficient of about 3.8, while both of *C. roxburghii* and *C. grandis* were clustered together at a distance of about 0.74 (Fig. 4).

The PCA analysis reflects the strength of the RAPD markers to classify the examined Taxa by plotting the PC1 and PC2. From the PCA scatter plot, a clearly discrimination between the two Taxa was observed revealing the characteristic grouping of *Cassia* and *Senna*. In addition, the species *Senna alata* and *Senna surattensis* were grouped together, but the species of *C. renigera*, *C. javanica*, *C. roxburghii* and *C. grandis* was grouped on a distinct group (Fig. 4C). The interspecific genetic divergence refers to the genetic variation within the species, with clear separation of the two genera *Cassia* and *Senna*, as revealed from the coincidence criteria of morphological and molecular features [3, 4]. The separation of *Cassia* and *Senna* species into two different clusters verify the segregation of the genus *Cassia* L. senso lato into two distinct genera namely *Senna* P. Mill., and *Cassia* L. senso stricto. The

![Figure 4](https://example.com/figure4.png)

**Fig. 4** UPGMA analysis of based on the RAPD Markers of seven different taxa of *Cassia* & *Senna* generated. A RAPD profile analysis of the experimented plants with the different primers, B PCA analysis of the tested plants based on the RAPD profile.
consistence of morphological and molecular taxonomic features of the subtribe Cassinae for grouping into two genera Cassia and Senna has been reported [86, 87]. Based on vegetative and reproductive characteristics, Cassia fistula was assigned to series Cassia while C. renigera, C. javanica subsp. nodosa and C. roxburghii were included in series Obolospermae and C. grandis to series Grandes. Senna surattensis in series Peiranisia while Senna alata to series Interglandulosae.

**GC-MS metabolic profiling analysis**

The metabolic profiling pattern of the tested plants was analyzed as metabolic marker for confirming the traditional taxonomical features and molecular DNA barcoding analysis [47]. Gas chromatography-mass spectrometry has been established as a key technological tool for metabolic profiling and taxonomical tools to confirm the traditional taxonomical features. The GC-MS metabolic profiling has been used frequently for taxonomic purposes 129 species belonging to 29 genera of the Convolvulaceae [88], six species of Salvia (Lamiaceae) [89], three species of the tree-fern Cyathea (Cyatheaceae) [90]. Eleven species of Solanum (Solanaceae) [91] Centaurea galicicae and C. tomodosii (Asteraceae) [92] and also for 14 species of that family [93]. GC-MS has immensely contributed to the detection of bioactive constituents from plants which might be very useful for drug research and discovery [30, 94].

An extensive survey of literature elucidated that there is no evidence for the utility of GC-MS screening of phytochemicals has been generated for the taxonomic investigation of genus Cassia from Egypt or anywhere else. Conversely, an immense phytochemical interest using GC-MS has been paid on Cassia sens. Lat. (including species of Senna) as a result of their excellent medicinal values [24, 67].

### Table 7

| Primers name | Primer Sequence (5’-3’) | Size Range of Amplified Product (bp) | Total no. of amplicon | Total number of bands | No of Monomorphic bands | Number of Polymorphic bands | (%) Polymorphism | PIC = 2 × fi × (1-fi) | MI=PIC × Number of Polymorphic bands |
|--------------|--------------------------|-------------------------------------|-----------------------|-----------------------|--------------------------|-----------------------------|----------------|-------------------------|---------------------------------|
| ABI-07       | GGTGAC GCAG              | 1000-100                            | 27                    | 8                     | 0                        | 8                           | 100            | 0.35                    | 2.8                             |
| ABI-08       | GTCCAC ACGG              | 1000-180                            | 27                    | 8                     | 1                        | 7                           | 87.5           | 0.33                    | 2.31                            |
| ABI-09       | TGGGGG ACTC              | 1200-250                            | 24                    | 9                     | 0                        | 9                           | 100            | 0.45                    | 4.05                            |
| ABI-10       | CTGCTG GGAC              | 1200-200                            | 24                    | 9                     | 0                        | 9                           | 100            | 0.40                    | 3.2                             |
| ABI-11       | GTAGAC CGGT              | 1200-200                            | 24                    | 9                     | 0                        | 9                           | 100            | 0.39                    | 2.34                            |
| ABI-12       | CCTGGA CGCA              | 900-100                             | 16                    | 8                     | 0                        | 8                           | 100            | 0.34                    | 2.72                            |
| Total        |                          | 130                                 | 47                    | 46                    |                           |                             | 0               | 0.37                    |                                 |

### Table 8

| Taxa                | Cassia fistula | Cassia grandis | Cassia renigera | Cassia roxburghii DC | Cassia javanica L. subsp. nodosa | Senna alata | Senna surattensis |
|---------------------|---------------|----------------|----------------|----------------------|---------------------------------|-------------|-------------------|
| Cassia fistula      | 100.00        |                |                |                      |                                 |             |                   |
| Cassia grandis      | 0.512         | 1              |                |                      |                                 |             |                   |
| Cassia renigera     | 0.297         | 0.518          | 1              |                      |                                 |             |                   |
| Cassia roxburghiiDC | 0.512         | 0.575          | 0.464          | 1                    |                                 |             |                   |
| Cassia javanica L. subsp. nodosa | 0.388 | 0.482 | 0.333 | 0.387 | 1 |
| Senna alata         | 0.105         | 0.166          | 0.142          | 0.093                | 0.181                           | 1           |                   |
| Senna surattensis   | 0.117         | 0.068          | 0.111          | 0.068                | 0.1                             | 0.076       | 1                 |

The GC-MS metabolic profiling has been used frequently for taxonomic purposes.
Table 9  The identified phytocompounds in the methanolic extracts of leaves of the taxa studied of *Cassia* and *Senna*

| RT  | Phytocompounds                                         | MF            | MW     | Chemical class            | Area % |
|-----|--------------------------------------------------------|---------------|--------|---------------------------|--------|
|     |                                                        |               |        |                           | sp1    | sp2    | sp3    | sp4    | sp5    | Sp6 | Sp7 |
| 17.97| Cyclooctasiloxane, hexadecamethyl-                      | C16H48O8Si8   | 592    | Organosiloxane            | 0.60   | 1.23   | –      | –      | –      | –   | –   |
| 21.01| 1-Hexadecanol                                          | C16H34O       | 242    | Cetyl alcohol (16 C fatty alcohol) | –      | 2.06   | –      | –      | –      | –   | –   |
| 22.85| 2,4-Di-tert-butylphenol                                 | C14H22O       | 206    | Alkyl benzene             | 2.57   | –      | –      | –      | –      | –   | –   |
| 23.24| 2,6-Difluoro benzoic acid, tridec-2-ynyl ester         | C20H26F2O2    | 336    | Ester                     | –      | –      | 1.55   | –      | –      | –   | –   |
| 23.55| Phenol, 2-propyl-                                       | C9H12O        | 136    | Propyl phenol             | –      | –      | –      | 1.70   | –      | –   | –   |
| 26.69| 1-Nonadecene                                           | C19H38        | 266    | Un-branched 19 C alkene   | –      | 5.48   | –      | –      | –      | –   | –   |
| 27.07| Guanosine                                              | C10H13N5O5    | 283    | Purine nucleoside         | –      | –      | –      | 6.38   | 2.60   | –   | –   |
| 28.08| Neophytadiene                                           | C20H38        | 278    | Diene Hydrocarbon         | 463    | 3.87   | 3.16   | –      | –      | –   | –   |
| 28.94| Tetradecanoic acid                                      | C14H28O2      | 228    | Fatty acid                | –      | –      | –      | 1.37   | –      | –   | –   |
| 32.54| Hexadecanoic acid, methyl ester                        | C17H34O       | 270    | Fatty acid ester          | 2.44   | 12.45  | 3.93   | 3.46   | 2.44   | –   | –   |
| 34.01| Hexadecanoic acid, ethyl ester                         | C18H36O2      | 284    | Fatty acid ester          | 459    | 0.63   | 5.13   | 3.67   | 4.14   | 5.5 | 5.7 |
| 36.04| Myo-Inositol, 2-C-methyl-                               | C7H14O6       | 194    | Carboxyclic sugar         | –      | –      | –      | 46.5   | 19.86  | 49.59| –   |
| 36.83| Phytol                                                 | C20H40O       | 296    | Acyclic diterpene alcohol | –      | 11.99  | –      | –      | 3.20   | 10.31| –   |
| 37.23| 16-Octadecenoic acid, methyl ester                     | C19H36O2      | 296    | Fatty acid ester          | 3.07   | –      | 3.96   | 3.61   | 4.06   | 2.24| 4.74|
| 37.26| 9-Octadecenoic acid, methyl ester (E)-                 | C19H36O2      | 296    | Fatty acid ester          | –      | 15.81  | –      | –      | –      | –   | –   |
| 37.61| 9,12-Octadecadienoic acid (Z,Z); methyl ester         | C19H40O2      | 294    | Fatty acid ester          | –      | 10.06  | –      | –      | –      | –   | –   |
| 38.54| 9-Octadecanoic acid (Z), ethyl ester                   | C20H38O2      | 310    | Fatty acid ester          | 7.13   | 1.15   | 9.30   | 8.63   | 3.58   | 5.25| 0.61|
| 40.60| Oleic acid                                             | C18H36O2      | 282    | Fatty acid                | 2.93   | 1.43   | 1.25   | 0.82   | –      | –   | –   |
| 43.80| Meadowlactone                                          | C20H28O2      | 310    | Delta-lactone             | –      | –      | 4.47   | –      | –      | –   | –   |
| 45.72| 9-Octadecenoic acid (Z); 2,3-dihydroxypropyl ester     | C21H40O4      | 356    | Monocacylglycerol         | 2.08   | –      | –      | –      | –      | –   | –   |
| 48.62| 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester  | C24H38O4      | 390    | Aromatic dicarboxylic acid ester (Ester) | 1.44 | 1.75 | 1.00 | – | 0.71 | – | – |
| 53.68| Docosanoic acid, 1,2,3-propanetriyl ester              | C68H134O6     | 1058   | Fatty acid ester          | 1.38   | –      | –      | –      | –      | –   | 0.38|
| 58.49| Vitamin E                                              | C29H56O2      | 430    | Fat-soluble vitamin       | 5.11   | 0.64   | 18.67  | 3.57   | 1.39   | 2.04| 0.57|

- Absent, MF Molecular formula, MW Molecular weight, sp1*Cassia fistula*, sp2*Cassia grandis*, sp3*Cassia javanica* subsp. *nodosa*, sp4*Cassia renigera*, sp5*Cassia Roxburghii*, *Senna alata*, sp6 *Senna surattensis*
metabolic compounds were identified in the methanol extracts of leaves of the taxa, with obvious fluctuation on their concentrations, as revealed from the area of the peaks of chromatograms of GC-MS. The identified compounds of the studied taxa with their retention times, molecular formula, molecular weight, chemical class and concentration were represented. The GC-MS chromatograms were shown in Figs. 5, 6 and 7. The identified compounds were assigned to various chemical classes such as organosiloxane, esters, fatty acid esters, fatty acids and alcohols, hydrocarbons, phenolic compounds, carboxylic sugar and fat-soluble Vitamin E. The first compound identified, in the leaf extracts, was Cyclooctasiloxane, hexadecamethyl at a retention time 17.97 min in *Cassia fistula* and *C. grandis*, while Vitamin E was the last compound at the retention time 58.49 min in all the taxa investigated except *S. surattensis*. The compounds Neophytadiene, Hexadecanoic acid, ethyl ester, 9-Octadeconoic acid, methyl ester, 9-Octadecenoic acid, ethyl ester and Vitamin E was highly dominant in *C. fistula*. While, the compounds 2,4-Di-tert-butylphenol, 1-Nonadecene, Hexadecanoic acid, methyl ester, 9-Octadecenoic acid, methyl ester, 9,12-Octadecadienoic acid (Z,Z), methyl ester were the most frequent in *C. grandis*. Vitamin E and phytol were the most dominant metabolites in *C. javanica* subsp. *nodosa* followed by 9-Octadecenoic acid, ethyl ester and Hexadecanoic acid. Myo-Inositol was the most frequent metabolite in *Cassia renigera* (46.5%) followed *C. roxburghii* (19.8%). Remarkably, the biological and chemical identities of the metabolites of the genera *Cas sia* was distinctly different from the genera of *Senna*. The most dominant compounds of *S. alata* and *S. surratensis* were different from that of *Cassia* sp., ensuring the metabolic difference of gene expression pattern on both genera. Out of the 23 phytochemical compounds identified, four compounds detected at different retention times and with varied concentrations displayed a consistent occurrence among the taxa investigated. These common
compounds comprised Hexadecanoic acid, methyl ester; Hexadecanoic acid, ethyl ester; 9-Octadecenoic acid (Z)-ethyl ester and Vitamin E (Table 9). Several of the scored phytochemical compounds were demonstrated as unique chemical traits for individual species; such for instances C. fistula, C. grandis, C. javanica subsp. nodosa and C. roxburghii. Furthermore, the absence of 16-Octadecenoic acid, methyl ester and Oleic acid at the retention time 37.2 and 40.6 min, respectively, could be characteristic for C. grandis, while the absence of 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester could be diagnostic for C. renigera, S. alata and S. sutattensis (Table 9).

The present study comparatively explores the taxonomic framework, phytochemical constituents of leaves of six species of Cassia and Senna and one subspecies of genus Cassia from Egypt via GC-MS screening for use as chemical markers for classification of plants, [91, 93], for the therapeutic agents, [30]. The chemical information of plants can provide new taxonomic diagnostic characters that help to improve classification of plants [95]. Hexadecanoic acid, methyl ester; Hexadecanoic acid, ethyl ester; 9-Octadecenoic acid (Z)-ethyl ester and Vitamin E displayed a consistent occurrence among the taxa investigated. Thus, they can be designated, here, as the chemotaxonomic markers for the taxa investigated of Cassia and Senna at the genus level. Besides, several of the identified were assigned as exclusive diagnostic chemical traits for individual taxa, for example, 9-Octadecenoic acid, 2,3-dihydroxypropyl ester and Docosanoic acid, 1,2,3-propanetriyl ester for C. fistula, while 1-Hexadecanol, 2,4-Di-tert-butylphenol, 1-Nonadecene and others are diagnostic for Cassia grandis. The lack of certain compounds and presence of other compound at the same retention times may be considered as chemotaxonomic guides for some species. For example, the absence of 16-Octadecenoic acid, methyl ester and oleic acid may be characteristic for Cassia grandis, while the absence of 1,2-Benzene dicarboxylic acid, bis(2-ethylhexyl) ester may be diagnostic for C. renigera. Based on vegetative and reproductive characteristics, C. fistula was assigned
to series *Cassia* and *C. grandis* to series *Grandes*, while *C. javanica* and *C. roxburghii* were included in series *Obolospermae*. Hence, the taxa studied of genus *Cassia* may find their sound to be utilized in identification of potential lead compounds very useful for discovery of novel pharmaceuticals. For instance, Hexadecanoic acid, methyl ester was reported to exhibit anti-inflammatory and antifibrotic activities [96]. 9-Octadecenoic acid (Z)-2,3-dihydroxypropyl ester was regarded as a magic lipid regarding its diverse application in pharmaceuticals, cosmetics, food and protein crystallization Powder-[97]. They added that this compound is known for its surfactant and emulsifying properties. Besides, its use as a drug delivery enhancer was documented [98]. Fatty acids are carboxylic acids with an aliphatic chain which are either saturated or unsaturated [99]. Monounsaturated and polyunsaturated fatty acids have been utilized to lower the risk of heart disease and also to enhance the immune system [100]. Herein, Oleic acid; is one of the unsaturated fatty acids, has been reported to exhibit various bioactivities such as anti-inflammatory, cancer preventive, hypcholeostrolemic and dermatitigenic [101]. The compound 1,2-Benzenedicarboxylicacid, bis(2-ethylhexyl) ester was isolated from twigs of the dicot flowering plant *Thevetia peruviana* as a potential biomarker [102]. They added that this compound was proved to be a strong immunomodulatory B-cell stimulant. Moreover, this compound revealed positive anticancer activity on PC3, MCF and other cancer cell lines. Phytol is an acyclic diterpene alcohol which is a precursor for vitamins E and K1 [66]. It results from the hydrolysis of chlorophyll and was found to be effective at different stages of arthritis [103]. Moreover, phytol was found to have antibacterial activities against *Staphylococcus aureus* [104]. Neophytadiene was reported as presenting antimicrobial and anti-inflammatory activities [105]. Vitamin E is a fat-soluble compound that functions as antioxidant in human body system [106]. 2,4-Di-tert-butyl phenol is a lipophilic

**Fig. 7** GC-MS chromatogram of methanol leaf extract of *Senna alata*, and *Senna surattensis*
phenol produced by various groups of organisms as a common toxic secondary metabolite [107].

Conclusions
Few taxonomical studies on the genus Cassia and Senna, were published regard to the biological identity of these plants as repertoire to various bioactive compound. Thus, the objective of the current was to revise and authenticate the phylogenetic relationship between studied taxa of the species of Cassia and Senna in Egypt using the recent tools of ITS barcoding, RAPD analysis and metabolic profiling, in comparing to the traditional taxonomical features. The taxonomical description of the studied taxa was confirmed from the molecular analysis of ITS sequences and RAPD analysis. Thus, from the molecular analysis, two clades were clearly separated into Clade I of Cassia and Clade II of Senna. The cluster I represented by C. fistula, C. renigera, C. roxburghii, and C. javanica sub nodosa, and the cluster II represented by S. alata and S. surattensis. The morphological, molecular traits of the studied plants were authenticated from the metabolic profiling by GC-MS analysis. The identified compounds were potentially useful for both the taxonomic purpose and pharmaceutical applications. The study highlighted the pharmacological significance of several of the identified phytochemicals. From the taxonomical view, the genetic links between members of the Cassiineae, namely the Cassia, Senna genus are solved and morphological observations support. Conclusively, the traditional morphological features, molecular barcoding using ITS sequences, RAPD analysis and metabolic traits by GC-MS analysis, authenticates the taxonomical diversity of the genus Cassia and Senna.

Supplementary Information
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Additional file 1: Table S1. List of 28 morphological character and their state in the seven studied taxa of Cassia and Senna.
Additional file 2.

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Authors’ contributions
M.M.E, H.A.H, R.S.S, and S.S.T conceptualize and write the original draft of the manuscript. A.S.E revise and edit the work. All of the authors read and approved the manuscript.

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Available data and materials
The datasets used and analyzed during the current study available from the corresponding author on reasonable request. The accession numbers of the deposited ITS sequences were listed on Table 4.

Declarations
Ethics approval and consent to participate
This article does not contain any studies with human participants or animals. The collection materials of the plants, complies the relevant institutional, national, and international guidelines and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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