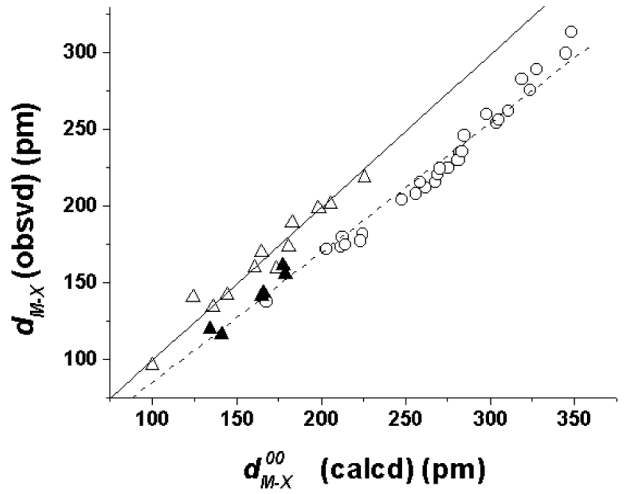

Figure 2. Plots of observed M–X distance for various MX_2 compounds (X = insulator). Triangles: M = insulator; Filled triangles M–X double bond; Circles: M = metal.

depend on the M–X bond order. More significantly, the M–X distance in MX_2 compounds in which M is a metal atom is also given by $d^{00}_{MX}/1.18$, even if they are nominally 'single' bonds. The simultaneous 'contraction' of M–X distance and 'expansion' of 1,3-X---X is contrary to that anticipated from eqs (5) and (7). This aspect will be treated in more detail in another communication in the context of atomic size and metallization of elements.

We further find that:

(1) The 1,3-X---X non-bonded distance is the more important structure-defining parameter (varying by ~ 1%) than the 1,2-M–X bonded distance (varying by ~ 4%) in an X–M–X linkage.

(2) Once the 1,2- and 1,3-distances are known, the geometry (linear or bent, say, in MX_2 halides) follows quite simply without requiring complex theoretical modelling[8] (such as participation of d electrons).

(3) Our methodology is (understandably) applicable to terminal linkages in any gas-phase molecules, but not always to the distance in ring systems, including, for example, bridged linkages[9] in M_2X_6 dimers.

(4) Changes in core size due to changes in valence or spin state are required to obtain 1,3- non-bonded X---X distance for transition metal elements in MX_n compounds (n = 3, 4).

In conclusion, we have shown that the 1,3-non-bonded distance in an X–M–X linkage is a fundamental and environment-independent property of the 'core' sizes of M and X atoms, without requiring inputs from quantum chemical (valence bond or molecular orbital) procedures. This follows from a simple mechanical model of molecular tensegrity, when various forces are balanced for a $\mu = 0$ condition for the chemical potential. Molecular geometry seems to be available *ab initio* from purely classical considerations, given a 'core' atomic size.

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ACKNOWLEDGEMENTS. I thank Prof. N. Kumar, RRI, Bangalore, for drawing attention to the concept of tensegrity in a discussion on tolerance factor and Prof. Bruno Samori, Bologna, for discussions and drawing attention to Ingber's work on cellular tensegrity. I also thank Council of Scientific and Industrial Research, New Delhi for subsistence grants and the National Chemical Laboratory, Pune for desk space.

Received 14 November 2005; revised accepted 1 April 2006

Molecular differentiation in *Murraya* Koenig ex L. species in India inferred through ITS, RAPD and DAMD analysis

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Two species of *Murraya*, viz. *M. koenigii* (L.) Spreng. ($2n$ = 18) and *M. paniculata* (L.) Jack. ($2n$ = 18), occurring in India and used in indigenous system of medicine, have a long-standing problem on systematic disposition of the wild and cultivated forms (*M. exotica*/

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***M. paniculata*), leading to different taxonomic opinions by previous workers. In the present work, ITS sequencing and PCR-based profiling with RAPD and DAMD methods were used to study the molecular differentiation in *Murraya* species collected from different geographical locations of India. The amplified ITS products were analysed using BLAST. The five RAPD and four DAMD primers resulted in discrete polymorphic fragment patterns across the compared taxa, while few other fragments were common to either of the *Murraya* taxa. All accessions of the two *Murraya* species could be separated broadly in two clusters, *M. koenigii* and *M. paniculata* respectively. The former were relatively more heterogeneous and had higher genetic diversity. Differences within the 5.8 S gene as well as in the rest of the ITS1 and ITS2 regions, differences in RAPD and DAMD profiles that resulted in separate clusters are all consistent with the differentiation of the two *Murraya* species. The present work demonstrates the usefulness of molecular analysis to improve the identification and differentiation of the *Murraya* species.**

Keywords: DAMD, ITS, *Murraya*, RAPD, UPGMA.

THE genus *Murraya* (Rutacaeae) comprises about 11 species of shrubs or small trees distributed in the tropics and subtropical regions of Sri Lanka, India, South China to Southeast Asia, Malaysia, New Guinea, Northeast Australia and New Caledonia[1]. Two species of *Murraya*, viz. *M. koenigii* (L.) Spreng. ($2n = 18$), and *M. paniculata* (L.) Jack. ($2n = 18$), are reported to occur in India[2,3]. Indian species of *Murraya* have long been used in the indigenous system of medicine as tonic, stomachic, carminative, to treat vomiting, diarrhoea and dysentery[4,5] and as an important ingredient of several anti-diabetes herbal formulations and drugs[6,7].

M. koenigii and *M. paniculata* have a wide distribution throughout India, both in wild and cultivated states. Wild or semi-wild plants of *M. koenigii* are more common in the sub-Himalayan tracts from West to East Himalayas, whereas in southern India this species is mostly cultivated[2]. Both wild and cultivated populations of this species exhibit enormous diversity in chemical constituents. An earlier study revealed that the chemical constituents of the essential oil from the leaves of *M. koenigii* vary with the locality, and specific constituents of the essential oil mark the genetically diverse stocks as chemotypes of *M. koenigii*[8]. At the morphological level also, the cultivated plants with a treelet habit with smaller leaves and very small, membranous and highly odoriferous leaflets, and a red-tinged petiole differ from those found in the wild with larger coriaceous and less aromatic leaves and leaflets. A number of 'landraces' of this species are widely cultivated in home gardens in southern India[2].

The other species, *M. paniculata* is also widespread with different habits, size and shape of leaflets, number and size of flowers and exomorphology of fruits, amongst both wild and cultivated forms[2]. The bushy shrubs with smaller, obovate, obtuse or obtusely acuminate leaflets, many-flowered (3 to 6) inflorescence, and slightly acuminoid berries found frequently cultivated were, however, treated under *M. exotica* by several earlier workers. The second form that is truly wild (*M. paniculata*), but also cultivated in gardens is a shrub or tree with slightly larger, ovate or oblong-elliptic, acuminate or caudate leaflets; few (3 or 4) flowered inflorescence, and acuminoid fruits. However, the frequent occurrence of intermediate forms in the wild as well as under cultivation makes the morphological distinction difficult[2]. The taxonomic complexity in *M. exotica/M. paniculata* complex is thus centred primarily on the question of the correct status of the wild and cultivated forms/taxa included under *M. paniculata* (for instance, *M. paniculata* var. *ovalifoliata* Engl. in Australia and Queensland; *M. paniculata* var. *zollingeri* Tanaka in Timor Islands; cultivated *M. exotica* and wild and cultivated forms of *M. paniculata* occurring in the Indian subcontinent), since according to some workers *M. exotica* is a separate and distinct species[9], while others[2] opined that it must be conspecific with *M. paniculata*. A few workers like Hooker[10] considered *M. paniculata* as a variety of *M. exotica*, while Huang[11] considered *M. exotica* as *M. paniculata* subsp. *exotica* (L.) Huang. Mabberley[12] suggested that *M. exotica* be best treated as a cultivar, but opined that the two forms of *M. paniculata* reported from Australia having differences in leaf size and aroma[13], need further elucidation and comparison with those found in neighbouring territories. It is this divergent disposition of the *M. exotica/M. paniculata* complex that makes it an interesting problem to attempt resolution using molecular evidences.

In recent years PCR-based methods have been used quite extensively due to their ease, rapidity and reliability, for analysis of molecular differentiation and for resolving taxonomic problems in plants. Internal Transcribed Spacer (ITS) analysis in several angiosperm groups has demonstrated that this region provides a valuable set of characters for addressing lower-level phylogenetic questions in flowering plants[14]. The Random Amplified Polymorphic DNA (RAPD)[15,16] and Directed Amplification of Minisatellite DNA (DAMD)[17] methods have been variously applied for molecular taxonomic studies in plants till date not only for inter-species, but also infra-specific resolution and differentiation[18–22].

In the family Rutaceae, chloroplast DNA has been analysed to construct an evolutionary history of the tribes and subtribes[23–25]. The only representative data for the complete ITS sequences in this family include sequences from *Ruta montana* and six species of *Haplophyllum*[26], while for a few species of genera *Acronychia*[27] and *Flindersia*[28], partial sequences of either ITS 1 or ITS 2 or 5.8 S are present in the nucleotide sequence databank. In case of *Murraya* species, earlier studies have reported partial to complete sequences of *atp*B–*rbc*L and chloroplast *rps*16

sequences[23,24] and no ITS sequence data were available for this genus. In the present work ITS, RAPD and DAMD techniques were used to study the molecular differentiation in *Murraya* species collected from different geographical locations of India.

Extensive field trips were undertaken to collect different species of *Murraya* from different geographical regions of the country. About 20 accessions representing the species were collected. Out of the total accessions collected, seven clearly identified accessions and one that was not identified because the tree was in the vegetative stage were considered for PCR-based analysis (Table 1). The remaining 12 accessions were either multiple accessions from the same location or were not clearly identified and so were not considered for the actual PCR analyses. Total genomic DNAs from individual plants were isolated from the silica gel-dried leaf tissue with a Dneasy Plant Mini Kit (QIAGEN), according to the instructions of the manufacturer. DNA concentrations were approximated by gel electrophoresis on 1% agarose, staining with ethidium bromide, and comparison with a set of concentration standards. The RAPD primers (U and G kits, 20 primers each) were procured from Qiagen-Operon Tech. Inc., CA, USA and after initial screening, primers OP-U13, OP-U17, OP-U02, OP-U11 and OP-G17 were selected on the basis of the best profiles with each of the template DNAs tested. All RAPD reactions were carried out as described earlier by Bhattacharya and Ranade[20]. The DAMD primers M13, 33.6, HBV and HVR were custom synthesized from Bangalore Genei, Bangalore, India. DAMD analysis was carried out as described earlier by Bhattacharya and Ranade[20]. All amplifications were performed on a PTC-200™ (MJ Research, Inc, USA) thermocycler and the amplified products were separated on 1.5% agarose gel in 0.5X TBE buffer at constant voltage of 5 V/cm for 3 h. After electrophoresis, the gel was stained in ethidium bromide, visualized, photographed and archived using NightHawk™ gel documentation system (pdi Inc, USA).

Fragment sizes of the amplification products obtained using RAPD or DAMD primers were estimated from the gel by comparison with standard molecular weight marker (low-range DNA ladder from Bangalore Genei). A pairwise matrix of Jaccard's similarity coefficient between accessions was determined for the band data from the two methods and a UPGMA tree was also computed after allowing a 500 replicate bootstrap test using the FreeTree program[29]. The trees were viewed, annotated and printed using TreeView program[30].

Table 1. *Murraya* accessions collected from different parts of the country

LWG herbarium accession number	Sample number	Location	State
226414	MX1	Baharaich	Uttar Pradesh
226099	MP1	Quilon	Kerala
225738	MP2	Quilon	Kerala
225642	MP3	Tirunelveli	Tamil Nadu
225644	MK4	Tirunelveli	Tamil Nadu
226039	MK5	Chessa	Arunachal Pradesh
226021	MK6	Umiam	Meghalaya
226430	MK7	Lonavala	Maharashtra
225750	ATA	Trivandrum	Kerala

The ribosomal RNA cistron internal transcribed spacer (ITS1, ITS2) region and the included 5.8S rRNA coding sequences were amplified from one sample each of *M. koenigii* (MK7) and *M. paniculata* (MP2) and two other Rutaceae field-collected samples (*Toddalia asiatica* accession #5 and #6) using universal primers[31]. The amplification conditions included 50 ng template, 4 pmol of each primer, 1.5 mM Mg^{2+}, 0.2 mM each dNTP, 5 units *Taq* DNA polymerase and 1X assay buffer in 100 µl reaction volume. The thermal cycler programme included a predenaturation at 96°C for 1 min followed by 35 cycles each at 94°C for 20 s, 50°C for 30 s and 72°C for 45 s. The final cycle allowed an extended incubation at 72°C for 4 min. The amplified ITS products were eluted from the agarose gels using Clean Genei kit (Bangalore Genei). Nucleotide sequences of the eluted DNAs were determined from both ends using primers P5 and P4 respectively. The sequences were analysed using BLAST program[32] for homology to other sequences already present in the EMBL/GenBank databases. This helped to determine whether the new sequences were actually from the ITS regions. The nucleotide sequence databanks were also searched for similar sequences from other Rutaceae genera and species. All complete ITS region sequences from Rutaceae plants along with the four new sequences determined in this work, two Sapindaceae plant sequences (family Sapindaceae is closer to Rutaceae) and *Morus alba* (family Moraceae) as outgroup for comparison were selected (Table 2), multiple-aligned and the phylogenetic tree of this alignment was determined. Multiple alignments were carried out using the program DIALIGN[33] and the resulting dendrogram was visualized and printed using the program TreeView[30], as described earlier for the RAPD and DAMD data tree.

RAPD and DAMD methods were used to analyse the genetic differentiation in *Murraya* species. Eight accessions belonging to the two species were considered. The five RAPD and four DAMD primers resulted in discrete patterns of bands, some of which were clearly polymorphic across the taxa compared, while few others were common to either of the *Murraya* taxa. Both RAPD and DAMD reactions were also carried out for another Rutaceae species, *Atalantia wightii* taken as an outgroup. The RAPD profiles obtained with primers OP-G17 and OP-U13, and DAMD profiles obtained with primers 33.6 and M13 are shown in Figure 1. The bands clearly differing between *M. koenigii* and *M. paniculata* in DAMD profile with primer M13 are indicated by two small arrowheads in Figure 1. The band data for all primers in the two methods were used for generating a UPGMA tree (Figure 2) after a 500 replicate bootstrap

Table 2. Summary of nucleotide sequences of ITS region selected for multiple alignment analysis

Taxon	Accession number	Sequence/region length (bases)				Reference
		Total	ITS 1	5.8 S	ITS 2	
Murraya koenigii	AJ 879084	677	259	164	254	This work
Murraya paniculata	AJ 879085	783	275	161	247	This work
Toddalia asiatica (#5)	AJ 879086	644	236	159	249	This work
T. asiatica (#6)	AJ 879087	643	237	164	242	This work
Haplophyllum blanchei	AY 484571	637	248	164	225	34
H. linifolium	AY 484572	628	244	164	220	34
H. coronatum	AY 484573	630	244	164	222	34
H. rosmarinifolium	AY 484574	626	244	164	218	34
H. suaveolens	AY 484575	625	244	164	217	34
H. bastetanum	AY 484576	627	244	164	219	34
Ruta montana	AY 484577	612	216	164	232	34
Serjania triquetra	AY 207571	646	236	164	236	Tian and Li (direct submission to GenBank)
Sapindus delavayi	AY 207570	596	222	164	209	Tian and Li (direct submission to GenBank)
Morus alba	AY 345157	626	167	99	309	Weiguo *et al.* (direct submission to GenBank)

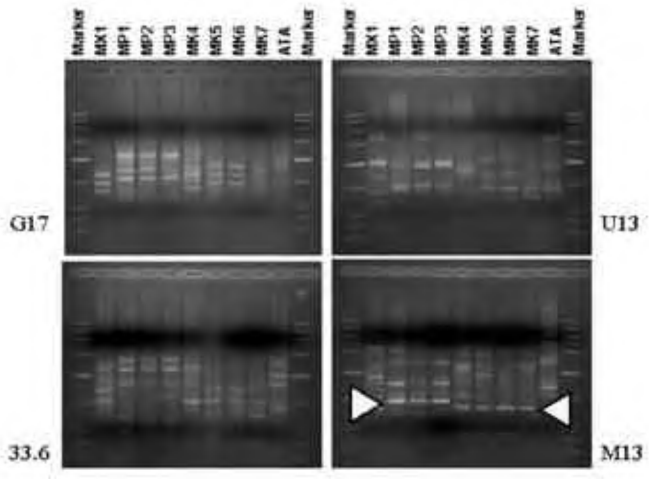

Figure 1. Agarose gel electrophoresis profiles of PCR with RAPD primers G17 and U13 (top) and DAMD primers 33.6 and M13 (bottom). Right arrowhead in profile with M13 primer indicates DAMD band common to *M. paniculata* accessions, while left arrowhead indicates the band common to *M. koenigii* accessions. Lanes are identified with taxa abbreviations as in Table 1.

test of robustness. This tree clearly indicates that the different accessions of *Murraya* species could be distinguished broadly into two groups consisting of MP1, MP2, MP3 in one and MX1, MK4, MK5 and MK7 in the other. Since MX1 accession of *Murraya* species, collected from Baharaich (Uttar Pradesh) was in a vegetative stage and could not be identified on the basis of morphological parameters, it was treated as an unidentified accession (MX1) and analysed along with other known samples. Banding pattern of MX1 though intermediate between *M. koenigii* and *M. paniculata*, was more closely related to the former and therefore, MX1 occurred in the cluster of *M. koenigii* accessions in the UPGMA tree. RAPD and DAMD profiles revealed that the different accessions of *Murraya* species could be distinguished broadly into two groups; however, these methods did not reveal any specific bias in the clustering of the different accessions of *Murraya* species collected from distinct geographical locales. This shows that geographical divergence of the accessions of the two species is not reflected in their RAPD and DAMD profiles.

Estimates of Shannon index[34] (*I*) and genetic diversity[35] (*H*) calculated using the POPGENE program[36] were used to infer heterogeneity of the two species. *M. koenigii* group was found to be more heterogeneous and had higher *I* and *H* values (0.1625 ± 0.1842 and 0.2500 ± 0.2669, respectively) compared to the corresponding values (0.0580 ± 0.1411 and 0.0864 ± 0.2068 respectively) in the *M. paniculata* group. Similarly, the *M. koenigii* group also had higher proportion of polymorphic bands and lower average Jaccard coefficient (about 52 and 0.57% respectively) compared to those in case of *M. paniculata* group (about 16 and 0.78% respectively), while the average Jaccard coefficient between the two groups was only 0.29, a value much lower than either of the intra-group values. This higher heterogeneity of the *M. koenigii* species group is consistent with the fact that each accession in this group was from a different state, while those in the *M. paniculata* group were from a narrow geographical region in two southern states of the country.

ITS region sequences (partial ITS 1, complete 5.8 S and partial ITS 2) were determined for the two *Murraya* species and two *Toddalia asiatica* accessions. These sequences were compared with those from the corresponding region in the case of seven other Rutaceae species, two Sapindaceae species and one *Morus alba* sequence already available in the databank (Table 2) by multiple alignment methods. The dendrogram of the multiple alignments is shown in Figure 3. The BLAST search results and multiple alignments of the determined sequences of two *Murraya* species and two *T. asiatica* accessions confirmed that the sequences were from the ITS region. On the basis of the

aligned sequences as well as with reference to the angiosperm consensus motif determined by Jobes and Thein[37], the putative start and end-points of the 5.8 S sequences were identified. This region, included within the two ITS regions, showed a strong conservation in its length in the family Rutaceae (Table 2) and in the determined sequences also. However, slight differences were noted between the two *T. asiatica* accessions and the two *Murraya* species sequences. In the latter case, the differences within the 5.8S gene as well as in the rest of the ITS1 and ITS2 regions are consistent with the differentiation of the two *Murraya* species.

All three PCR-based methods (ITS, RAPD and DAMD) were effective in discriminating between species of *Murraya*. Furthermore, this study allowed the recognition of *M. koenigii* and *M. paniculata* as distinct species. Since the *M. paniculata* group of accessions was relatively less heterogeneous, it appears that this group may not include any other divergent sub-group and so the taxa included as *M. exotica* are either not a distinct third species or are only morphologically distinct forms of *M. paniculata*. Thus *M. exotica* can be treated as a synonym of *M. paniculata*, as has been suggested earlier[2]. The genetic divergence found between species may also be sufficient to agree with the morphological findings. However, more extensive samplings of the species are needed to study the genetic diversity across a range of cultivated and natural populations of *Murraya* species. This study is an attempt on molecular analysis to improve the identification and differentiation of the *Murraya* species.

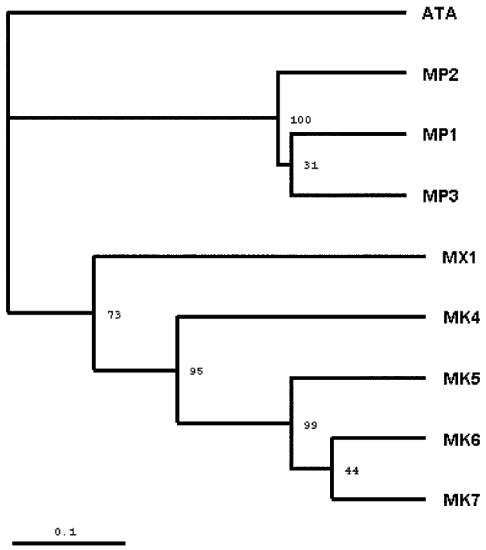

Figure 2. Jaccard coefficient/UPGMA tree for cumulative RAPD and DAMD band data. Numbers to the right of the branches are taxa (abbreviated sample numbers) as in Table 1. Smaller numbers at the node are bootstrap percentages after a 500 replicate bootstrap test was carried out. The scale at the bottom left corner of the tree is the Jaccard coefficient scale.

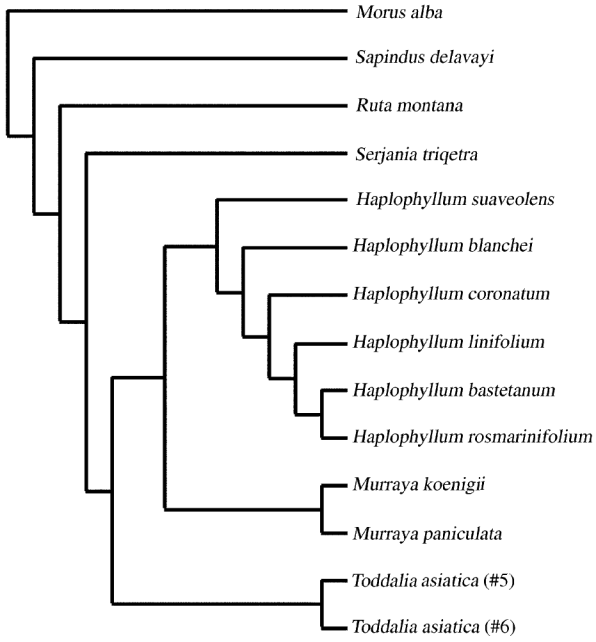

Figure 3. DIALIGN alignment results of *Murraya*, *Toddalia*, other Rutaceae and selected sequences where ITS1, complete 5.8 s and ITS2 sequences are available. The tree has the mulberry (*Morus alba*) sequence as the outgroup.

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ACKNOWLEDGEMENTS. We thank Jyoti Srivastava and Sushma Verma for help with plant collection. The work was partially supported by DBT grant-in-aid projects. We also thank the Director, NBRI, Lucknow for support.

Received 14 February 2005; revised accepted 27 January 2006

ATP levels and adenylate energy charge in soils of mangroves in the Andamans

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Adenosine 5′-triphosphate (ATP) is considered to be a useful indicator of life in soil and the adenylate energy charge (AEC) indicates the energetic status of soil microorganisms. ATP concentration and AEC levels have been extensively studied in a diverse group of soils. However, little knowledge is available on the levels of ATP and AEC in soils of mangroves. We report here the levels of adenylates ATP, adenosine di-phosphate (ADP) and adenosine monophosphate (AMP)) and AEC in soils of undisturbed mangroves of South-, Middle-, North- and Little-Andamans. Relevant soil physico-chemical and microbial parameters and their relationship to ATP and AEC were also examined. Averaged across various mangrove sites, total N level was 1.44 ± 0.13 g kg^{-1}, organic C 15.6 ± 1.5 g kg^{-1}, microbial biomass C 410 ± 35 μg kg^{-1}, microbial biomass N 34 ± 2 μg kg^{-1} and qCO_2 41.1 ± 4.4 mg CO_2 (g biomass C)$^{-1}$ d^{-1}. Among the adenylates, ATP ranged

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