

Article

Whole-Genome Evolutionary Analyses of Non-Endosymbiotic Organelle-Targeting Nuclear Genes Reveal Their Genetic Evolution in 12 Representative Poaceae Species

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Abstract: Chloroplasts and mitochondria, descendants of ancient prokaryotes via endosymbiosis, occupy a pivotal position in plant growth and development due to their intricate connections with the nuclear genome. Genes encoded by the nuclear genome but relocated to or being functional within these organelles are commonly referred as organelle-targeting nuclear genes (ONGs). These genes are essential for maintaining cytonuclear coordination, thereby determining the stability of the life cycle. While molecular function and cytonuclear coordination of some endosymbiosis-derived ONGs (E-ONGs) have been extensively studied, the evolutionary history and characteristics from a more widespread range of non-endosymbiosis-derived ONGs (NE-ONGs) remain largely enigmatic. In this study, we focused on 12 representative species within the Poaceae family to systematically identify NE-ONGs and investigated their evolutionary history and functional significance on a phylogenetic timescale. Upon aligning these 12 species' evolutionary histories, we observed the following phenomena: (i) an exploration of NE-ONGs between the BOP and PACMAD clades unveiled dynamic compositions, potentially influencing their photosynthetic divergence; (ii) the majority of the abundant species-specific NE-ONGs exist in a single-copy status, and functional enrichment analysis further underscored their specialized roles, which could be crucial for species adaptation; and (iii) comparative analyses between plasmid- and mitochondria-related NE-ONGs (pNE-ONGs and mNE-ONGs) revealed a prevalence of pNE-ONGs, indicating tighter control for chloroplast function in Poaceae. In summary, this study offers novel insights into the cytonuclear co-evolutionary dynamics in Poaceae speciation and draws attention to crop improvement by using NE-ONGs.

Keywords: non-endosymbiosis-derived ONGs (NE-ONGs); genetic evolution; cytonuclear coordination; Poaceae



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1. Introduction

Plant chloroplasts and mitochondria are endosymbiotic descendants of ancient prokaryotic cyanobacteria and alpha-proteobacteria, establishing intricate information connections with the eukaryotic nucleus [1,2]. Throughout endosymbiosis, organellar genomes have undergone substantial reduction, transferring portions of their genetic material into the host nucleus via nuclear chloroplast DNA (NUPTs) and/or nuclear mitochondrial DNA (NUMTs) [3,4]. Conversely, the nuclear genome encodes proteins with N-terminal transit peptides, facilitating their import into respective organelles [5]. These genes are commonly known as organelle-targeting nuclear genes (abbreviated as ONGs). Genomic information allows systematic characterization of ONG composition by predicting N-terminal transit peptides [6,7]. ONGs play vital roles in the proper development, functional maintenance and propagation of respective organelles, including Cytb6f of photosynthetic chain, the

key rate-limiting enzyme Rubisco of photosynthesis [8], most of the components in the TOC-TIC super-complexes [9], as well as subunits of the cytochrome *b* and cytochrome *c* oxidase complex [10]. This predictive capability significantly contributes to the in-depth analysis of protein sorting and transport [6], molecular functions and practical applications in plant immune responses [11], including fungal and oomycete infection [12].

Additionally, these ONGs can be categorized into two groups based on their evolutionary origin: the first group are endosymbiosis-derived ONGs (E-ONGs) that returned to organellar endosymbionts; and the second group comprises intrinsic eukaryotic genes without homology to either prokaryotic ancestor, namely, non-endosymbiosis derived ONGs (NE-ONGs). Evolutionary analyses of ONGs have primarily focused on E-ONGs in *Arabidopsis thaliana*, where 18% of protein-coding genes of the whole genome were acquired from the cyanobacterial ancestor of plastids and mostly returned to respective organelles [13]. Through homology searching against the genomes of existing relatives of prokaryotic endosymbionts (cyanobacteria and alpha-proteobacteria), the complete profile of NE-ONGs in species, which are also able to participate in various important biological processes [1], can also be characterized. However, the compositions and evolutionary dynamics of NE-ONGs within closely related plant species, along with their functional participation, remain largely unexplored.

Poaceae, encompassing approximately 11,000 species within 12 subfamilies, includes many cereal crops and herbaceous plants and plays a crucial role in human sustenance and the biodiversity of grassland ecosystems [14–16]. Due to the economic and ecological significance of Poaceae, there is widespread interest in sequencing and assembling species' nuclear genomes, as well as their evolution and classification [17,18]. Considering that the phylogenetic framework for Poaceae is well established, this plant family encompasses two primary phylogenetic clades, known as the BOP and PACMAD clades: the BOP clade encompasses C_3 plants (such as wheat, rice, barley, and bamboo), and the PACMAD clade encompasses C_4 plants (such as maize, millet, and sorghum) and some C_3 – C_4 intermediate species (Neurachninae in Panicoideae) [17,19]. Furthermore, significant progress has been made in obtaining comprehensive genomic information for many economically important grass taxa. Therefore, it becomes possible to explore the potential functional participation of NE-ONGs in their photosynthetic divergence.

In this study, we characterize the profiles of NE-ONGs within representative Poaceae species and explore their dynamic evolutionary features in the context of phylogenetic speciation. We also compare the functional participation of specialized NE-ONGs in different species. Our results provide novel insights into cytonuclear coordination in plants and emphasize its potential significance for adaptation and speciation among grasses.

2. Materials and Methods

2.1. Data Collection

For this evolutionary analysis of NE-ONG, we employed 12 representative Poaceae species, encompassing 7 BOP and 5 PACMAD species, that have high-quality sequenced genomes and comprehensive gene annotations. Additionally, *Arabidopsis thaliana* served as an outgroup species to provide a comparative framework for further evolutionary analysis. Pertinent genome sequences and annotation files were obtained from various resources. Specifically, the data of *Arabidopsis thaliana* (TAIR10) [20], *Brachypodium distachyon* (v3.1) [21], *Oryza sativa* (v7_JGI) [22], *Setaria italica* (v2.2) [21,23], *Sorghum bicolor* (v3.1.1) [24], and *Miscanthus sinensis* (v7.1) [25] were downloaded from Phytozome (<https://phytozome-next.jgi.doe.gov/>; last accessed on 9 June 2023). Genomic data for *Hordeum vulgare* (goldenpromise), *Triticum urartu* [26], and *Zea mays* (AGPv3) were retrieved from Ensembl (<https://www.ensembl.org>; last accessed on 9 June 2023). The genomic information for *Olyra latifolia* and *Bonia amplexicaulis* [27] was accessed from the Germplasm Bank of Wild Species (<http://www.genobank.org>; last accessed on 15 September 2020). Lastly, genomic data for *Eragrostis curvula* (GCA_007726485.1, version CERZOS_EC1.0) and *Aegilops tauschii* (GCA_002575655.2, version Aet v5.0) were downloaded from NCBI [28,29].

2.2. Identification of ONGs and NE-ONGs

Based on the protein sequences of 12 representative Poaceae species and *A. thaliana*, de novo subcellular targeting prediction programs and the CyMIRA database [8] were employed to identify ONGs and NE-ONGs. First, the combination of TargetP and LOCALIZER were utilized to predict ONG candidates de novo [6,7,30]. After excluding genes with inconsistent or dual-targeting annotations (predicted to target both chloroplasts and mitochondria), BLASTP (v2.6.0; e-value cutoff of 10^{-3}) [31] was employed to search for the ONG candidates against the CyMIRA database to validate prediction accuracy. Furthermore, to distinguish NE-ONGs from other ONGs, we conducted sequence alignment between the ONGs and prokaryotic ancestors of chloroplast and mitochondria using BLASTP (E-value cutoff of 10^{-3}). This alignment encompassed 15 cyanobacterial species and strains, as well as 40 alpha-proteobacteria species and strains, as detailed in Tables S1 and S2. Genes that lack orthologous sequences in comparison to prokaryotic ancestors were defined as NE-ONGs; correspondingly, those associated with plasmids and mitochondria were designated as pNE-ONGs and mNE-ONGs, respectively (Table S3).

2.3. Phylogenetic Analysis of NE-ONG Groups in 12 Representative Poaceae Species

The integration of NE-ONG orthologous groups (NE-ONG groups) into respective phylogenetic species trees allows for the characterization of the evolutionary birth/death dynamics of ONGs throughout speciation [17]. First, OrthoFinder v2.3.12 [32] was used to identify orthologous groups among sampled Poaceae species and *A. thaliana*, as summarized in the last two columns of Table 1. The maximum parsimony criterion was utilized to identify birth and death events (marked as “+” and “−,” respectively) of NE-ONG groups on respective phylogenetic nodes and each species tip (labeled with letters). If the NE-ONG group was uniquely identified or not identified in a specific species, it was defined as a species-specific birth or death and labeled at the appropriate species node. Accordingly, if an NE-ONG group was consistently shared or lost among species in a unique species clade, the respective birth or death event was labeled at the common ancestral node (Tables S4–S6). To authentically trace the evolutionary footprint of each gene shared with respective species and common ancestors, they were similarly recorded and mapped to the species tree (Tables S7 and S8).

Table 1. Content of NE-ONGs and NE-ONG orthogroups of 12 Poaceae species.

Species	Clade	Number and Percentage in Total Gene	Orthogroups	Single-Copy Gene	Gene per Group (NE-ONGs)	Single Copy (Whole Genome)	Gene per Group (Whole Genome)
<i>Oryza sativa</i>	BOP clade	3445 (8.87%)	3022	1822 (52.89%)	1.14	17,474 (44.96%)	1.43
<i>Olyra latifolia</i>	BOP clade	2624 (7.17%)	2161	1330 (50.69%)	1.21	14,335 (39.19%)	1.83
<i>Bonia amplexicaulis</i>	BOP clade	3700 (7.86%)	2846	1289 (34.84%)	1.30	12,008 (25.52%)	2.01
<i>Hordeum vulgare</i>	BOP clade	2274 (6.94%)	1840	1174 (51.63%)	1.24	13,185 (40.21%)	1.76
<i>Triticum urartu</i>	BOP clade	3865 (10.16%)	3199	2148 (55.58%)	1.21	17,557 (46.14%)	1.55
<i>Aegilops tauschii</i>	BOP clade	3276 (8.45%)	2522	1366 (41.70%)	1.30	14,689 (37.87%)	1.84
<i>Brachypodium distachyon</i>	BOP clade	3263 (9.51%)	2974	1718 (52.65%)	1.10	14,408 (41.99%)	1.41
<i>Zea mays</i>	PACMAD clade	8370 (13.18%)	6717	3713 (44.36%)	1.25	24,782 (39.04%)	1.43
<i>Sorghum bicolor</i>	PACMAD clade	3276 (9.59%)	2915	1745 (53.27%)	1.12	14,624 (42.85%)	1.41
<i>Miscanthus sinensis</i>	PACMAD clade	6705 (9.89%)	5154	2498 (37.26%)	1.30	16,522 (24.37%)	1.92
<i>Setaria italica</i>	PACMAD clade	3056 (8.83%)	2594	1493 (48.85%)	1.18	13,923 (40.26%)	1.52
<i>Eragrostis curvula</i>	PACMAD clade	5077 (9.20%)	3586	1515 (29.84%)	1.42	13,746 (24.91%)	2.15

2.4. Function Annotation and Enrichment Analysis of NE-ONGs

To ensure uniformity in the functional annotation of all studied species, we employed eggNOG [33,34] to annotate the NE-ONG proteins. Subsequently, the R package clusterProfiler (v3.12.0) was used to complete function enrichment analysis based on a hypergeometric distribution test [35].

2.5. Selection Pressure Analysis of Ancient NE-ONG Groups

Genes from each ancient NE-ONG group were split into BOP and PACMAD clades. After that, protein and CDS sequences were extracted from the relevant genome datasets. ParaAT [36] version 2.0 was employed to calculate the Ka/Ks values of BOP and PACMAD clades for each ancient NE-ONG group by “-m muscle -f axt” and other default parameter settings.

2.6. Gene Duplication Analysis in *Z. mays*, *S. bicolor* and *M. sinensis*

To identify various duplication events involving protein-coding genes in *Z. mays*, *S. bicolor* and *M. sinensis*, including singletons, dispersed duplicates, proximal duplicates, tandem duplicates and segmental/WGD duplicates, BLASTP was used initially (E-value cutoff 10^{-5}), followed by independent analysis with the duplicate_gene_classifier module of MCScanX [37]. Function enrichment analysis of WGD-related NE-ONGs was performed as described above in Section 2.4.

3. Results

3.1. Characterization of NE-ONGs and NE-ONG Groups in Representative Poaceae Species

By conducting homology searches against 15 cyanobacteria-related species and 40 alpha-proteobacteria-related species (Tables S1 and S2) and predicting organelle targeting, we characterized NE-ONG compositions in 12 representative Poaceae species grouped into phylogenetic BOP (*Oryza sativa*, *Olyra latifolia*, *Bonia amplexicaulis*, *Hordeum vulgare*, *Triticum urartu*, *Aegilops tauschii*, *Brachypodium distachyon*) and PACMAD clades (*Zea mays*, *Sorghum bicolor*, *Miscanthus sinensis*, *Setaria italica*, *Eragrostis curvula* from Chloridideae) (see Materials and Methods). We identified 2274 (6.94% of total protein-coding genes in *H. vulgare*) to 8370 (13.18% in *Z. mays*) NE-ONGs across sampled species. The average content was lower in the BOP clade than in the PACMAD clade (8.42% vs. 10.14%, Table 1), suggesting compositional divergence of NE-ONGs between the two clades. After categorizing NE-ONGs into orthologous groups (NE-ONG groups, see Materials and Methods), we identified 1840 (*H. vulgare*) to 6717 (*Z. mays*) NE-ONG groups. Notably, within these groups, a significant proportion ranging from 29.84% to 55.58% was associated with single-copy genes. Intriguingly, when compared to the background orthogroups across the whole genome, which comprised 2.15 genes per group in *E. curvula* and 1.41 genes per group in *S. bicolor* (Table 1), NE-ONG groups exhibited a notably lower gene copy number, averaging at 1.10 genes per group in *B. distachyon* and 1.42 genes per group in *E. curvula* (Table 1, *t*-test, $p < 0.001$). Notably, the average percentage of NE-ONG groups associated with single-copy genes, specifically 48.57% in BOP and 42.72% in PACMAD, did not exhibit a statistically significant difference between the two clades; similarly, the average number of genes per group, 1.21 in BOP and 1.25 in PACMAD, showed no difference either (*t*-test, $p > 0.05$; Table 1). Furthermore, NE-ONGs harbor fewer exons than other nuclear background genes (*t*-test, $p < 0.05$; Figure S1), which on average means that NE-ONGs have more simple gene structure.

Subcategorizing NE-ONGs by their targeting organelles (pNE-ONGs, plastid-targeting NE-ONGs; mNE-ONGs, mitochondria-targeting NE-ONGs) revealed a greater number of pNE-ONGs (1242–5107) than mNE-ONGs (1032–3262) in Poaceae species (*t*-test, $p < 0.05$; Table 2). At the orthogroup level, we discovered that the percentage of groups associated with single-copy genes in pNE-ONG groups (ranging from 31.31% to 57.49%) is comparable to that of mNE-ONG groups (ranging from 27.92% to 52.81%) (*t*-test, $p > 0.05$), but statistically significant relative to other background nuclear genes (*t*-test, $p < 0.001$). Furthermore, the number of genes per group also exhibited similarity between pNE-ONG

groups (varying between 1.06 and 1.33) and mNE-ONG groups (varying between 1.10 and 1.39; Paired Mann–Whitney U test, $p < 0.05$).

Table 2. Content of pNE- and mNE-ONGs and orthogroups of 12 Poaceae species.

Species	Clade	pNE-ONGs				mNE-ONGs			
		Total Gene	Single-Copy Gene	Orthogroups	Gene per Group	Total Gene	Single-Copy Gene	Orthogroups	Gene per Group
<i>Oryza sativa</i>	BOP clade	1989	1124 (56.51%)	1824	1.09	1456	698 (47.94%)	1272	1.14
<i>Olyra latifolia</i>	BOP clade	1494	777 (52.01%)	1266	1.18	1130	553 (48.94%)	958	1.18
<i>Bonia amplexicaulis</i>	BOP clade	2081	769 (36.95%)	1668	1.25	1619	520 (32.12%)	1260	1.28
<i>Hordeum vulgare</i>	BOP clade	1242	638 (51.37%)	1017	1.22	1032	536 (51.94%)	859	1.20
<i>Triticum urartu</i>	BOP clade	2284	1313 (57.49%)	1958	1.17	1581	835 (52.81%)	1326	1.19
<i>Aegilops tauschii</i>	BOP clade	1847	819 (44.34%)	1533	1.20	1429	547 (38.28%)	1116	1.28
<i>Brachypodium distachyon</i>	BOP clade	1848	1033 (55.9%)	1744	1.06	1415	685 (48.41%)	1288	1.10
<i>Zea mays</i>	PACMAD clade	5107	2222 (43.51%)	4204	1.21	3262	1491 (45.71%)	2789	1.17
<i>Sorghum bicolor</i>	PACMAD clade	1923	1053 (54.76%)	1769	1.09	1353	692 (51.15%)	1213	1.12
<i>Miscanthus sinensis</i>	PACMAD clade	3760	1463 (38.91%)	3046	1.23	2944	1035 (35.16%)	2278	1.29
<i>Setaria italica</i>	PACMAD clade	1759	919 (52.25%)	1581	1.11	1297	574 (44.36%)	1087	1.19
<i>Eragrostis curvula</i>	PACMAD clade	2878	901 (31.31%)	2168	1.33	2199	614 (27.92%)	1583	1.39

The compositional and structural features of these NE-ONG groups show that the majority of component NE-ONGs exist in a single-copy state (31.31–57.49% for pNE-ONGs and 27.92–52.81% for mNE-ONGs), statistically significant relative to other background nuclear genes (t -test, $p < 0.001$). Furthermore, NE-ONGs harbor fewer exons than other nuclear background genes (t -test, $p < 0.05$, Figure S1).

3.2. Unstable NE-ONGs in Ancient NE-ONG Groups Shared by BOP and PACMAD Clades

To understand the evolutionary dynamics of NE-ONG groups, we mapped the birth and death events of NE-ONGs (including pNE-ONGs and mNE-ONGs) on the phylogenetic species tree (see Materials and Methods). Although there are differences in different branch nodes (from B to Y nodes), the evolutionary pattern of pNE-ONG and mNE-ONG groups were similar (Figure 1A,B): the majority of NE-ONG groups were shown to be species-specific (97.31% pNE-ONG groups vs. 96.55% mNE-ONG groups).

In sum, 75 ancient pNE-ONG and 50 mNE-ONG groups were identified (from 1094 pNE-ONGs and 927 mNE-ONGs, respectively), which were generated before the BOP and PACMAD clades diverged (Figure 1A,B; B node). Gene Ontology (GO) enrichment analysis revealed conserved functions for those ancient NE-ONGs within their respective clades (Figure 2A,B): for pNE-ONG groups, these include starch-binding domain, vitamin B6 photoprotection and homeostasis, and a photosystem II 10 kDa polypeptide; for mNE-ONG groups, these include Tim21, ripening-related protein, and protein Rfl. Notably, while these ancient NE-ONG groups were conserved, their compositions were not maintained stably in terms of variable gene numbers. Within the PACMAD clade, pNE-ONG groups harboring multiple ONGs were more abundant than in the BOP clade (multiple ONG groups 86 and 104 in BOP and PACMAD clades, respectively) (Figure S2A; Fisher’s exact test, $p < 0.001$). Similar instability was also observed in mNE-ONG groups (multiple ONG groups 102 and 105 in BOP and PACMAD clades, respectively) (Figure S2B; Fisher’s exact test, $p < 0.001$). Several illustrative instances of amplified mNE-ONG group include phosphate-induced protein 1 conserved region (OG0000708), solute carrier family (OG0003917), SNARE-associated Golgi protein (OG0004855) and unknown function (OG0003805), whereas pNE-ONG groups do not have significant differences between the

two clades. Notably, these three GO terms also showed significant differences in gene numbers and gene numbers in corresponding mNE-ONG groups between the two clades (Figure S2D, blue stars, t -test, $p < 0.05$). These results suggest that the amplification of genes involved in key biological processes plays latent roles reflected in divergence between the BOP and PACMAD clades.

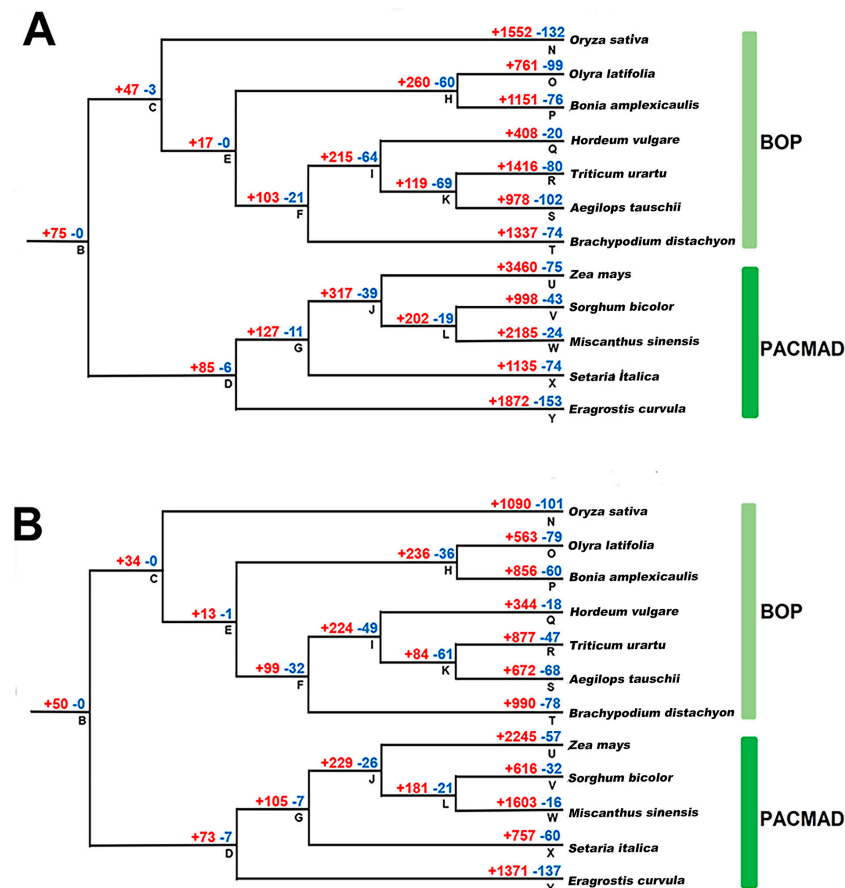


Figure 1. The dynamic evolution pattern of NE-ONG groups in 12 representative Poaceae species. (A,B) Evolutionary pattern of pNE-ONGs (A) and mNE-ONGs (B). Each node in the graph is represented by a letter, the shared nodes (at least two species) in the species tree are B~L, while the species-specific nodes are N~Y. The number above each corresponding node signifies the quantity of NE-ONG groups either gained (highlighted in red) or lost (blue) by the node.

To test whether these unstable NE-ONGs were under the constraint of natural selection, we conducted a comparative analysis of the K_a/K_s ratios between the pNE-ONG and mNE-ONG groups within the BOP and PACMAD clades (Figure 2C). We found that most pNE-ONG and mNE-ONG groups were under negative selection ($K_a/K_s < 1.0$). Compared to pNE-ONG groups, the K_a/K_s ratios are significantly lower in mNE-ONG groups (Figure 2C; t -test, $p < 0.01$). Notably, the K_a/K_s ratios of pNE-ONGs within the PACMAD clade were lower than those in the BOP clade, whereas the converse was observed in mNE-ONGs (Figure 2C). These results indicate that the selection signal between ancient negative selection on pNE-ONGs and mNE-ONGs is significantly biased.

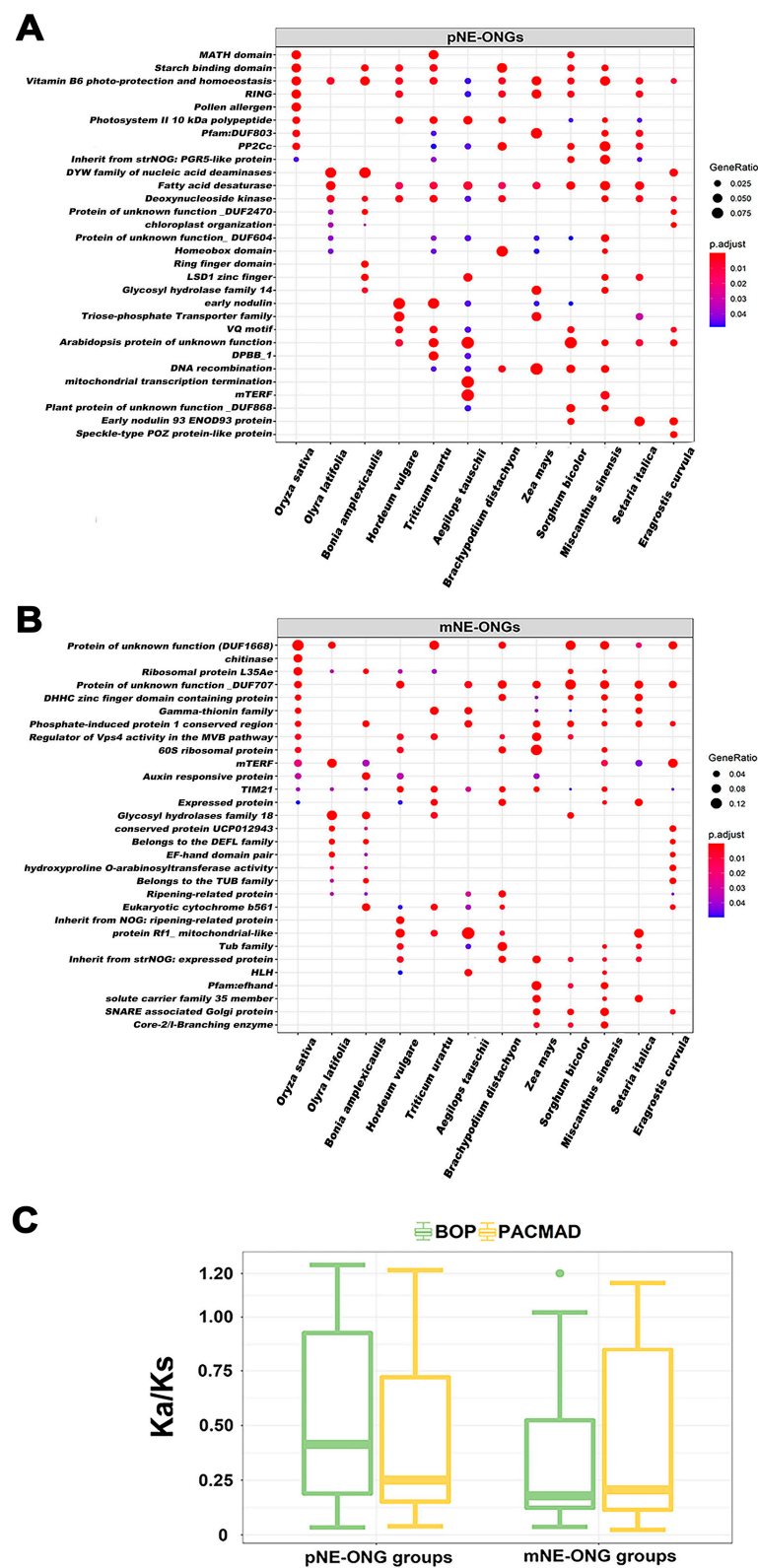


Figure 2. The GO enrichment results and comparisons of Ka/Ks values. (A) GO enrichment of B-node-related pNE-ONGs. (B) GO enrichment of B-node-related mNE-ONGs. (C) Comparisons of Ka/Ks values among different NE-ONG groups of B node in both the BOP (left) and PACMAD (right) clades.

3.3. Different Functions of BOP- and PACMAD-Specific NE-ONG Groups

Aside from the ancient NE-ONG groups commonly found in both the BOP and PACMAD clades, we identified distinct clade-specific gain and loss of pNE- and mNE-ONG groups within each clade (Figure 1A,B; C and D nodes). Considering the limited number of lost NE-ONG groups within each clade (three and six pNE-ONG groups and seven mNE-ONG groups were lost; Figure 1A,B), we focused on characterizing functions of those clade-specific gained NE-ONG groups. Genes of gained BOP-specific pNE-ONG groups were enriched in the exostosin family; PACMAD-specific pNE-ONG groups were enriched in UDP-glycosyltransferase, Hsp20/alpha crystallin family, GRAS family and pentatricopeptide repeat-containing protein (Figure S3A,B). On the other hand, genes belonging to BOP-specific pNE-ONG groups were enriched in C2 domain, mitochondrial-like; PACMAD-specific pNE-ONG groups were enriched in glycerol-3-phosphate acyltransferase and the PPR repeat family (Figure S3C,D). Overall, the PACMAD clade gave rise to a greater number of clade-specific NE-ONG groups, and these BOP- and PACMAD-specific groups exhibited distinct and unique functionalities.

3.4. Species-Specific NE-ONG Groups with Specialized Unique Functions

Notably, within each species following speciation, 408–3460 and 344–2245 species-specific pNE-ONG and mNE-ONG groups were identified, respectively (Figure 1A,B). The species-specific pNE-ONG content was significantly higher than that of species-specific mNE-ONG groups (Paired *t*-test, $p < 0.001$), consistent with the overall dominance of pNE-ONG groups (Table 2). Furthermore, we investigated the functional features of these species-specific NE-ONGs. pNE-ONG and mNE-ONG groups showed similar patterns. As expected, these species-specific NE-ONG groups were mostly enriched in non-overlapping or unique GO terms, indicating their functional specialization in respective species (Figure 3A,B). Notably, pNE-ONG groups in some species were enriched in transmembrane transporter activity, transmembrane amino acid transporter protein, glycosyl hydrolase family 14; mNE-ONG groups in some species were enriched in X8 domain, retrotransposon protein and NB-ARC domain.

3.5. aWGDs Contributed to the Ultra-Amplification of Species-Specific NE-ONG Groups in *Z. mays* and *M. sinensis*

Among the Poaceae species studied, *Z. mays* and *M. sinensis* exhibited the highest number/proportion of both species-specific pNE-ONG and mNE-ONG groups, surpassing even their sister species, *S. bicolor* (Figure 1A,B). Considering that both *Z. mays* and *M. sinensis* have undergone respective recent ancient whole-genome duplications (aWGD) after their divergence from *S. bicolor* [38,39], we explored the potential contribution of aWGD to the abundance of species-specific NE-ONG groups. Employing syntenic analysis with MCScanX (see Section 2) to characterize the composition of NE-ONG groups in terms of their duplication history (singletons, dispersed gene, tandem, etc.), we discovered that both species-specific pNE- and mNE-ONGs in *Z. mays* (6.71% and 9.34% for pNE- and mNE-ONGs, respectively) and *M. sinensis* (13.18% and 13.88% for pNE- and mNE-ONGs, respectively) exhibited a larger proportion of WGD duplicates than *S. bicolor* (2.09% and 5.04% for pNE- and mNE-ONGs, respectively; Figure 3C,D). This trend was not observed in other duplication categories. Intriguingly, singletons showed an opposite trend when compared to WGD duplicates (Figure 3C,D). After performing GO analysis, we discovered that WGD-related NE-ONGs are enriched in ER lumen proteins, ubiquitin-conjugating enzymes, and 60S ribosomal proteins (Figure 3E,F), potentially associated with organelle morphology and secondary metabolites.

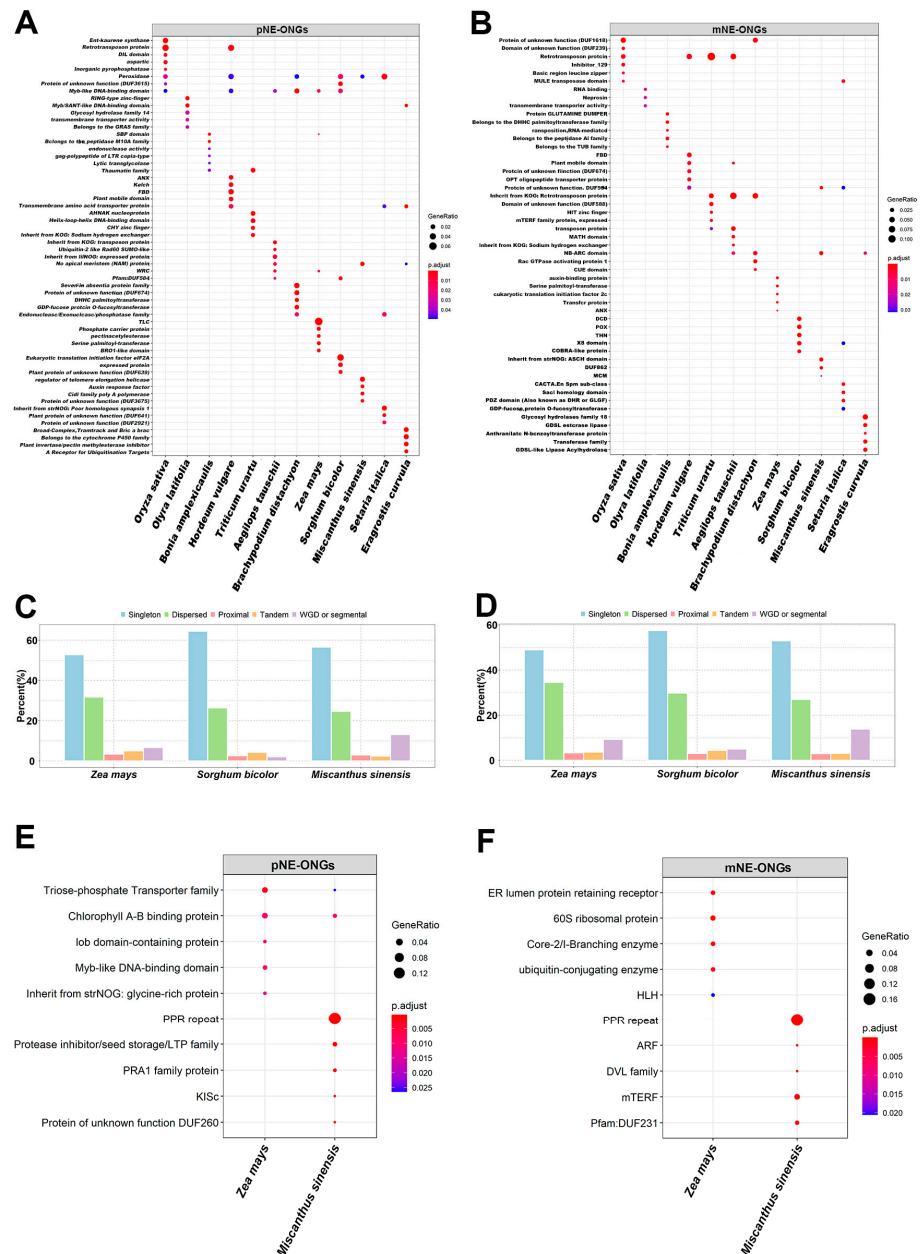


Figure 3. The characteristics of NE-ONGs from species-specific nodes (N~Y). **(A,B)** GO enrichment of pNE-ONGs **(A)** and mNE-ONGs **(B)** among species. **(C,D)** Content of five duplicated gene types in *Andropogoneae* species (*Z. mays*, *S. bicolor* and *M. sinensis*) for pNE-ONGs **(C)** and mNE-ONGs **(D)**. **(E,F)** GO enrichment of WGD-related pNE-ONGs **(E)** and mNE-ONGs **(F)** in *Z. mays* and *M. sinensis*.

4. Discussion

Mitochondria and chloroplasts, descendants of ancient prokaryotes [40–44], have undergone substantial genomic reduction during endosymbiosis, resulting in the transfer of genes to the nuclear genome. The proper development and function of these organelles depend on the activity of organelle-targeting nuclear genes (ONGs) as proteins back to respective organelles [30,45–47], which can be categorized into two groups: those with an ancient endosymbiotic origin (E-ONGs) and those without such characteristics (NE-ONGs). The central focus of this study was to investigate the dynamic composition, evolutionary history, and functional implications of NE-ONGs during the speciation of representative Poaceae species.

The predominant features of NE-ONGs involves single-copy composition, species-specific generation, and functional specialization, all consistently connected. First, the predominantly single-copy status of Poaceae NE-ONG groups aligns with their species-specific evolutionary history (Figure 3C,D). Species-specific NE-ONGs, presumed to be nascent genes generated post-speciation, show limited family amplification. Notable exceptions include *Z. mays* and *M. sinensis*, which experienced separate ancient whole-genome duplications (aWGDs) after speciation. However, to maintain the cytonuclear dosage balance, since organelle-targeting genes are prone to restore to single copy within following diploidization after aWGD [48,49], the relatively lower but significant frequency of single-copy NE-ONGs in these cases still supports the general trend (Table 1). In addition, the specialized and non-overlapping functions of species-specific NE-ONGs (Figure 3A,B) could facilitate nuclear control, fine-tuning chloroplasts and mitochondria in photosynthesis, energy supply, stress responses and even metabolism [50–54]. Unique NE-ONGs within each species provide a genetic basis for species adaptation to distinct and changing habitats.

Additional comparisons shed light on differences in NE-ONGs between chloroplasts and mitochondria (pNE-ONGs vs. mNE-ONGs) and between Poaceae clades (BOP vs. PACMAD). The dominance of pNE-ONGs over mNE-ONGs (Table 2) suggests a tighter control requirement for proper chloroplast function than for mitochondria, at least in Poaceae. Extending this inquiry to other plant families compared to animal species (which have only mNE-ONGs) would be valuable. Regarding NE-ONGs in BOP vs. PACMAD, ancient NE-ONGs shared across Poaceae (Figure 1A,B; B node) maintain dynamic compositions in these two clades; additionally, clade-specific NE-ONGs were also identified (Figure 1A,B; C and D nodes). These NE-ONGs could be linked to the anatomical and physiological divergence of these two types of species in photosynthesis [55–57]. Future experimental and evolutionary confirmations are needed to delve more deeply into these dynamics.

Whole-genome duplication (WGD) has been particularly prevalent in angiosperms species [58], recognized as an important evolutionary mechanism of plant diversification [59]. Here, we found that ancient WGD events contributed to the composition of NE-ONGs in *Z. mays* and *M. sinensis* (Figure 3C,D). A higher proportion of WGD-related NE-ONGs might suggest their crucial molecular functions and intricate cytonuclear coordination patterns, enabling better adaptation to diverse environmental changes. Indeed, our GO analysis suggested that WGD-related NE-ONGs were majorly related to organelle morphology and secondary metabolites (Figure 3E,F). Notably, previous research has reported that co-regulated gene sets related to ribosome biogenesis (locating both nuclear and cytosolic) are identified as being adjacent gene pairs [60], which also provides us with a framework for analyzing expression networks among NE-ONGs (especially for tandem duplicates of NE-ONG) in the future to further elucidate the evolutionary trajectory of these genes. With the advancement of genetics and plant physiology and a deeper understanding of the mechanism of photosynthesis, efforts to enhance photosynthetic efficiency in crops have been taking new approaches. One of them involves the transfer or establishment of C_4 photosynthetic machinery into C_3 crops [61–63], which necessitates characterizing related essential genes. The Poaceae include many of the world's most important C_3 and C_4 cereal crops, which are concentrated in the BOP and PACMAD clades, respectively [57]. Our characterized C_4 -enriched PACMAD clade-specific pNE-ONGs provide an interesting set of essential gene candidates (Figure S3). Their exact functions in C_4 photosynthetic pathways need to be confirmed by further molecular experiments. Further explorations of those NE-ONGs may be valuable for the sustainability of agricultural production.

5. Conclusions

In summary, we systematically identified NE-ONGs, characterized their evolutionary history, and explored their potential functional significance in a clear, well-established phylogenetic framework in the Poaceae. We observed the dynamic evolutionary patterns of ancient NE-ONGs composition in the BOP and PACMAD clades, indicating variability that

potentially influenced photosynthetic divergence. Additionally, our findings implicated that NE-ONGs played a pivotal role in speciation. Notably, the NE-ONGs were closely associated with ancient whole-genome duplication (aWGD) events in *Z. mays*, *S. bicolor*, and *M. sinensis*. Altogether, the composition of NE-ONGs in the Poaceae are consistent with their unique species evolutionary trajectories, which provides a unique perspective for analyzing cytonuclear co-evolution as well as for implying potential applications of NE-ONGs in improving the photosynthesis of C₃ Poaceae crops.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy14061177/s1>. Figure S1: Exon numbers in NE-ONGs and whole-genome nuclear genes in 12 Poaceae species; Figure S2: Gene numbers in each NE-ONG group and each GO term from B node; Figure S3: The function enrichment of NE-ONG groups from C and D nodes; Table S1: Genomes and assembly numbers of cyanobacteria-related species and strains; Table S2: Genomes and assembly numbers of alpha-proteobacteria-related species and strains; Table S3: NE-ONGs of 12 representative Poaceae species; Table S4: Nodes' establishment conditions and condition codes of Poaceae species; Table S5: Criteria of judging "+" between Poaceae species; Table S6: Criteria of judging "-" between Poaceae species; Table S7: Evolutionary dynamic trajectory of pNE-ONG groups from 12 Poaceae species; Table S8: Evolutionary dynamic trajectory of mNE-ONG groups from 12 Poaceae species.

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